BIOLOGICAL CHARACTERIZATION OF *TRITRICHOMONAS FOETUS*

OF BOVINE AND FELINE ORIGIN

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BIOLOGICAL CHARACTERIZATION OF *TRITRICHOMONAS FOETUS*

OF BOVINE AND FELINE ORIGIN

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A Dissertation

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Doctor of Philosophy

Auburn, Alabama
May 10, 2008

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VITA

Heather Dawn Stockdale is the daughter of Ernest Neil and Penny Stockdale of Milton, Kentucky. She was born on November 19, 1976 in Madison, Indiana and graduated with honors from Trimble County High School in 1995. She began her undergraduate studies at the University of Kentucky, graduating in 1999 with a Bachelor's of Science degree in Biology. In 2002, she enrolled in the graduate program at Appalachian State University. She graduated summa cum laude in 2004 with a Master's of Science degree in Biology and was nominated and accepted to the Alpha Epsilon Lambda Graduate and Professional Honor Society. In 2004, she began her Doctoral work in Parasitology at Auburn University College of Veterinary Medicine, graduating cum laude with a Doctorate degree in Biomedical Sciences on May 10, 2008.

DISSERTATION ABSTRACT

BIOLOGICAL CHARACTERIZATION OF *TRITRICHOMONAS FOETUS* OF BOVINE AND FELINE ORIGIN

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Doctor of Philosophy, May 10, 2008
(M.S., Appalachian State University, 2004)
(B.S., University of Kentucky, 1999)

120 Typed Pages

Directed by Byron L. Blagburn

Tritrichomonas foetus is a causative agent of venereal trichomoniasis in cattle characterized by early fetal death and post-coital pyometra. Reports have suggested that *T. foetus* (or a similar organism) is also the causative agent of large-bowel diarrhea in cats, characterized by large bowel inflammation, flatulence, tenesmus and fecal incontinence. Diagnosis of feline trichomoniasis is based upon observation of live organisms in direct smears, cultured feces or by amplification of specific genes using polymerase chain reaction (PCR). No documented treatment successfully eliminates *T. foetus* consistently from naturally infected cats. Certain drugs may reduce clinical signs and numbers of trichomonads from feces, but relapses of diarrhea commonly occur.

In the first study I attempted to estimate the prevalence of feline trichomoniasis within the pet population in the United States. To do so, 173 fecal samples were collected from cats in 16 states. Feces were scored for consistency and subjected to

culture and PCR analysis. Seventeen of 173 (10%) were positive for *T. foetus* by both fecal culture and PCR. Results indicate that *T. foetus* is prevalent in the pet population and that its presence correlates with the presence of diarrhea.

Experimental infections were conducted to determine if *T. foetus*, whether of bovine or feline origin, are biologically distinct. In the second study, two groups of virgin Angus heifers were inoculated with either *T. foetus* isolated from a pyometritic cow or a naturally infected cat. Vaginal, cervical, and uterine mucus samples were analyzed over an 11-week period and a single transcervical uterine biopsy sample was obtained from each animal, revealing severe damage to the endometrium in heifers infected with the bovine isolate of *T. foetus.* This was not observed in heifers infected with the feline isolate.

In the third study, 6 cats were inoculated with a bovine (D-1) isolate of *T. foetus* and one cat was inoculated with a feline (AUTf-1) isolate of *T. foetus*. Fecal samples from each cat were collected and subjected to culture over a period of five weeks. By PI day 15 the cat infected with the feline (AUTf-1) isolate had become culture positive for trichomonads while only one of six cats infected with the bovine (D-1) isolate was positive by PI day 32. At necropsy, the intestine of each cat was divided into five sections and the contents were collected and subjected to culture. The cat that received the feline (AUTf-1) isolate was positive in 4 of 5 intestinal sections and two cats infected with the bovine (D-1) isolate were positive in only one intestinal section. The combined results of studies two and three indicate that the disease caused by feline and bovine isolates of *T. foetus* in cattle are not identical and the susceptibility of cats to the feline (AUTf-1) and bovine (D-1) isolates *T. foetus* also appears demonstrably different.

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Byron L. Blagburn, for his guidance and support during my time at Auburn University. Additionally, thanks are due to my committee members, Dr. M. Daniel Givens, Dr. Jennifer A. Spencer, Dr. Christine C. Dykstra and Dr. David S. Lindsay. The experimental infection studies would not have been possible without the help of Dr. Soren Rodning, Dr. Ron H. BonDurant, Dr. Ray Dillon, Ellie Gripshover, Charles Price, Jamie Butler, Tracey Land, Sharon Barney, Dr. David Stringfellow and the caretakers at the North Auburn Beef Unit, Dr. Nancy Cox and Dr. Henry Baker. I would also like to thank Dr. Mark Carpenter for assistance with statistical analysis and Drs. Stephen Lenz, Joseph Newton and Elizabeth Welles for assistance with histopathological analysis. Thanks are due to Dr. Richard Bird, Patricia Deinnocentes and Scott Lenaghan for assistance with molecular analysis and photomicrographs taken throughout the studies were possible with the assistance of Drs. David Miller, Maria Toivio-Kinnucan, John Dennis and Pete Christopherson.

Finally, thanks and gratitude are due to my parents, Neil and Penny, my brother Jonathan and fiancé Trey for their unconditional love and support during this challenging time in my life.

Journal used

The Journal of Parasitology

Computer software used

Microsoft® Windows® XP Professional

SAS® Statistical Software

Vector NTI™ Advance 10

Phylogeny Inference Package (PHYLIP) ver. 3.67

Phylodraw ver. 0.8

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CHAPTER I.

LITERATURE REVIEW: *TRITRICHOMONAS FOETUS* AND TRICHOMONIASIS

The Trichomonads

The trichomonads are anaerobic protozoan parasites placed in the phylum Parabasalia, order Trichomonadida and family Trichomonadidae (Brugerolle and Lee, 2000). A new rank system classifies trichomonads as [Excavata: Parabasalia: Trichomonadida] (Adl, et al., 2005). They are comprised of a unique complement and arrangement of organelles and intricate cytoskeletal features (Figure 1.1). Trichomonads have a single nucleus located at the anterior end of the cell body, which is pyriform, or pear shaped. They replicate via binary longitudinal fission, undergoing closed mitosis in which there is no breakdown of the nuclear envelope; the spindle is extranuclear. The Golgi apparatus is quite large, and does not divide during replication. The cytoskeleton includes an axostyle, pelta and costa. The axostyle, comprised of microtubules, supports the cell body, and extends beyond the length of the cell. The pelta serves as the originating point and supportive structure for the flagella. The flagella include three to five anterior flagella, and often a posterior recurrent flagellum, each emerging from basal bodies composed of nine microtubule triplets. The basal bodies are made up of contractile centrin fibers, allowing internalization of flagella during formation of the pseudocyst. The costa is a structure unique to trichomonads, and supports the posterior

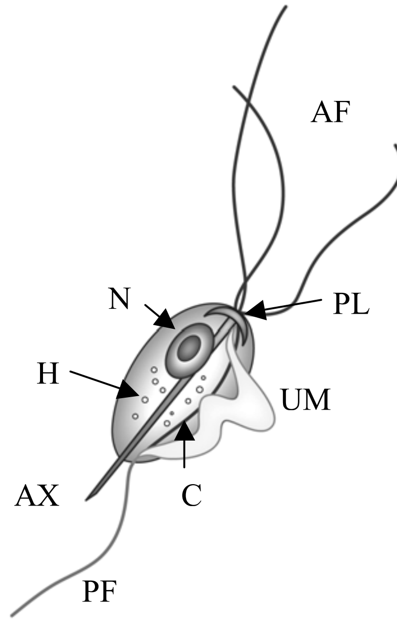


Figure 1.1. *Tritrichomonas foetus* morphological characteristics. External features and internal organelles include anterior flagella (AF), posterior recurrent flagellum (PF), axostyle (AX), costa (C), hydrogenosomes (H), undulating membrane (UM), nucleus (N) and pelta (PL). Note sketch is not to scale.

recurrent flagellum and the resulting undulating membrane which extends one-half to three-fourths the length of the cell body. Trichomonads do not have traditional mitochondria, and rely on spherical organelles known as hydrogenosomes to synthesize ATP (Honigberg, 1963, Roberts and Janovy, 2000, Benchimol, 2004).

There are several genera and species of trichomonads affecting humans and animals (Table 1.1). The trichomonad most commonly encountered in veterinary medicine is *Tritrichomonas foetus*. The cytoskeleton and internal organelles of *T. foetus* are similar to other trichomonads; however this genus has three anterior flagella, a recurrent flagellum that extends from an undulating membrane three-fourths the length of the cell body. Additionally, the axostyle continues beyond the posterior end of the cell body. The approximate size of *T. foetus* is 10µm x 6µm (Kofoid, 1920, Wenrich and Emmerson, 1933, BonDurant and Honigberg, 1994, Benchimol, 2004).

Tritrichomonas foetus is the causative agent of venereal trichomoniasis in cattle, documented on six continents (Morgan, 1947) and intestinal trichomoniasis in cats (Kessel, 1928, Jordan, 1956, BonDurant and Honigberg, 1994, BonDurant, 1997, Gookin, et al., 1999, Levy, et al., 2003). There has also been a report of *T. foetus* causing diarrhea in dogs (Gookin, et al., 2005) and a questionable report of human infection (Okamoto, et al., 1998). Recently, attempts were made to synonymize *Tritrichomonas suis,* a commensal trichomonad that infects the stomach, colon and nasal passages of swine, with *T. foetus* after molecular analysis of shared genetic sequences and detailed comparisons of morphological characteristics (Tachezy, et al., 2002).

Table 1.1. Selected trichomonads of human and veterinary importance (Jordan, 1956, BonDurant and Honigberg, 1994, Honigberg and Burgess, 1994, Gookin, et al., 1999, Roberts and Janovy, 2000, Levy, et al., 2003, Gookin, et al., 2005).

Trichomonad species	Host	Location	Disease
Trichomonas tenax	humans	oral cavity	none
Trichomonas vaginalis	humans	vagina, urethra vaginitis	urethritis
Pentatrichomonas hominis	humans	intestine	none
	dogs	large intestine	diarrhea
Trichomonas gallinae	birds	upper digestive tract	caseous nodules, fluid in crop
Trichomonas canistomae	dogs	oral cavity	none
Trichomonas felistomae	cats	oral cavity	none
Tritrichomonas foetus	cattle	vagina, uterus prepuce	abortion, metritis balanoposthitis
	swine	stomach, colon nasal passages	none
	cats	large intestine	diarrhea
	dogs	large intestine	diarrhea

Bovine Trichomoniasis

Trichomonas foetus (Riedmüller, 1928) syn. *Trichomonas* spp. reported by Kunstler (1888) syn. *Trichomonas utero-vaginalis vitulae* reported by Mazzanti (1900) was first described in cows and aborted fetuses in Europe, however pathogenicity was not fully realized until the research of Riedmüller between 1928 and 1933 (Morgan, 1944, Laing, 1956). Kunstler described *Trichomonas* in the vagina of a cow and intestine of a pig, while Mazzanti found trichomonads in the reproductive tract of sterile cows in a slaughterhouse and described a uterus containing "fluid resembling sour milk". Riedmüller (1928) later published results describing aborted bovine fetuses containing trichomonads. In 1929, Abelein demonstrated evidence that *Trichomonas foetus* had the ability to cause reproductive disease in cattle (Morgan, 1944).

Infection with *T. foetus* results in venereal disease in cattle; trichomonads are found in the reproductive tract of the cow and prepucial cavity of the bull. A *T. foetus* infection may result in infertility, abortions and pyometra in the cow, as well as vaginitis, cervicitis, endometritis and salpingitis (Laing, 1956, Parsonson, et al., 1976, BonDurant, 1997, Felleisen, 1999). Infected bulls are usually mature, greater than three to four years of age, and are commonly asymptomatic (BonDurant, et al., 1990, Rae, et al., 1999). However, inflammation of the prepuce and glans penis (balanoposthitis) can occur since trichomonads are found in smegma within the crypts of the penile midshaft and caudal regions, and prepucial cavity of infected bulls, (Laing, 1956, Parsonson, et al., 1976, BonDurant, 1997, Felleisen, 1999, Rae, et al., 1999, Rhyan, et al., 1999).

The abortions are usually early in the pregnancy, but can be midterm or late-term, and it is unclear how they occur (BonDurant, 1997). Hypotheses include (1) the

possibility of large organism numbers in the maternal uterus, (2) surface antigens released from the trichomonad that bind to neighboring host cells, triggering opsonization and phagocytosis or antibody-dependent cell cytotoxicity (ADCC), (3) adherence to either the fetus or maternal endometrium resulting in severe cytotoxicity previously thought to not exist, and (4) trichomonad enzymes may affect surface proteins of host cells that provide communication between the fetus and maternal endometrium (BonDurant, 1997).

Trichomonads have been found in the chorionic stroma of the placentas of aborted fetuses, in addition to the alveoli and bronchioles (Rhyan, et al., 1988). Trichomonads are lumen-dwellers, actually adhering to vaginal epithelial cells (Corbeil, et al., 1989) and in order to enter the fetus they would have to cross one or more membranes. It is believed that the trichomonads are caught up in the amniotic fluid and amniotic sac during development. The fetus then ingests this fluid, allowing the trichomonads to enter areas such as the fetal stomach, intestine and lung, but the trichomonads would still have to penetrate the chorion (BonDurant, 1997). *In vitro* research using bovine oocytes, zygotes and embryos in the presence of *T. foetus* showed no effect on mobility or function of spermatozoa. Fertilization and embryonic development still occurred and there were no significant differences in the number or percentage of hatched embryos when compared to *T. foetus*-free controls (Bielanski, et al., 2004). These conclusions are supported by previous work in which virgin heifers were mated with infected bulls. This resulted in a 61% pregnancy rate, with two heifers demonstrating signs of future abortion when examined at necropsy 60 and 95 days after mating (Parsonson, et al., 1976).

However recent *in vitro* studies indicate that *T. foetus* severely damages and infiltrates the zona pellucida, reaches the oocyte and induce apoptosis (Benchimol, et al., 2007).

Many cattle infected with *T. foetus*, both bulls and cows, may be asymptomatic. Sometimes the most prominent symptom is low fertility rates or an increase in aborted fetuses. However, *T. foetus* can be diagnosed using samples from a bull, cow or even a freshly aborted fetus. Diagnosis involving bulls is accomplished by a prepucial scraping, allowing any parasites collected to proliferate at room temperature in a culture media such as the InPouch TF® culture system (Biomed Diagnostics, White City, OR) or trypticase-yeast-maltose (TYM) Diamond's medium (Diamond, 1983). After a period of 1-3 days, flagellates, if present in the original sample, will be visible under a microscope. When diagnosing trichomoniasis in cows there is usually a muco-purulent vaginal discharge which can be collected and examined for flagellates (Figure 1.2). Since trichomoniasis can also cause pyometra, the uterine discharge can also be used (Figure 1.3). Lastly, in instances where infection has spread throughout a herd and there are an increased number of aborted fetuses, necropsy of a fresh fetus can aid in diagnosis of this disease (BonDurant, 1997). Observing flagellates under the microscope will allow a morphological assessment. Once the organisms are cultured, *T. foetus* DNA can be extracted and a PCR assay can be performed to verify the cause of the infection (Grahn, et al., 2005).

There is no approved treatment for *T. foetus* in cattle. Some have suggested that imidazoles can be used; however, they are not FDA approved compounds. The best treatment is prevention through artificial insemination and herd management (Laing, 1956, BonDurant, 1997). The use of virgin bulls, maiden cows or the acquisition of

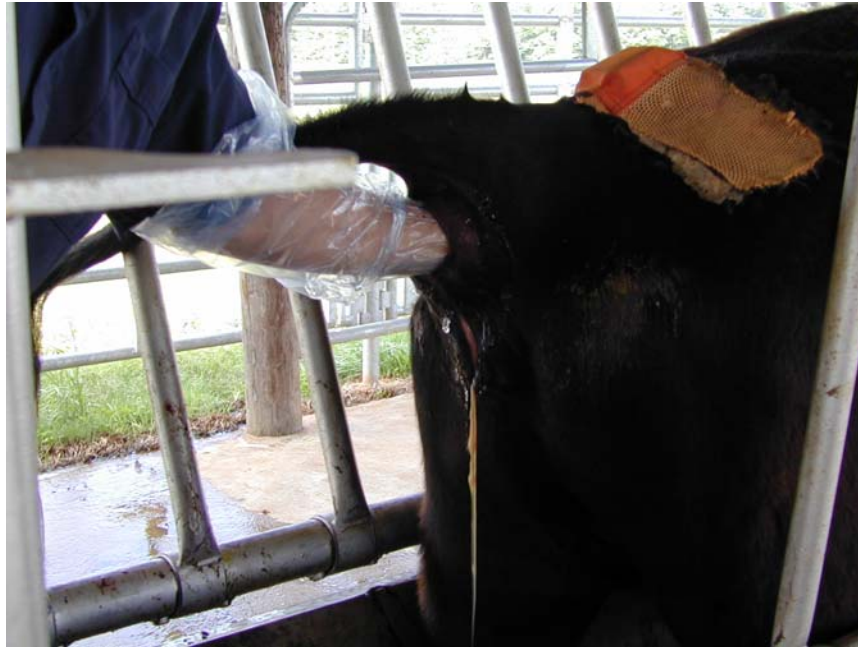


Figure 1.2. Muco-purulent vaginal discharge from a heifer experimentally infected with *Tritrichomonas foetus*. The vaginal discharge can be collected and used for diagnosis of venereal trichomoniasis. Detection of trichomonads can be accomplished by direct smear or culture and viewing organisms using light microscopy at 100X magnification.

Figure 1.3. Material collected from the uterus of a heifer experimentally infected with *Tritrichomonas foetus*. The vial on the right is the uterine contents of a negative control heifer, not infected with *T. foetus*. The vial on the left is the uterine contents of a heifer infected with *T. foetus*. The uterine material can be collected and used for diagnosis of venereal trichomoniasis. Detection of trichomonads can be accomplished by direct smear or culture and viewing organisms using light microscopy at 100X magnification.

pregnant cows can reduce the prevalence of infection (Laing, 1956). It is also important to keep herds confined as much as possible (BonDurant, 1997). A vaccine has been introduced for use in heifers (Trich Guard® V5L, Fort Dodge Laboratories, Overland Park, KS). It contains killed *T. foetus, Campylobacter* and *Leptospira* cells (BonDurant, 1997), and while it is not a cure for bovine trichomoniasis, studies indicate that it may shorten the duration of infection and decrease the severity of the disease (Kvasnicka, et al., 1999) .

Many infected bulls are lifelong carriers of the parasite, while cows may clear the infection after a few months (Skirrow and BonDurant, 1990a, Felleisen, et al., 1998). Once infected with *T. foetus*, cows develop a strong humoral immune response. After seven to nine weeks post-infection, research has shown that IgA and IgG1 levels increase in vaginal, cervical and uterine secretions (Skirrow and BonDurant, 1990b). Transient IgM responses were also detected, mainly in cervical secretions. Transient IgG2 responses were detected throughout the reproductive tract during later stages of the infection (Skirrow and BonDurant, 1990b). Research has also shown that trichomoniasis in bulls induces a humoral response. As with infected cows, the primary antibodies detected in the serum, smegma and prepucial washings of infected bulls were IgA, IgG1, IgG2 and IgM (Campero, et al., 1990, Rhyan, et al., 1999). Chronic infections in bulls are believed to result from larger trichomonad populations in the smegma verses those adhering to the surface epithelium of the penis which can be readily accessed by antibodies (Campero, et al., 1990).

It is estimated that the beef industry in the United States loses millions of dollars each year due to trichomoniasis (Fitzgerald, et al., 1958, Wilson, et al., 1979). In one

epidemiological simulation model of disease dynamics in *T. foetus* infected herds, calf revenues decreased 4-10% due to lower calf numbers and weaning weights. Additionally, cow revenues decreased 5-35% when compared to uninfected herds (Rae, 1989). Infection by *T. foetus* is predominantly found in the western United States due to large, free-roaming herds that are allowed to mate freely (BonDurant, 1997). However, studies have shown a mean prevalence of *T. foetus* infection in approximately 11.9% of bulls on beef cattle ranches in central Florida, with a prevalence of 35.9% in one of eleven ranch units (Rae, et al., 1999).

Feline Trichomoniasis

Trichomonas felis was the name given to trichomonads recovered from a South American cat in 1922 by Da Cunha and Muniz (Kessel, 1928). In 1926, Tanabe referred to these trichomonads as *Pentatrichomonas felis* since they were found in both dogs and cats (Kessel, 1928). There was debate regarding the number of anterior flagella, and appeared to be mixed populations of trichomonads with three, four or five anterior flagella (Kessel, 1928). Differences in the numbers of anterior flagella were attributed to either the loss of extra flagella, adherence to debris or obscuring of flagella by the cell body.

In 1956, Jordan published the first case report documenting *Trichomonas* spp. as a possible cause of diarrhea in cats. The cat in this case suffered from chronic diarrhea containing blood and mucus, anorexia, weight loss and lethargy. The cat died within days of admission. Years later, there were reports of *Pentatrichomonas hominis* infecting kittens and young cats. These cats presented with similar symptoms including

malodorous mucoid diarrhea and frequent defecation outside the litter box, lethargy and dyschezia (Romatowski, 1996, 2000). It was later suggested that *Tritrichomonas foetus* was the causative agent of chronic diarrhea in cats, and not *P. hominis* (Levy, et al., 2003). Light microscopy confirmed the presence of three anterior flagella and rRNA genes from both *T. foetus* and *P. hominis* were compared and found to be dissimilar.

The results indicating that *T. foetus* was the causative agent of chronic diarrhea in cats led to further investigative studies. Results of research indicated that cats infected with *T. foetus* suffered from large-bowel diarrhea that was pasty, malodorous and usually associated with blood or mucus. Incontinence, flatulence and tenesmus were also common symptoms (Gookin, et al., 1999). The diarrhea persisted from two days to three years. The average age of diagnosis was nine months, with the majority less than one year. There also appeared to be no differences in susceptibility between males or females, or pure or mixed breed cats (Gookin, et al., 1999). Additional studies found that diarrhea usually resolved within two years, but relapses were common (Foster, et al., 2004). A prevalence survey of 117 cats representing 89 catteries at an international cat show found that 31% (36/117 cats and 28/89 catteries) were positive for *T. foetus* infection (Gookin, et al., 2004). Many of the positive cats were from multi-cat households however the exact route of transmission remains unknown. The majority of positive samples were confirmed by polymerase chain reaction (PCR) of fecal specimens utilizing primers specific for rRNA (Gookin, et al., 2002, Gookin, et al., 2004). Additional methods used for *T. foetus* detection include identification of motile trophozoites by direct examination of fresh feces, culture using TYM Diamond's medium

and the InPouch TF® culture system (Biomed Diagnostics, White City, OR) (Diamond, 1983, Gookin, et al., 2003, Gookin, et al., 2004).

Treatment of feline trichomoniasis is problematic and often unsuccessful. One of the initial treatments attempted was 0.5% carbarsone in a 2.5% sodium bicarbonate solution (Jordan, 1956). In this case trichomonads were no longer detected in feces, but hemorrhagic diarrhea developed followed, by death only days after treatment. Other attempted treatments with limited success include metronidazole alone or in combination with enrofloxacin, resulting in resolution of diarrhea and trichomonads only to be followed by relapse (Romatowski, 1996, 2000). Treatment with fenbendazole, furazolidone and metronidazole was found to control bacterial growth in the colon however trichomonads remained detectable in feces (Gookin, et al., 1999). Treatments which utilized prescription diets or home remedies including plain turkey and rice were unsuccessful. Supplements including yogurt, slippery elm, pumpkin, or glutamine, frequent bathing and litter box changes also did not resolve diarrhea (Foster, et al., 2004).

Some treatment regimes have demonstrated efficacy against feline trichomoniasis. Metronidazole, fenbendazole and enrofloxacin used in combination has been shown to resolve diarrhea and trichomonad infection in several cases without relapse (Stockdale, et al., 2006). Ronidazole has shown greater efficacy than metronidazole for treatment of feline trichomoniasis, effectively eradicating trichomonads and resolving diarrhea without relapse (Gookin, et al., 2006). The results of ronidazole were dosage dependent, and cats receiving the lowest concentration of drug relapsed within 20 weeks (Gookin, et al., 2006). Tinidazole has shown some efficacy against feline trichomoniasis, decreasing detection of *T. foetus* in fecal samples and increasing resistance to later infections in

experimentally infected cats. However, tinidazole was not able to eradicate *T. foetus* infections in all cats (Gookin, et al., 2007).

Research Objectives

There is still little known regarding *T. foetus* infection in cats. Is this organism identical to *T. foetus* found in the reproductive tract of cattle? If so, how is does it gain entry into and survive the large intestine of a cat? The reproductive tract and large intestine are two distinctly different environments and the ability to thrive in both would require unique adaptive mechanisms. In addition to extensive characterization of the trichomonads recovered from the feces of naturally infected cats, it is necessary to conduct cross-transmission studies to determine host susceptibilities using trichomonad organisms isolated from both cattle and felids.

It also remains unclear whether bacteria or other flora/fauna within the large intestine contribute to the ability of *T. foetus* to establish infection or induce diarrhea in cats, or whether concurrent infections with other parasites or infectious agents such as feline immunodeficiency virus (FIV) or feline leukemia virus (FeLV) could enhance susceptibility to *T. foetus* infection. There is an additional possibility that certain breed susceptibilities to *T. foetus* infection exist in the feline population. Many reported clinical cases of feline trichomoniasis include Pixie-bob tails, Siamese, and Russian blue breeds (Romatowski, 1996, Felleisen, 1999, Lappin, 2000). However, infections also were reported from domestic long-hair and domestic short-hair breeds. The cases documented to date appear to represent an equal mix of males and females, as well.

The objectives of this research are to first, obtain information on the occurrence of *T. foetus* in the pet cat population via arbitrary fecal samples obtained by practicing

veterinarians from client-owned cats in different geographic regions in the United States. Second, characterize the comparative infectivity and pathogenicity of bovine and feline isolates of *T. foetus* in virgin heifers. Third, determine the infectivity of a bovine isolate of *T. foetus* in cats and fourth, compare genetic and morphologic characteristics of bovine and feline isolates of *T. foetus* using light microscopy, electron microscopy and gene sequencing methodologies.

CHAPTER II.

TRITRICHOMONAS FOETUS INFECTIONS IN SURVEYED PET CATS

Introduction

Feline trichomoniasis is a large-bowel disease in cats thought to be caused by *Tritrichomonas foetus* (Gookin, et al., 2001, Levy, et al., 2003, Foster, et al., 2004). Reported clinical signs of trichomoniasis may include chronic diarrhea associated with blood or mucus, flatulence, tenesmus and anal irritation (Gookin, et al., 1999, Foster, et al., 2004, Stockdale, et al., 2006). Since 1996, reports of feline trichomoniasis have increased dramatically. The increase in diagnosis is most likely due to increased awareness and improved diagnostic techniques. *Tritrichomonas foetus* is often misdiagnosed as *Giardia* spp. (Gookin, et al., 2004) or *Pentatrichomonas hominis* (Romatowski, 1996, 2000, Levy, et al., 2003) or underdiagnosed in an outdoor cat whose daily bowel movements are usually not observed by the owner.

A prevalence study of purebred cats at an international cat show revealed that 31% of 117 surveyed cats and 89 catteries sampled were positive for *T. foetus* infection (Gookin, et al., 2004). However this represented only 12% of total cats and 16% of total catteries present at the cat show. Among the cats testing positive for *T. foetus*, only 24 had reported loose stools or diarrhea and 36 were co-infected with *Giardia* spp. or coccidia (5). Specific breeds for each positive cat were not given.

Statistics reported by the 2007-2008 National Pet Owners Survey (APPMA, 2008) indicate that there are over 90 million pet cats in American households (HSUS, 2008). These pet cats include both pure and mixed breeds living in multi- or single cat households. These cats can also harbor additional pathogens that may cause or contribute to large-bowel disease. The goal of this survey was to estimate the prevalence of *T. foetus* in pet cats representing three geographic regions of the United States. Additionally, the prevalence of cats concurrently infected with other pathogens will also be noted along with presence of reported clinical signs of feline trichomoniasis.

Materials and Methods

Study Population

Cats were chosen arbitrarily by participating veterinarians who were instructed to choose cats as they were presented to the clinic and not based on presence or absence of disease however there is the possibility of inherent bias in the selection process. Selected cats presented with or without signs of trichomoniasis. The study population included male and female cats, purebred and mixed breed varieties, and comprised all age groups.

Sample Collection

Fresh fecal samples were taken from each cat directly from the litter box or by use of a fecal loop. Samples were added to the InPouch TF® culture system (BioMed Diagnostics, White City, OR) and shipped overnight to the Auburn University College of Veterinary Medicine. The following information was obtained from each cat when possible: age, breed, sex, geographic location, origin of acquisition by owner (i.e. shelter, stray, cattery), concurrent infections and past treatments. Veterinarians were asked to score fecal consistency using a scale of 0, 33, 66, and 100 as follows: 0=diarrhea, watery,

loose or possibly blood; 33= no form, loose, puddles or piles; 66= formed, soft or wet; 100= formed and hard (Purina® Fecal Scoring System for Cats, Nestlé Purina PetCare Co., St. Louis, MO) (Figure 2.1). Some positive cats were successfully treated with fenbendazole 50mg/kg PO q24h for 5 days, metronidazole 75mg PO q24h for 10 days and enrofloxacin 5mg PO q24h for 21 days, taken concurrently (Stockdale, et al., 2006), or ronidazole 30-50mg/kg PO q12h for 14 days (Gookin, et al., 2006). When possible, veterinarians were contacted for a follow-up sample after completion of the selected treatment regimen.

Sample analysis

Fecal specimens were examined immediately upon arrival at the Auburn University College of Veterinary Medicine. The contents of the InPouch TF® were examined by light microscopy at 100X magnification and cultured at 37°C in trypticase-yeast-maltose (TYM) media without agar (Diamond, 1983). Cultured samples were evaluated two days after incubation at 37°C for the presence of motile trophozoites. Negative cultures were kept for 10 days, and reevaluated every two days. Positive cultures were verified by polymerase chain reaction (PCR) (Grahn, et al., 2005) using DNA that was extracted from each sample (Billeter, et al., 2007). Conserved 5.8S rRNA gene sequences and internal transcribed spacer (ITS) regions were used to verify the presence of *T. foetus*. This PCR procedure utilized the primers TFR-3 and TFR-4 (Felleisen, et al., 1998, Grahn, et al., 2005). Amplified PCR gene sequences from each positive sample were compared against other acquired positive samples of both bovine and feline origin.

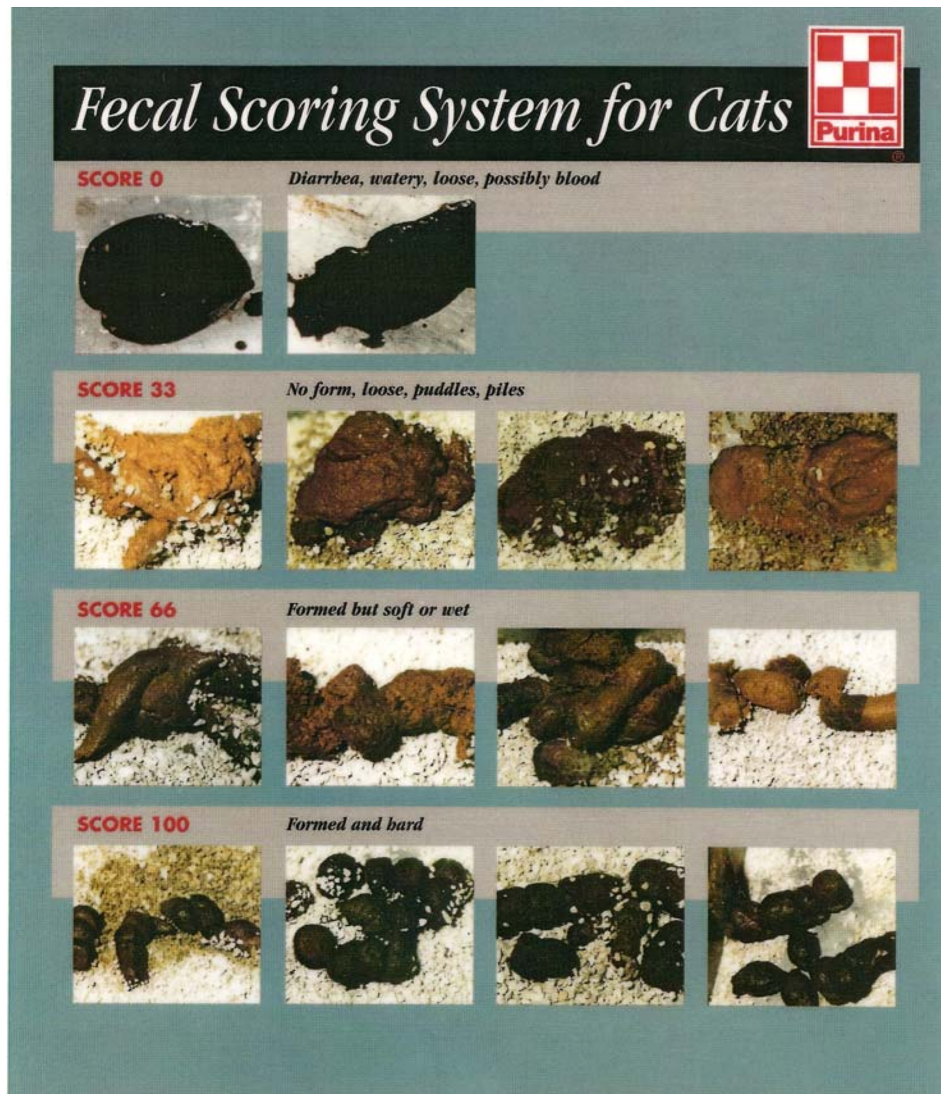


Figure 2.1. Fecal scoring system used to determine consistency of stool samples acquired from surveyed cats. Veterinarians were advised to use this as their evaluation guide when needed.

The analysis included gene sequences from feline isolates collected from naturally infected cats, (accession numbers EU569301-EU569312), and gene sequences in GenBank from trichomonads isolated from felines (accession number AF466750) (Levy, et al., 2003) and bovines (accession number AF339736) (Walker, et al., 2003). Additionally, the analysis included trichomonad DNA sequences isolated from porcine, *Tritrichomonas suis* (accession number AY349190) (Kleina, et al., 2004), a lizard, *Anolis bartschi, Tritrichomonas nonconforma* (accession number AY886845) and a tree shrew, *Tupaia belangeri*, *Tritrichomonas mobilensis* (accession number AY886842) (Cepicka, et al., 2006)

Results

One hundred seventy-three feline fecal samples were submitted for examination from veterinarians throughout the United States. Total samples included pure (32) and mixed breed (141) cats. Samples were received from Alabama (69), California (10), Delaware (4), Florida (2), Georgia (2), Hawaii (14), Indiana (1), Kentucky (20), Louisiana (10), Missouri (1), New Jersey (1), North Carolina (22), Ohio (2), Tennessee (13), Texas (1) and Virginia (1). Seventeen of the 173 (10%) samples were positive for *T. foetus* using both culture and PCR procedures (Tables 2.1 and 2.2). Positive cats were between the ages of 6 weeks and 12 years and were reported to have chronic diarrhea, with blood or mucus. Positive surveyed cats were pure (12) and mixed breed (5), male (10) and female (7). Positive pure bred cats included Siamese (1), Russian Blue (1), Bengal (1), Japanese Bobtail (1), Scottish Fold (1), Toyger (5), Himalayan-x (1) and Abyssinian (1). Several cats were infected concurrently with *Giardia* spp. (5),

Table 2.1. Signalment and treatment outcomes for cats positive for *Tritrichomonas foetus*.

Isolate	Age	Sex	Breed	Location	Treatment*	Outcome	Concurrent Infection
1	11mo	M	Siamese	Alabama	met, fen, enro	clear; *T. foetus* culture (-)	ringworm
2	2yo	M	Russian Blue	Alabama	met, fen, enro	*T. foetus* culture (-); diarrhea	*Cryptosporidium* spp.
3	17wo	F	Bengal	Alabama	met, fen, enro, ron	euthanized	FIP
4	2yo	F	Japanese Bobtail	North Carolina	met, fen, enro	clear; *T. foetus* culture (-)	
5	6wo	M	DSH	Hawaii	ron	clear; *T. foetus* culture (-)	*Giardia* spp., ringworm
6	7wo	M	DSH	Hawaii	ron	clear; *T. foetus* culture (-)	*Giardia* spp., ringworm
7	7wo	M	DSH	Hawaii	ron	clear; *T. foetus* culture (-)	*Giardia* spp., ringworm
8	12wo	M	Scottish fold	California	ron	clear; *T. foetus* culture (-)	
9	7mo	M	Toyger	California	unknown	unknown	
10	1.5yo	F	Toyger	California	unknown	unknown	
11	7mo	F	Toyger	California	unknown	unknown	
12	8mo	M	Toyger	California	unknown	unknown	
13	10mo	M	Toyger	Texas	met, fen, enro, ron	unknown	*Isospora* spp.
14	9mo	F	Himalayan –X	Alabama	met, fen, enro	clear; *T. foetus* culture (-)	*Giardia* spp.
15	11mo	F	DSH	Tennessee	met, fen, enro	clear	*Giardia* spp.
16	4yo	M	DMH	Ohio	unable to treat	euthanized	coccidia
17	12yo	F	Abyssinian	New Jersey	met, fen, enro	unknown	

*Treatment abbreviations: met = metronidazole, fen = fenbendazole, enro = enrofloxacin, ron = ronidazole (Stockdale et al., 2006; Gookin et al., 2006)

Table 2.2 Numerical breakdown of survey results collected from pet cats by participating veterinarians. Population percentage is also given. Complete results are given in Appendix A.

Total cats surveyed	173	
Total cats with diarrhea*	77	45%
culture positive	17	10%
culture negative	60	35%
Total cats without diarrhea*	69	40%
no information available (NIA)	27	15%
culture positive	0	0%
culture negative	96	55%
Total pure bred cats	30	17%
pure bred cross	2	
culture positive	12	38%
culture negative	20	62%
Total mixed breed cats	141	82%
culture positive	5	4%
culture negative	136	96%
Total culture positive cats		
with concurrent infection	9	53%
Giardia	5	
Cryptosporidium	1	
coccidia	2	
ringworm	4	
FIP	1	
without concurrent infection	7	41%

Age range culture positive cats 6 weeks old – 12 years old
Age range culture negative cats 4 weeks old – 13 years old

*If only the fecal scoring system was used in stool evaluation, out of 0, 33, 66 or 100, 0-33 was considered diarrhea; 66-100 was considered normal stool (Figure 2.1).

Cryptosporidium spp. (1), coccidia (2) or dermatophytes (4) or also diagnosed with Feline Infectious Peritonitis (FIP) (1) (Tables 2.1 and 2.2).

Sixty surveyed cats were culture negative and reported to have diarrhea. The remaining 96 surveyed cats that were culture negative had no reports of diarrhea (69) or no information was given (27). Negative cats were between the ages of 4 weeks and 13 years old, representing both pure (20) and mixed (136) breeds (Table 2.2). Negative pure bred cats included Himalayan (2), Himalayan-x (1), Ragdoll (1), Persian (7), Siamese (2), Toyger (3), Scottish Fold (3) and Bengal (2). Negative surveyed cats were also reported to be diagnosed with diabetes (3), gingivitis (1), tapeworms (3), allergies (1), roundworm (4), upper respiratory infection (2), coccidia (7), FIV (1), *Giardia* spp. (3) and fleas (1) at the time of fecal collection (Appendix A).

Eight of the 17 positive surveyed cats cleared the trichomonad infection concurrent with resolution of diarrhea. Four of these cats were treated with a combination of enrofloxacin (5 mg PO q24h for 21 days), metronidazole (75 mg PO q24h for 10 days) and fenbendazole (50 mg/kg PO q24h for 5 days) (Stockdale, et al., 2006), and four cats were treated with ronidazole (30-50 mg BID for 14 days) (Gookin, et al., 2006). One cat was *T. foetus* negative after treatment and *Cryptosporidium* spp. positive but did not resolve diarrhea. Two cats were euthanized, one due to an additional FIP diagnosis and one due to an inability to administer treatment. The outcome of six cats is unknown (Table 2.1).

Of the 17 positive surveyed cats, genetic DNA was successfully extracted from 12 fecal samples (GenBank accession numbers EU569301-EU569312) collected at Auburn University. The PCR products amplified from conserved 5.8S rRNA, ITS-1 and ITS-2

genes were compared to similar known trichomonad sequences in GenBank. These comparisons showed 97-100% sequence identity between the feline trichomonad isolates collected at Auburn University and published bovine and feline *T. foetus* isolates (AF339736 and AF466750, respectively). Further genetic sequence analyses between these feline trichomonad isolates and various bovine, feline, porcine and reptilian trichomonads are described in detail in Chapter V.

Discussion

This survey revealed that 10% (17/173) of the surveyed feline pet population was positive for *T. foetus.* Of the 17 positive cats, 12 were pure bred and included Siamese (1), Russian Blue (1), Bengal (1), Japanese bobtail (1), Scottish fold (1), Toyger (5), Himalayan-x (1) and Abyssinian (1). This prevalence rate is lower than the prevalence rate reported by Gookin et al., 2004 at an international cat show, 31%. However, cats in the Gookin et al. study were all purebred representing only 12% of total cats and 16% of total catteries present at the cat show. Among the 36 cats testing positive for *T. foetus* at the international cat show, only 24 had reported loose stools or diarrhea and 14 were also co-infected with *Giardia* spp. or coccidia (5) (Gookin, et al., 2004). All 17 of our positive cats presented with chronic diarrhea, but not always associated with blood or mucus (Appendix A). Many of the cats surveyed by Gookin et al. were kept in densely populated catteries (1-59 cats/household) and of 89 catteries sampled, 62 reported cats with loose stool while only 28 were *T. foetus* positive. Another survey conducted in the United Kingdom reported 16 out of 111 cats positive for *T. foetus*, 14 of which were pure bred. Breeds included Siamese (6), Bengal (6), Ragdoll (1) and Persian (1) (Gunn-Moore, et al., 2007). Additionally, a survey conducted in the United States found that of

32 cats presenting with diarrhea, 12 were pure bred and included Abyssinian (7), Bengal (2), Persian (1), Tokinese (1) and Pixie Bob (1) (Gookin, et al., 1999). It is evident that feline trichomoniasis is not a disease of only pedigreed cats however the possibility that these cats are at greater risk has not been ruled out.

The infected cats in our survey displayed some common signs of trichomoniasis, including chronic large-bowel diarrhea associated with blood or mucus, vomiting and weight loss. Concurrent infections in our surveyed cats infected with *T. foetus* reported by the veterinarian included *Cryptosporidium* spp (1), *Giardia* spp. (5), *Isospora* spp. (1), coccidia (1), dermatophytes (ringworm) (4) and FIP (1). The question still remains as to whether *T. foetus* is the single cause of large-bowel diarrhea in infected cats or if a concurrent infection or impaired immune system leave younger cats more vulnerable to a potentially opportunistic parasite. In our survey, of the 17 positive cats 10 had additional pathogens or disease present. Studies investigating the pathogenicity and infectivity of *T. foetus* in cats have observed concurrent infection or disease when *T. foetus* is present. In one study, 32 cats presented with diarrhea associated with blood or mucus and 8 had coexisting enteric infections. Two were diagnosed with severe or mild lymphoplasmacytic colitis, typhlitis and enteritis, additional infections included *Isospora* spp. (3), *Toxascaris leonina* (1), *Giardia* spp. (1) and *Giardia* spp. and *Cryptosporidium* spp. (1) (Gookin, et al., 1999).

In an experimental infection study, eight cats were inoculated with a feline isolate of *T. foetus* (Gookin, et al., 2001). Four cats were already infected with *Cryptosporidium* spp. and four were specific pathogen-free (SPF). The investigators reported increased frequency and intensity of diarrhea in *Cryptosporidium* spp. positive cats with stool

samples ranging from semi-formed (score, 2) to cow-pat or liquid (score, 3 and 4). The SPF cats were reported to have stool samples ranging from formed (score, 1) to semi-formed (score, 2) and were labeled diarrhea. Comparing this fecal scoring system to the one used in our survey, samples that were formed (score, 100) to semi-formed (score, 66) were considered normal since stress or small dietary changes could cause softer stool, while samples that were cow-pat (score, 33) to liquid (score, 0) were considered to be diarrhea. Differences in stool consistency can differ between individuals' opinions or between score systems utilized in the experiments. With this in mind, the experimental infection of SPF cats with *T. foetus* demonstrated disease that was significantly less severe than that exhibited by cats concurrently infected with *Cryptosporidium*. According to our scoring system, SPF cats exhibiting semi-formed stools would have been considered normal. The authors report no increase in *Cryptosporidium* oocysts shed in feces and that cats had normal stools before infection with *T. foetus*. Trichomonads were also recovered from feces at an earlier time point and from more cats in those concurrently infected with *Cryptosporidium* spp. (3 of 4 by post-inoculation day 4) than the SPF cats (2 of 4 by post-inoculation day 7). Although the authors state the addition of *T. foetus* did not contribute to symptoms of cryptosporidiosis, differences in experimental outcomes between the two groups suggest *T. foetus* alone may not be sufficient to cause chronic diarrhea with the close association of blood or mucus. Again, differences in opinion regarding fecal consistency can sway results.

The average age of the infected cats in this study (12 months) was similar to the average age (9 months) of infected cats previously reported (Gookin, et al., 1999). Studies to date support that feline trichomoniasis appears to be a disease of younger cats

and kittens, with the rare exception of a cat older than six years (Gookin, et al., 1999, Foster, et al., 2004, Stockdale, et al., 2006). Infection with *T. foetus* and resulting symptoms have been shown to resolve after two years in some cats (Foster, et al., 2004). At present, there is no approved treatment for feline trichomoniasis and responses to therapies mentioned previously may vary. Many cats infected with *T. foetus* may resolve diarrhea only to relapse weeks or months later (Foster, et al., 2004). Consequently, elimination of the parasite is problematic for those cats that do not spontaneously recover. The method(s) of transmission of *T. foetus* infection in cats remains unknown. It is unclear whether it is transmitted through shared litter boxes or between queens and kittens. It also remains unclear whether *T. foetus* is the only pathogen causing disease in these cats or playing the role of opportunist in cats on the fringe of disease. The methodologies used in this study may leave room for inherent bias however does give limited insight into the prevalence of *T. foetus* in the pet cat population.

CHAPTER III.

EXPERIMENTAL INFECTION OF CATTLE WITH A FELINE ISOLATE OF *TRITRICHOMONAS FOETUS*

Introduction

Tritrichomonas foetus is a flagellated, protozoan parasite that causes bovine trichomoniasis, a venereal disease resulting in infertility and abortion, pyometra, endometritis, vaginitis, and cervicitis in infected cows. In bovines, *T. foetus* inhabits the vagina, cervix, and uterus of cows and the prepucial cavity of bulls (BonDurant, 1985, 1997, Felleisen, 1999). *Tritrichomonas foetus* appears to be a cosmopolitan organism. Experimental data demonstrate that *T. foetus* infections can be sustained in mice for at least 26 wk (Hook, et al., 1995) and may be a causative agent of diarrhea in dogs (Gookin et al., 2005). There has been an attempt to synonymize *T. foetus* and *Tritrichomonas suis*, a gastrointestinal trichomonad of pigs (Tachezy et al., 2002). One case report described trichomonad organisms resembling *T. foetus* in the cerebral spinal fluid of a human patient that underwent a peripheral blood stem cell transplant (Okamoto et al., 1998). Numerous reports have appeared recently in the scientific literature in which a species of *Tritrichomonas* was reported as a cause of large bowel diarrhea in cats (Stockdale et al., 2006). Structural, morphological, and molecular data suggest the causative agent is *T. foetus* (Gookin et al., 2002; Levy et al., 2003; Yeager and Gookin, 2005). Cats infected with *T. foetus* may present with flatulence, tenesmus, and chronic

diarrhea that may contain blood or mucus (Gookin et al., 1999; Gookin et al., 2001; Foster et al., 2004). These cats also come from a variety of backgrounds, which may or may not include contact with cattle (Gookin et al., 1999; Gookin et al., 2004; Stockdale et al., 2006). *Tritrichomonas foetus* is found in the large intestine of cats, commonly along the mucosal surface of the colon; it may also be found in the ileum and cecum (Gookin et al., 2001, Yeager and Gookin, 2005). Determination of the species of *Tritrichomonas* responsible for diarrhea in cats may help clarify host susceptibilities and routes of transmission for parasites within this genus. This research reports the results of experimental infection of heifers with *T. foetus* obtained from naturally infected cats. We also attempted to infect a cohort of heifers with *T. foetus* obtained from naturally infected cattle to serve as a positive control. Results from this study may help support or refute the synonymy of species infecting the 2 different hosts.

Materials and Methods

Heifers and estrus synchronization

Twenty virgin Angus heifers, ranging in age from 15- to 29-mo-old, were obtained from a reproductive herd maintained at the Auburn University College of Veterinary Medicine. These heifers were randomly divided into 2 groups of 10, and a vaginal sample was taken from each heifer and cultured to ensure no prior infection with *T. foetus*. The average age of heifers in the first group was 19.8 mo; heifers in this group were inoculated with a bovine isolate of *T. foetus* obtained from a naturally infected cow. The average age of heifers in the second group was 23 mo; heifers in this group were inoculated with a feline isolate of *T. foetus* obtained from a naturally infected cat. The group receiving the bovine isolate was inoculated and monitored first. After sampling

completion, the group receiving the feline isolate was inoculated and monitored. These were not concurrent experiments. Infections with the bovine isolate were conducted April 2006 thru September 2006, and infections with the feline isolate were conducted October 2006 thru April 2007.

To improve the chances of successful infection, inoculations with *T. foetus* were carried out during estrus, which was synchronized among heifers in each group to mimic natural infection during coitus (Skirrow and BonDurant, 1990a, BonDurant, 1997). Estrus synchronization was achieved using an EAZI- BREED™ CIDR® (controlled vaginal drug release) insert (Pfizer, InterAg, Hamilton, New Zealand), delivering 1.38 g of progesterone over a **7-**day period. At the time of progesterone device insertion, each heifer also received 2 ml (100 µg) of Cystorelin® (gonadorelin diacetate tetrahydrate) (Merial, Duluth, Georgia) by intramuscular injection with an 18-gauge, 40-mm needle. After 7 days, the CIDR inserts were removed and the heifers were given 5 ml (25 mg) of Lutalyse® (dinoprost tromethamine) (Pfizer, Pharmacia & Upjohn Co., New York, New York) by intramuscular injection with an 18-gauge, 40-mm needle. Time of estrus for each heifer was determined using the HeatWatch® Estrus Detection System (CowChips, LCC, Denver, Colorado). The radio frequency transmitters for HeatWatch® were placed in nylon patches and glued to the caudal dorsal midline of each heifer on day 7 using cattle backtag cement (H.B. Fuller Company, St. Paul, Minnesota) as described in the HeatWatch® user's manual. The system data, i.e., mounting frequency, was evaluated during the first 5 days to determine inoculation times for each heifer with *T. foetus*. When estrus was confirmed, 8 heifers in each group of 10 were inoculated with 10^6 *T. foetus* organisms of either bovine or feline origin. The 2 remaining heifers in each group

were used as negative controls and were inoculated with sterile, parasite-free phosphate buffered saline solution (PBSS).

Organisms and inoculation

A previously cloned (Skirrow and BonDurant, 1990a) bovine isolate of *T. foetus* (D-1) was originally cultured from a naturally infected cow. A feline isolate of *T. foetus* (AUTf-1) was obtained from the feces of a naturally infected cat that presented with diarrhea to the Auburn University College of Veterinary Medicine (Stockdale, et al., 2006). The feline isolate was cultured in Trypticase-Yeast Extract-Maltose Medium (TYM) without agar (Diamond, 1983). A single cell clone of the feline isolate was obtained by limiting dilution and both the feline and bovine isolates were stored in freezing media in liquid nitrogen until 1 wk prior to infection. The freezing media was made under sterile conditions using FCS, DMSO and TYM at 3:2:5, respectively. Immediately prior to inoculation, each isolate was thawed and maintained in TYM at 37° C. Isolates were washed and suspended in sterile PBSS at a concentration of 10^6 trichomonads/ml. Approximately 1 ml of this suspension was inoculated into the cranial lumen of the vagina of 8 of the 10 heifers in the group using a 52.5-cm infusion pipette equipped with a flex adaptor (Continental® Plastic Co., Delavan, Wisconsin). The 2 remaining heifers in each group were inoculated with 1 ml of sterile, parasite-free PBSS.

Sampling procedures

At 24 hr post-inoculation (PI), samples were taken from the cranial vagina and cervix of each heifer. One control heifer was sampled first to assure the absence of cross-infections between the heifers. The second control heifer was randomly placed among the remaining 8 infected heifers to assure trichomonads were not transmitted by our

sampling techniques. Vaginal mucus samples were collected from each heifer using a sterile 52.5-cm infusion pipette with a flex adaptor and 20-ml syringe. Cervical mucus samples were collected using a sterile, stainless steel ½ ml French-style straw gun (Nasco, Fort Atkinson, Wisconsin) covered by a plastic French-style sheath (Nasco), further covered by a plastic 52.5-cm oversleeve sanitary chemise (Agtech, Inc., Manhattan, Kansas) to prevent contamination with vaginal contents. Cervical mucus was aspirated using a 20-ml syringe. Vaginal and cervical mucus samples were each suspended in 2-ml TYM. Vaginal and cervical mucus samples were collected daily for 7 days, and then bi-weekly for 10 wk. Beginning 1 wk PI, samples were collected from one uterine horn of each heifer once weekly for 10 wk. Prior to uterine sampling, each heifer was administered a low caudal epidural of 2% lidocaine HCl (0.5 ml/45.5 kg) (Hospira, Inc., Lake Forest, Illinois) using an 18-gauge, 35-mm needle to facilitate sample collection. Also prior to uterine sampling, the perineal region was washed 3 times with water and T-Scrub© povidone-iodine cleansing solution (Thatcher Co., Salt Lake City, Utah). A sterile 14-, 16-, 18-, or 20-F catheter was passed through the cervical canal and into the uterine horn. Uterine mucus samples were collected by flushing approximately 20 ml of sterile PBSS into and out of the uterine horn. Samples were evaluated by culture as described below. All heifers were sampled until trichomonads were no longer recovered from cultured samples.

During wk 6 PI, a transcervical uterine biopsy was performed and endometrial samples were obtained from each heifer after administering low caudal epidural anesthesia as previously described. This procedure was performed using Jackson uterine

biopsy forceps. The endometrial biopsy samples were fixed in 10% neutral buffered formalin and processed using routine histologic techniques.

Sample evaluation

Vaginal and cervical samples were returned to the laboratory and maintained in TYM at 37° C for 1 wk. Uterine samples (2 ml) were resuspended in TYM and maintained at 37° C for 1 wk. All samples were examined after 2 days in culture using standard light microscopy at 100**x** magnification. Samples were designated as positive (+) or negative (-) based on the presence of motile *T. foetus* trophozoites. All samples were discarded after *T. foetus* was observed or after 2 consecutive negative examinations.

Biopsy specimens were scored in a blinded fashion for periglandular and interstitial inflammation, the presence of an intact epithelium, and endometrial edema. Inflammation was characterized based on the presence of neutrophils, lymphocytes, plasma cells, macrophages, and eosinophils, and their distribution, along with edema, throughout the sample. Each specimen was scored using a scale of increasing severity: 1=minimal, 2=slight, 3=moderate, 4=marked, 5=severe, extensive. Surface epithelium and uterine glands were noted as present (P) or absent (A). Overall scores were calculated by taking the product of a leukocyte score and the distribution score, followed by addition of the edema score.

Statistical analysis

All statistical analyses were performed using SAS® Statistical Software (SAS Institute, Inc., Cary, North Carolina). Statistical test results with a *P*-value less than 0.05 were reported as significant. Two-sample *t*-tests were used to analyze total vaginal and cervical positives between each experimental group during the first wk of sampling, as

well as total days vaginal, cervical, and uterine positives during sampling wk 1 through 10. This test was also used to compare inflammatory scores between the 2 groups. A log-rank test, using the Lifetest procedure in SAS®, was used to analyze the time until a positive uterine sample was detected. Fisher's exact tests, using the Freq procedure in SAS®, were used to compare the numbers of heifers of each group retaining an intact surface epithelium and the numbers of each group that had cleared the trichomonad infection by wk 19 PI.

Results

Trichomonads recovered from samples

Motile trichomonads were detected in cultures of vaginal and cervical mucus from heifers infected with both bovine D-1 (Fig. 3.1) and feline AUTf-1 (Fig. 3.2) *T. foetus* isolates. We observed both vaginal and cervical positive cultures in both groups beginning from day 1 PI through day 7 PI. The mean numbers of vaginal positive and cervical positive days were 4.5 and 3.9**,** respectively, for heifers inoculated with the bovine isolate of *T. foetus*, and 4.3 and 3.0 respectively, for heifers inoculated with the feline isolate of *T. foetus*. These means were not statistically significantly different between the groups infected with bovine or feline *T. foetus* isolates with respect to the number of vaginal (P=0.587) or cervically positive (P=0.213) days during the first wk. During the remaining 10 wk of sampling, motile trichomonads were detected in cultures of vaginal and cervical mucus in 7 of 8 inoculated heifers from both groups (Figs. 3.1, 3.2). There were no statistically significant differences in the total number of vaginal days positive (P=0.3495) or cervical days positive (P=0.4313) for infected heifers

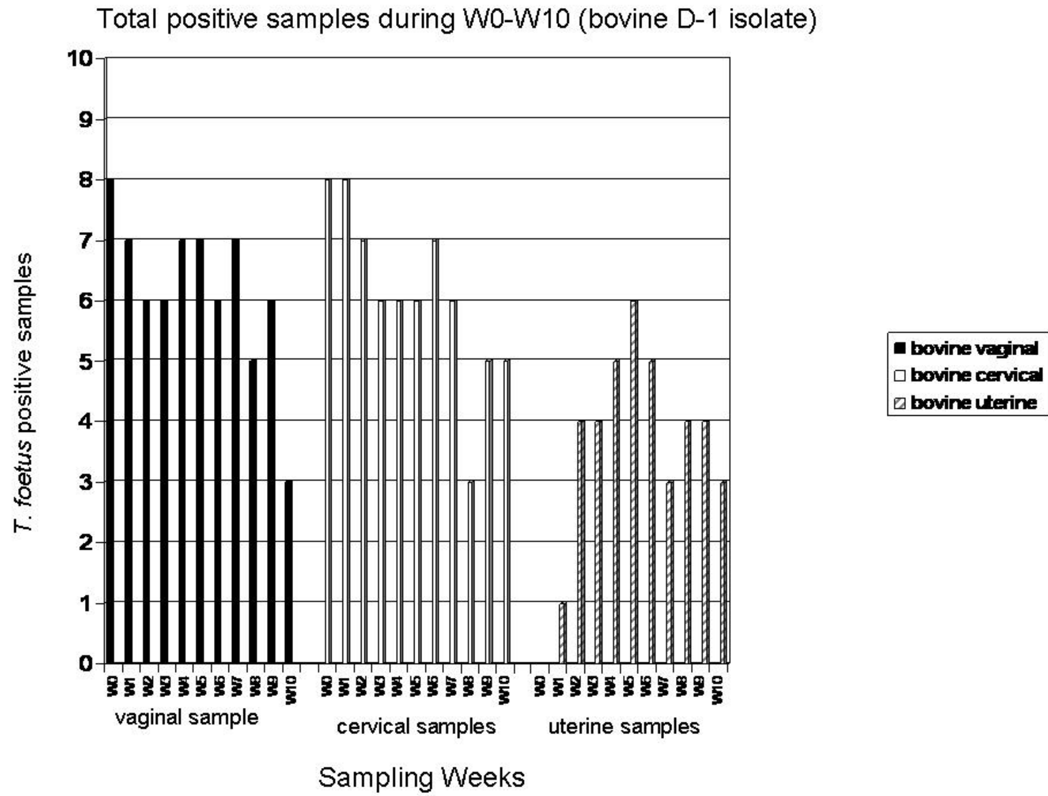


Figure 3.1. Cumulative weekly vaginal, cervical and uterine positive cultures taken from heifers inoculated with the bovine isolate of *Tritrichomonas foetus*. Sampling was performed daily during the initial week (W0) and bi-weekly for the remaining ten weeks (W1-W10).

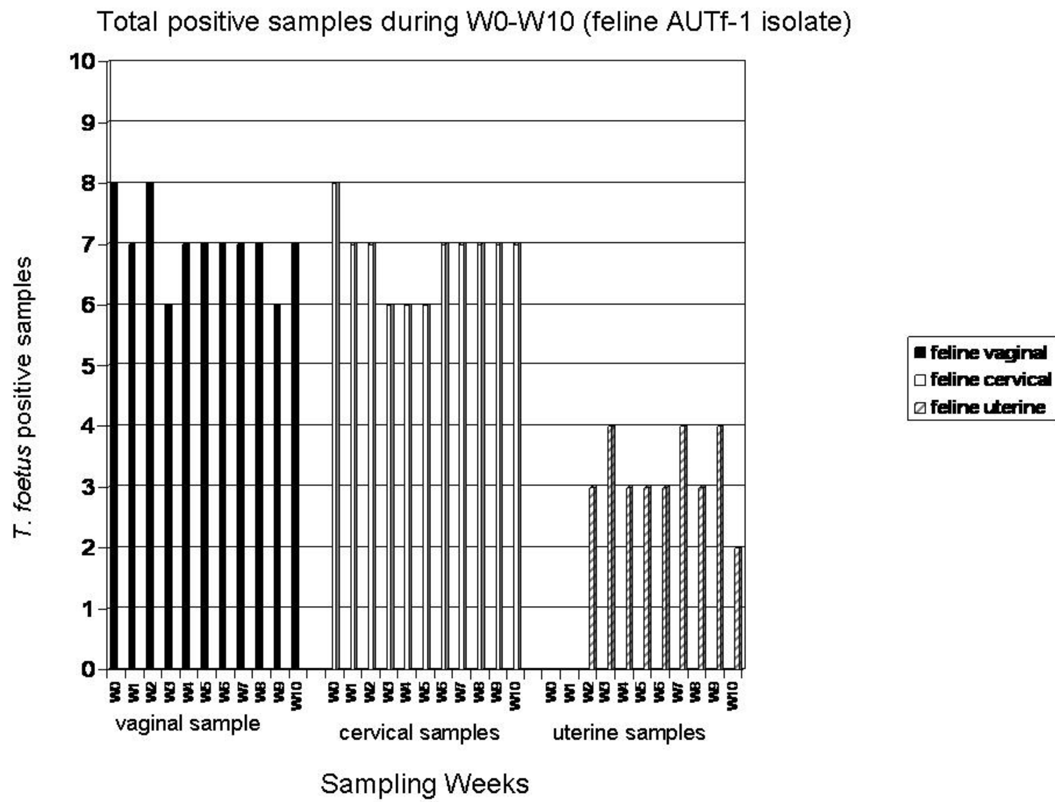


Figure 3.2. Cumulative weekly vaginal, cervical and uterine positive cultures taken from heifers inoculated with the feline isolate of *Tritrichomonas foetus*. Sampling was performed daily during the initial week (W0) and bi-weekly for the remaining ten weeks (W1-W10).

between each group during the 10 sampling weeks. A thick, yellow vaginal discharge was observed in many of the infected heifers. Heifers from each group had uterine samples positive for motile trichomonads. Positive uterine samples also contained a thick, yellow muco-purulent exudate. Uterine samples were positive in 7 of 8 heifers inoculated with the bovine *T. foetus* isolate at 1, or more, collection points during the 10 wk sampling period (Fig. 3.1), while uterine samples were positive in 5 of 8 heifers inoculated with the feline *T. foetus* isolate (Fig. 3.2). There were no statistically significant differences in the number of uterine positive heifers between each group (P= 0.4980). The time until development of a uterine infection in heifers inoculated with the bovine isolate ranged from 1 wk PI to 6 wk PI, while heifers inoculated with the feline isolate ranged from 2 to 3 wk PI. There were no statistically significant differences in time required to establish a uterine infection between the 2 groups (P=0.385). The mean time to detection of positive uterine samples was 3 wk PI for both groups.

Uterine biopsies

Examination of endometrial biopsies revealed endometritis, with both periglandular and interstitial inflammation, in heifers from each infection group. A mixed infiltrate of inflammatory cells was present, along with varying degrees of edema and decreased glandular density. Heifers infected with the bovine *T. foetus* isolate demonstrated more variability in severity of endometritis than those infected with the feline *T. foetus* isolate with respect to periglandular (P=0.004) and interstitial inflammation (P=0.009). This was evident in calculated histologic scores of each uterine biopsy sample (Table 3.1). Overall mean histological scores for heifers infected with the

Table 3.1. Inflammatory response of *Tritrichomonas foetus* infection determined from transcervical, uterine biopsies of endometrial tissue. Uterine samples were taken from heifers infected with the bovine isolate (D-1) and feline isolate (AUTf-1) of *T. foetus*. Scores from each heifer (1=minimal, 2=slight, 3=moderate, 4=marked, 5=severe, extensive; data not shown) were taken and individual leukocyte scores were calculated by taking the product of each leukocyte score and the distribution score given for each sample. Individual scores are shown below. The maximum and minimum overall scores are calculated by taking those individual scores and adding the edema score. A (-) denotes that samples were not available due to inability to penetrate cervix.

Leukocyte	Heifer # (D-1 isolate)										Heifer # (AUTf-1 isolate)									
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
Periglandular																				
neutrophils	4	0	0	2	0	-	0	0	-	0	0	0	0	0	0	-	0	2	0	0
lymphocytes	6	0	0	1	0	-	0	1	-	1	1	1	1	0	1	-	0	1	1	2
plasma cells	4	0	0	1	-	-	0	1	-	1	1	1	1	0	2	-	0	1	1	2
macrophages	0	0	0	0	-	-	0	0	-	0	0	0	0	0	0	-	0	0	0	0
eosinophils	4	0	0	0	-	-	0	0	-	0	0	1	0	0	1	-	0	0	0	0
edema	4	0	4	2	1	-	3	2	-	3	2	3	2	2	2	-	2	3	2	2
Maximum	10	0	4	4	1	-	3	3	-	4	3	4	3	2	4	-	2	5	3	4
Minimum	4	0	4	3	1	-	3	2	-	3	2	3	2	2	2	-	2	3	2	2
Average	7.6	0	4	2.8	1	-	3	2.4	-	3.4	2.4	3.6	2.4	2	2.8	-	2	3.8	2.4	2.8
Interstitial																				
neutrophils	1	1	0	6	12	-	0	0	-	4	1	0	0	0	2	-	0	0	0	2
lymphocytes	0	0	0	3	12	-	1	1	-	2	1	2	1	1	4	-	1	1	1	4
plasma cells	1	1	0	9	16	-	1	2	-	2	1	2	1	1	4	-	1	2	2	6
macrophages	0	0	0	0	0	-	0	0	-	0	0	2	0	0	2	-	0	0	0	2
eosinophils	1	0	0	0	4	-	0	0	-	2	0	0	0	0	4	-	1	0	0	2
edema	4	0	4	2	1	-	3	2	-	3	2	3	2	2	2	-	2	3	2	2
Maximum	5	1	4	11	17	-	4	4	-	7	3	5	3	3	6	-	3	5	4	8
Minimum	4	0	4	2	1	-	3	2	-	3	2	3	2	2	4	-	2	3	2	4
Average	4.6	0.4	4	5.6	9.8	-	3.4	2.6	-	5	2.6	4.2	2.4	2.4	5.2	-	2.6	3.6	2.6	5.2

bovine isolate ranged from 0.0 to 7.6 for periglandular inflammation, and 0.4 to 9.8 for interstitial inflammation. In contrast, the overall mean histologic scores for heifers infected with the feline isolates ranged from 2.0 to 3.8 for periglandular inflammation, and 2.4 to 5.2 for interstitial inflammation (Table 3.1). There were no statistically significant differences in mean histologic scores between heifers infected with either isolate with respect to periglandular (P=0.731) and interstitial inflammation (P=0.40). In addition to periglandular and interstitial inflammation, presence or absence of surface epithelium was also noted. Surface columnar epithelium was intact in 7 of 8 heifers infected with the feline isolate of *T. foetus* (Figs. 3.3A, B), while none of 6 heifers infected with the bovine isolate of *T. foetus* had intact surface epithelium present (Figs. 3.3C, D) (epithelial scores were not available for 2 heifers). Epithelial scores were statistically significantly different between the 2 groups (P=0.005).

Trichomonad infection clearance times were also evaluated between each group. Clearance times were based on positive or negative vaginal and cervical mucus samples. Samples were cultured weekly after the 10 wk study period was complete. Heifers were considered free of infection if 5 consecutive negative vaginal and cervical samples were observed. After 20 wk PI, trichomonads were no longer detectable in heifers infected with the bovine isolate. In contrast, trichomonads were no longer detectable in only 2 of the heifers infected with the feline isolate by 20 wk PI. There is a statistically significant difference in clearance times between each group (P=0.021).

Discussion

Heifers inoculated with both bovine and feline *T. foetus* isolates that sustained trichomonad infections had pathologic changes in the reproductive tract consistent with

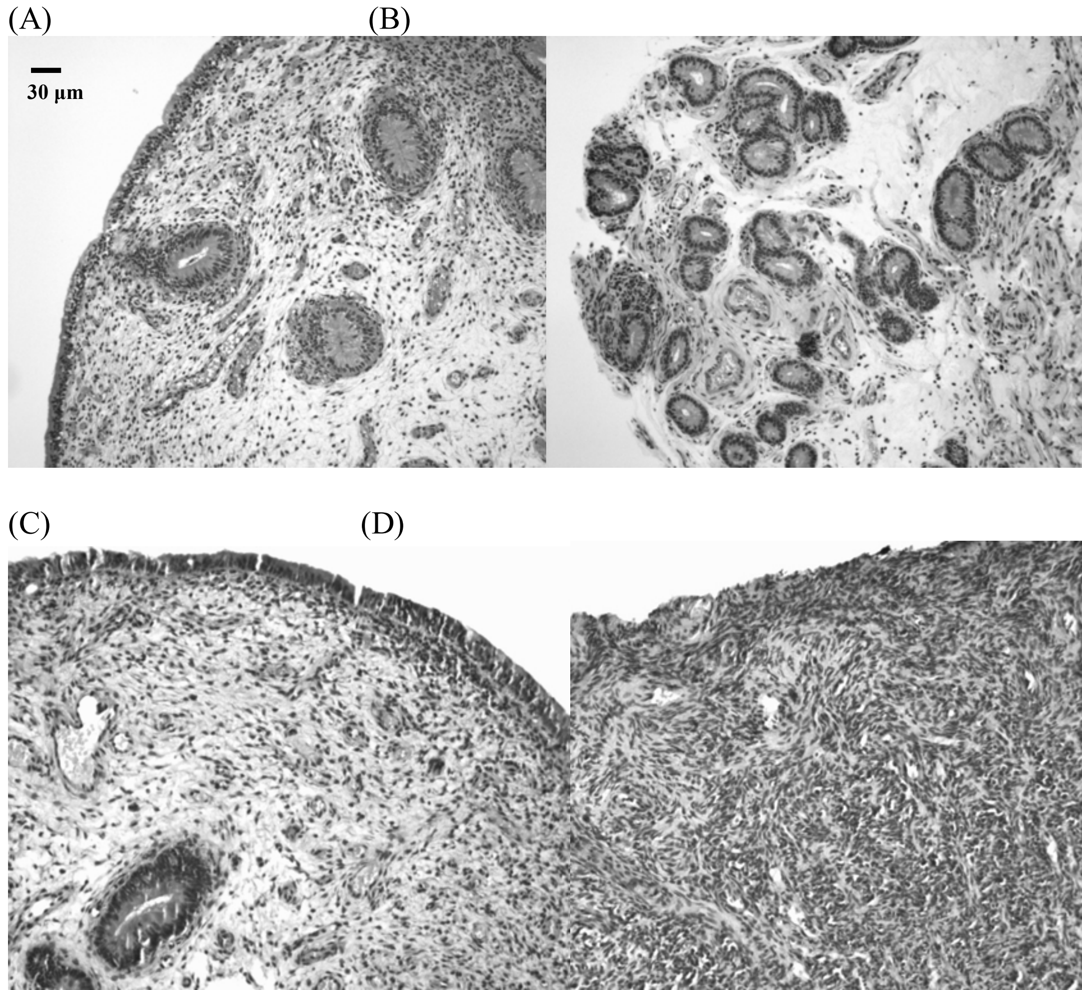


Figure 3.3. Transcervical uterine biopsy samples from three heifers taken six weeks PI. (A) A normal uterine sample, from control heifer ID#6-feline isolate. Note the presence of the epithelial border and glands. (B) A sample from heifer ID#4, infected with the AUTf-1 feline isolate of *Tritrichomonas foetus*. Note the absence of the epithelial border and the presence of periglandular inflammation and edema. This is the only sample in which the epithelial border was not intact. (C) A normal uterine sample, from control heifer ID#4-bovine isolate. Note the presence of the epithelial border and glands. (D) A sample from heifer ID#5, infected with the D-1 bovine isolate of *T. foetus*. Note the absence of glands and epithelial border and an infiltrate of mixed inflammatory cells.

previous reports of bovine trichomoniasis (Skirrow and BonDurant, 1990a, Anderson, et al., 1996, Felleisen, 1999). Since the experiments were not concurrent, the age of each. heifer was also considered when evaluating infections. The average age of heifers inoculated with the bovine or feline *T. foetus* isolate was 19.8 and 23 mo, respectively Age did not appear to impact susceptibility to trichomonad infection. Biweekly mucus aspirations revealed vaginitis and cervicitis in infected heifers, and endometritis was evident from uterine washes and uterine biopsy samples. Trichomonads were recovered from vaginal and or cervical samples from heifers in both groups during the first wk of infection, beginning 24 hr PI. Previous studies resulted in similar findings (Skirrow and BonDurant, 1990a). All heifers infected with the bovine *T. foetus* isolate cleared the infection by wk 20, while 5 heifers infected with the feline *T. foetus* isolate remained culture positive at wk 20 and 3 heifers remained culture positive at wk 30. Persistence of infection with both isolates in this study remains within range of persistent infection with bovine *T. foetus* in previous studies (Skirrow and BonDurant, 1990a). In both groups in the present study, 7 of 8 heifers maintained trichomonad infections for the duration of the study and, while not all infected heifers developed uterine infections, there were no statistical differences between the 2 groups.

Infertility and abortions due to bovine trichomoniasis can impose economic losses to cattle producers (BonDurant, 1997). Embryonic death may result from endometritis, which can potentially disrupt the implantation of the embryo due to inflammation and loss of surface epithelium. Other protozoan agents, as well as viral or bacterial pathogens may also cause abortion and infertility (Vanroose, et al., 2000). Experimental infection in the present study resulted in endometritis among heifers in both experimental groups.

Trichomonads were present in uterine washes and were accompanied by muco-purulent discharge and histologic evidence of inflammation. Biopsy samples of the uterine endometrium contained an influx of mixed inflammatory cells, i.e., plasma cells, macrophages, neutrophils, and eosinophils, as well as edema and loss of surface epithelium. Heifers within the group inoculated with the bovine *T. foetus* isolate had a significantly higher variability of inflammation than heifers within the group inoculated with the feline isolate. However, mean levels of inflammation between the 2 groups were comparable. This suggests that the trichomonads induce the same pathology, regardless of origin.

A significant difference was observed between the 2 isolates regarding the presence or absence of endometrial surface epithelium. All samples from heifers infected with the bovine *T. foetus* isolate were devoid of an intact surface epithelial border, while only 1 of 7 samples from heifers infected with the feline isolate was without an intact surface epithelial border. Although the feline isolate is capable of inducing trichomoniasis and many of the resulting pathologic effects, the reduced ability to cause the loss of the surface epithelium may suggest different parasite-host interactions. This could represent genetic divergence, and the introduction of a parasite into a host to which it is not readily adapted. This may also suggest that infection with the feline isolate of *T. foetus* may not cause infertility or abortion, a potential result of endometritis that may cause the loss of surface epithelium and disruption of embryonic implantation.

Tritrichomonas foetus may be a multi-host parasite capable of infecting a variety of hosts. Many livestock parasites (as many as 77% by one estimate) are indeed multi-host parasites (Haydon, et al., 2002). All hosts for *T. foetus* are mammalian and the site

of infection is the lumen of either the gastrointestinal or the reproductive tract. *Tritrichomonas foetus* recovered from cats may have adapted to the new host environment by exploiting the similarities of the cow's reproductive tract and large intestine. Both are luminal environments, which require the parasite to develop a means of adherence to epithelial cells, a common characteristic of *T. foetus* in both cows and cats (Felleisen, 1999; Yeager and Gookin, 2005).

The mechanism of *T. foetus* transmission between different host species remains unknown, as does the definitive routes of transmission between feline hosts. Additional cross-transmission studies, particularly attempts to transmit bovine isolates of *T. foetus* to cats are needed. Additional molecular comparisons are also a necessary component of future research. To date, the internal transcribed spacer region (ITS1 and ITS2) and the 5.8S and 18S rRNA genes are the standards used for genetic comparisons between the bovine and feline isolates of *T. foetus* (Gookin et al., 2002; Grahn et al., 2005; Levy et al., 2003). Future studies must combine genetic information, biological and structural data, along with host specificity and parasite-host interactions, to conclusively define the taxonomy of *T. foetus.*

CHAPTER IV.

EXPERIMENTAL INFECTION OF CATS (*FELIS CATUS*) WITH *TRITRICHOMONAS FOETUS* ISOLATED FROM CATTLE

Introduction

Tritrichomonas foetus is the causative agent of bovine trichomoniasis, a disease of the reproductive tract resulting in infertility and abortions in infected cows (BonDurant, 1985, BonDurant, 1997, Felleisen, 1999). Infected bulls become chronic carriers, while infected cows may clear the infection within 2-6 months (BonDurant, 1997, Stockdale, et al., 2007). In addition, *T. foetus* has recently been recognized as an agent of feline large bowel disease (Foster et al., 2004; Gookin et al., 1999; Gookin et al., 2001; Levy et al., 2003). Over the past decade, the numbers of reports of *T. foetus*-induced large bowel diarrhea in cats has increased (Stockdale et al., 2006). Surveys of cats from the United States and other countries have demonstrated that *T. foetus* is found in both purebred and mixed breed cats that may or may not have been in contact with cattle (Gookin et al., 1999; Gookin et al., 2004). Clinical signs of disease in infected cats include diarrhea with mucus, lethargy, anorexia and weight loss (Gookin et al., 1999; Gookin et al., 2001; Jordan, 1956; Stockdale et al., 2006). Additional signs may include diarrhea with blood, tenesmus, flatulence and malodorous feces. Experimental infection of cats with feline-derived *T. foetus* has reproduced many of these signs (Gookin et al., 2001). In some reports, the diarrhea associated with trichomonad infection had been attributed to *Pentatrichomonas hominis* (Romatowski, 1996, 2000). However, it was later shown that

T. foetus was the causative agent and not *P. hominis* (Levy et al., 2003).

Previous research has demonstrated that a feline isolate of *T. foetus* can successfully infect bovines. However, the resulting disease differs from that caused by a bovine isolate of *T. foetus* (Stockdale et al., 2007). To our knowledge, there exist no published reports of attempts to experimentally infect cats with a bovine isolate of *T. foetus*. In this study, we report the results of experimental infection of cats with the D-1 bovine isolate of *T. foetus*.

Materials and Methods

Protozoal Isolates

Two isolates of *T. foetus,* a feline isolate (AUTf-1) isolated from a naturally infected cat presented to Auburn University College of Veterinary Medicine (Stockdale et al., 2006) and a bovine isolate (D-1) originally collected from a naturally infected, pyometritic cow (Skirrow and BonDurant, 1990a), were obtained and cultured in Trypticase-Yeast-Maltose (TYM) media without agar (Diamond, 1983). These isolates were kept in freezing media in liquid nitrogen until one week before use. Freezing media consisted of fetal calf serum (FCS), dimethyl sulfoxide (DMSO) and TYM media at 3:2:5, respectively. The isolates were then thawed and subcultured in TYM media at 37°C.

Cats

Eight domestic shorthair cats, 6 females and 2 males, ranging in age from 8-12 months, were obtained from the Scott-Ritchey Research Center, Auburn University College of Veterinary Medicine. Fecal samples were collected from each cat to verify the absence of *T. foetus* by culture and polymerase chain reaction (PCR) (Grahn et al.,

2005). Cats were housed in the same room in separate stainless steel cages. Lighting and temperature were automatically controlled and daily care and maintenance was provided by the Division of Laboratory Animal Health, Auburn University College of Veterinary Medicine. The cats were acclimated to the cages and environment for 10 days prior to experimental infection.

DNA isolation and PCR

Tritrichomonas foetus DNA was isolated from fecal samples by first washing the feces 3 times with Tris-EDTA (TE) buffer (1mM EDTA, 10mM Tris, pH 8.0). After the final wash, the pellet was resuspended in 100µL TE and DNA extraction for PCR analysis was carried out as described in Billeter, et al., 2007. The PCR protocol, with modifications, described in Grahn, et al., 2005 was used to identify *T. foetus* from culture and fecal samples by amplifying the internal transcribed spacer 1 (ITS1) region. Modifications included 1mM 10X PCR buffer II, 1mM $MgCl_2$, 0.2mM dNTP and 0.05 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Additionally, the annealing temperature was decreased to 56 °C for 30 s.

Inoculation

Cats were fasted for twenty-four hours prior to inoculation. For experimental infection, cats were sedated using a mixture of medetomidine hydrochloride (Domitor®, Pfizer Animal Health, Exton, PA, USA) (68mg/ml), butorphanol tartrate (Torbugesic®, Fort Dodge, KS, USA) (1.8mg/ml) and ketamine (Ketaset®, Fort Dodge) (0.14mg/ml) at a rate of 0.075ml/kg. The AUTf-1 and D-1 isolates were washed and resuspended in antibiotic-free, fetal bovine serum-free TYM media without agar approximately one hour prior to inoculation. Six cats received 1.5 X 10^6 trichomonads (D-1 isolate) in 10 ml of

antibiotic-free, fetal bovine serum-free TYM media without agar via orogastric intubation. One cat was inoculated with 1.5 X 10^6 trichomonads (AUTf-1 isolate) in similar media. One cat was inoculated with media only. Cats were allowed to recover naturally.

Collection of fecal samples and culture

Fecal samples were collected from each cat three times a week for five weeks. Samples were obtained per rectum using a plastic loop or from a freshly voided sample in the litter box. Each sample was immediately suspended in 2ml of TYM media and incubated at 37° C. In addition to sample collection, fecal consistency was noted using a scale of 1-4 (1=diarrhea, watery, loose or possibly blood; 2= no form, loose, puddles or piles; 3= formed, soft or wet; 4= formed and hard) (Purina® Fecal Scoring System for Cats, Nestlé Purina PetCare Co., St. Louis, MO, USA). Emesis or other signs were also noted. All samples were examined at 100X magnification after two days in culture. Samples were scored as positive (+) or negative (-) based on the presence of motile *T. foetus* trophozoites, and were verified using PCR (Grahn et al., 2005). Negative samples were held for 10 days and rechecked every two days.

Euthanasia and necropsy

At five weeks PI, all cats were sedated as described above. Following sedation, the cats were euthanized by intravenous administration of sodium pentobarbital (Euthasol®, Delmarva Laboratories, Midlothian, VA, USA) at the dose of 17.7mg/kg. At necropsy, the ileum, cecum and colon were removed as one section. The anterior ileum and posterior colon were ligated with cotton twine. Ligatures were also placed at the posterior ileum, base of the cecum and the anterior colon. The colon was then

sub-divided into three equal sections using two additional ligatures. This yielded a total of five separate sections of bowel: terminal ileum, cecum, anterior, medial and posterior colon. Approximately 2-5ml of TYM media was injected into each section using a sterile 18-guage needle and 10ml syringe. The sections were then incised and the contents of each section were collected into a separate specimen cup. Approximately 1ml of contents from each section of intestine was resuspended in 5ml of TYM media and incubated at 37° C. Samples were examined as previously described for visible trophozoites and verified using PCR procedures (Grahn et al., 2005).

Tissues for histopathologic examination were fixed in 10% buffered formalin. Following fixation, the intestinal segments were trimmed and sections of ileum, cecum, and three sections of colon (proximal, middle and distal) submitted for routine paraffin embedding and tissue sectioning. Five micron sections of tissue from these five locations were cut with a microtome, affixed to glass slides and stained with hematoxylin and eosin stain.

Inflammation in the lamina propria of the ileum, cecum and colon was subjectively evaluated, in a blinded fashion, with light microscopic examination of tissue sections stained with hematoxylin and eosin. A grading scale of 0 -3 was followed with 0 being no to few lymphocytes and plasma cells present and 3 being moderate to large numbers of lymphocytes and plasma cells present. The size of Peyer's patches was also evaluated. Additionally, other histopathologic lesions including crypt abscesses and the amount of mucus present were evaluated and assigned lesion scores.

Results

Fecal cultures

Motile trichomonads were successfully observed in cultures from three fecal samples obtained from the positive control cat (cat no.7), inoculated with the feline (AUTf-1) isolate, beginning day 16 post-inoculation (PI) (Table 4.1). Of the six cats inoculated with the bovine (D-1) isolate, only one cat (cat no.2) was culture positive on day 32 PI (Table 4.1). The remaining five cats inoculated with the bovine (D-1) isolate remained culture negative throughout the study. Neither of the two cats (no.2 bovine [D-1] isolate and no.7 feline [AUTf-1] isolate) that were culture positive developed diarrhea or demonstrable vomiting, loss of appetite or fever during the study. On day 9 PI, one cat (cat no.5) inoculated with the bovine (D-1) isolate had soft stool with small amounts or blood and mucus, and cat no.4 had episodes of vomiting and a fever of 102.8 F on day 21 PI (Table 4.1), but neither cat was culture positive for *T. foetus*.

Intestinal cultures

Motile trichomonads were successfully observed in cultures from the intestinal contents of both fecal culture positive cats (see above) (Table 4.2). Cat no.2, bovine (D-1) isolate, was culture positive in the cecum. Cat no.7, feline (AUTf-1) isolate, was culture positive in the ileum, cecum, medial colon and posterior colon (Table 4.2). Additionally, cat no.4 bovine (D-1) isolate was culture positive in the cecum. Cat no.8, (media only), was culture positive in the ileum. However this was an erroneous result due to improper labeling of tubes during subculture. That a negative diagnosis was appropriate for this culture is supported by the fact that PCR results in all samples

Table 4.1. Results of the fecal sample cultures and health observations of cats over the 5 week study period. Cats were inoculated with either the bovine D-1 isolate (nos. 1-6) or feline AUTf-1 isolate (no. 7) of *Tritrichomonas foetus*. One cat (no. 8) was not inoculated with trichomonads (media only) and used as a negative control. Over the five week period, 15 samples were taken from each cat, beginning post-inoculation (PI) day 2 (2d). All cats had fecal scores ranging from 3-4 (formed and either soft and wet or hard) unless otherwise noted.

Cat ID	**Isolate**	**No. Positive Fecal Cultures/ Total No. Samples**	**Signs of Disease**
1	D-1	0/15	none
2	D-1	PI 32d*; 1/15	none
3	D-1	0/15	none
4	D-1	0/15	PI 21d; fever (102.8 F) vomiting
5	D-1	0/15	PI 9d, blood and mucus in loose stool; fecal score = 1
6	D-1	0/15	none
7	AUTf-1	PI 16d*; 3/15	none
8	Media only	0/15	none

* day of first positive culture sample

Table 4.2. Culture results of material washed from individual sections of intestine from each cat. A (-) indicates an intestinal section that was culture negative for the presence of *Tritrichomonas foetus*, and a (+) indicates an intestinal section that was culture positive for the presence of *T. foetus*. Concentrations of *T. foetus* recovered from culture samples of each intestinal section are listed in parenthesis as trichomonads/ml.

	Cat ID no.							
Intestinal section	1	2	3	4	5	6	7	8
ileum	-	-	-	-	-	-	+ ($1.73x10^5$)	+*
cecum	-	+ ($1.75x10^4$)	-	+ ($2.75x10^4$)	-	-	+ ($3.15x10^6$)	-
anterior colon	-	-	-	-	-	-	-	-
medial colon	-	-	-	-	-	-	+ ($2.5x10^3$)	-
posterior colon	-	-	-	-	-	-	+ ($5.0x10^3$)	-

* contamination due to mislabeled tubes during subculture, negative result confirmed by PCR

obtained from the original specimen cups at necropsy, and from weekly fecal samples obtained throughout the study, were negative.

Histopathologic analysis

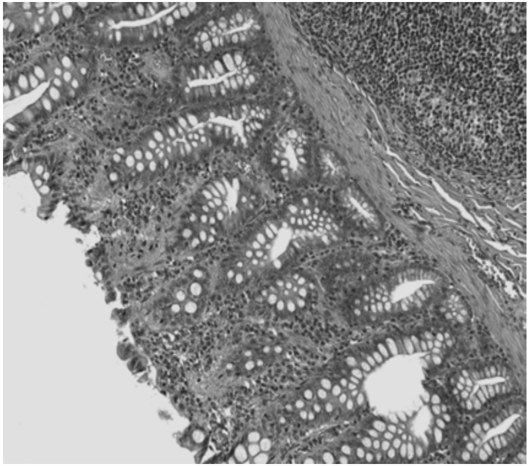
Tissue samples were obtained and scored as described above from each intestinal section (Table 4.3). All intestinal samples taken from the cat inoculated with the feline (AUTf-1) isolate showed an increase of lymphocytes and plasma cells in the mucosa, which appeared to be migrating from moderately hyperplastic Peyer's patches (Figure 4.1A). Often grade 3 sections also contained aggregates of lymphocytes and plasma cells in addition to those lymphocytes and plasma cells scattered throughout the proprial tissue. There was also an increase in the presence of crypt abscesses and mucus production. All samples taken from cats inoculated with the bovine (D-1) isolate demonstrated similar lesions (Figure 4.1B). Each cat had some level of lymphocyte and plasma cell infiltration in the sections of intestinal mucosa, although some were more pronounced than others (Table 4.3). Samples collected from the non-infected control cat displayed similar levels of lymphocytes and plasma cells however Peyer's patches and surrounding mucosa appeared normal (Figure 4.1C).

Discussion

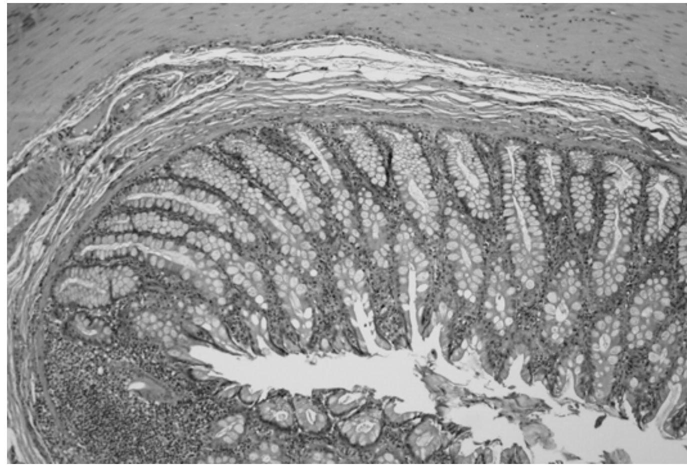
Results of this study suggest that differences exist in both infectivity and pathogenicity for feline (AUTf-1) and bovine (D-1) isolates in experimentally infected cats. These results are similar to those of our previous study in which heifers infected with the AUTf-1 isolate of *T. foetus* demonstrated temporal differences in infection and severity of disease when compared to heifers infected with a bovine (D-1) isolate of *T. foetus* (Stockdale et al., 2007). Of the 6 cats inoculated with the bovine (D-1) isolate,

Table 4.3. Histopathological analysis results of intestinal sections recovered from each cat at necropsy. Intestinal sections were stained with hematoxylin and eosin and the presence of lymphocytes and inflammatory cells were scored on a scale of increasing severity (1=minimal, slight, 2=moderate, 3=severe, marked). Individual scores for each intestinal section recovered from each cat are shown along with overall mean scores.

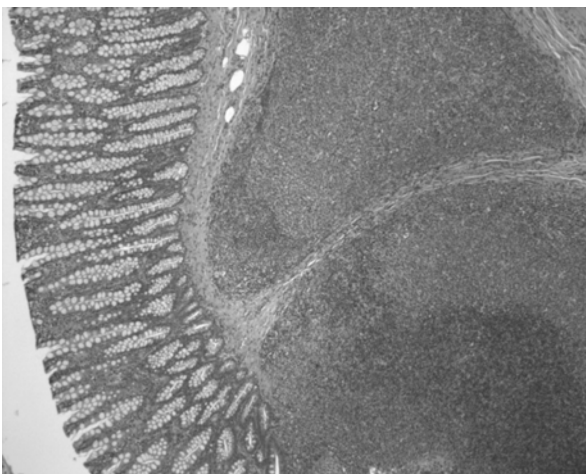
bovine D-1 isolate	**ileum**	**cecum**	**anterior colon**	**medial colon**	**posterior colon**	**mean score**
1	2	3+	2+	3+	2+	2.4
2	1-2	1	1-2+	2+	2+	1.6
3	2	2+	2+	1	2+	1.8
4	2	1+	1	1	1-2+	1.3
5	1	2+	2	2+	2-3	1.9
6	1-2	1+	1+	2-3+	3+	1.8
feline AUTf-1 isolate						
1	1-2	2+	2+	3+	2-3+	2.4
media only						
1	2	2	2+	2+	3+	2.2



(A)



(B)



(C)

Figure 4.1. Tissue samples taken at necropsy from the intestine of cats infected with *Tritrichomonas foetus*. The feline isolate, AUTf-1 (A), the bovine isolate, D-1 (B) and the non-infected control (C). Images are taken at 100X magnification.

only 1 was positive by fecal culture during the 5 week study. This occurred on the last day of sampling. The cat inoculated with the feline (AUTf-1) isolate was positive by fecal culture at 15 days PI. PCR results supported that negative fecal cultures were truly negative and not the result of too few organisms to culture successfully. Positive fecal cultures were also positive by PCR, supporting the sensitivity of the fecal culture method (Gookin et al., 1999; Gookin et al., 2003; Gookin et al., 2004; Grahn et al., 2005). Similar results were obtained from culture samples from intestinal contents of the ileum, cecum and colon of each cat at necropsy. The cat inoculated with the feline (AUTf-1) isolate was again culture positive in the ileum, cecum, medial and distal colon. Of the 6 cats inoculated with the D-1 isolate, a single cat that was fecal culture positive was also positive when cecal contents were collected. One additional cat was culture positive when cecal contents were collected but was negative based on fecal culture. These results were again verified using PCR (Grahn et al., 2005).

It is possible that trichomonads are passed in feces intermittently or in low numbers only during episodes of diarrhea. Consequently, *in situ* sampling techniques would likely be more sensitive than fecal culture techniques. The use of PCR procedures for detection of trichomonads in organ contents or in feces would be expected to be more sensitive than culture, only requiring a minute amount of DNA and able to easily distinguish between different trichomonads easily {Grahn et al., 2005). This is important when trichomonad populations are low or a mixed infection is suspected.

Interpretation of histologic changes in the colon can be difficult. Lymphocytes and plasma cells normally respond to antigens within the colonic lumen. Wide variation in numbers of lymphocytes and plasma cells is common place and can even be

interpreted as a normal response to the ever changing enteric microenvironment (Wilcock, 1992). Analysis of histopathologic changes in each feline intestinal section revealed aggregates of lymphocytes and plasma cells of approximately equal frequency and intensity in both treatment groups. Based on prior research (Yeager and Gookin, 2005), the experimental infection results suggest that longer infection times may be necessary to show demonstrably different pathogenic events in cats. These cats were not specific pathogen-free animals and the pathologic changes observed in the intestinal mucosa could be in response to any intraluminal antigen such as food antigens or antigens from one or several of the many species of bacteria and other microorganisms living in the colonic contents.

The results of this study together with the results of our previous research in bovines suggest that there are differences in biological or pathogenic behavior of the feline and bovine isolates that cannot be ignored. The two studies provide compelling evidence that fundamental differences exist between the different isolates of *T. foetus*. In the opinion of the authors, these differences exceed what one would expect to observe in normal intra-specific variation.

CHAPTER V.

FURTHER BIOLOGICAL CHARACTERIZATION OF *TRITRICHOMONAS FOETUS* OF FELINE ORIGIN

Introduction

Tritrichomonas foetus was originally described in Europe and is the causative agent of venereal trichomoniasis in cattle. Clinical signs in cows include vaginitis, cervicitis, pyometra, and endometritis. Infertility and early to mid-term abortions can also result from *T. foetus* infection. In bulls, inflammation of the prepuce and glans penis (balanoposthitis) can occur however infected bulls are often asymptomatic (BonDurant, 1985, BonDurant, 1997, Felleisen, 1999).

The first report of trichomoniasis in cats in the early 1900s described nine kittens infected with trichomonads detected in diarrheic stool (Kessel, 1928). Clinical signs included yellow-brown diarrhea with or without blood. Years later, trichomoniasis was reported in a young cat brought to a veterinary clinic displaying similar clinical signs (Jordan, 1956). The reported symptoms of feline trichomoniasis include chronic, malodorous large-bowel diarrhea, that may contain blood or mucus, flatulence, tenesmus, and fecal incontinence leading to soiling outside the litter box (Gookin, et al., 1999, Foster, et al., 2004). Over the past decade, there have been increasing reports of clinical feline trichomoniasis (Gookin, et al., 1999, Gookin, et al., 2004, Stockdale, et al., 2006).

Published comparisons of the genomic sequences of two internal transcribed spacer (ITS) regions, ITS-1 and ITS-2, along with highly conserved 18S, 5.8S and 28S rRNA genes has suggested that the causative agent of bovine and feline trichomoniasis is *T. foetus* (Felleisen, 1997, Felleisen, et al., 1998, Gookin, et al., 2002, Levy, et al., 2003, Grahn, et al., 2005).

I believe that host specificity, along with morphological and genetic information should be included in any taxonomic evaluation. Differences were observed in disease and host specificity in heifers infected with *T. foetus* isolated from a cat and also from cats infected with *T. foetus* isolated from cows (Stockdale, et al., 2007, Stockdale, et al., 2007). The purpose of this study was to further characterize and compare feline and bovine isolates of *T. foetus* using information from experimental infections in both cattle and cats in addition to genetic sequencing and phylogenetic analysis and acquired morphological data.

Materials and Methods

Sources of organisms and maintenance

Each of the twelve feline trichomonad isolates used in this study were obtained from naturally infected cats presented to the Auburn University parasitology laboratory. Only one feline isolate, AUTf-1, was observed by electron microscopy and used for morphological comparisons. The bovine isolate, D-1, was obtained from a naturally infected, pyometritic cow, donated by Dr. R. H. BonDurant. Additional bovine isolates (no. 25, 33, 34, 36) were obtained from naturally infected cows culled from a herd in Florida. All organisms were cultured in trypticase-yeast-maltose (TYM) Diamond's medium (Diamond, 1983) without agar and incubated at 37 °C during experimental

observations. Organisms were stored in liquid nitrogen in freezing medium made under sterile conditions using FCS, DMSO and TYM at 3:2:5, respectively.

Light Microscopy

Direct smears from cultured solutions of AUTf-1 were prepared using TYM Diamond's medium. Organisms were examined with an Olympus bifocal microscope (Olympus America Inc., Center Valley, PA) at 100X. Differential interference contrast (DIC) photomicrographs were taken using a Nikon TE-2000 C1 (Nikon Instruments Inc., Melville, NY) at 60X. Prior to viewing, organisms were gently pelleted and the majority of supernatant media was removed. Pelleted organisms were resuspended in a 1:1 solution of paraformaldehyde-cacodylate. A direct smear was made from this mixture and sealed using Paramount® histological mounting medium (Fisher Scientific Co, Fair Lawn, NJ).

Scanning Electron Microscopy (SEM)

Suspensions of AUTf-1 in TYM Diamond's medium without agar were concentrated from 15ml to 1ml by gentle centrifugation and removal of excess medium. Round glass slides were cleaned with 1% HCl in 70% EtOH and coated with 0.1% poly-L-lysine for 5 minutes. Slides were dried at 60°C for 1 hour, washed with distilled water, and coated with the concentrated trichomonad-TYM medium solution. This was allowed to set for 15 minutes and excess media was removed. The slides were then fixed in 2.5% glutaraldehyde, 0.1M cacodylate buffer for 2 hours, washed with 0.2M cacodylate buffer for 2-3 minutes. Slides were then fixed in 1% OsO_4 in distilled water for 5 minutes and washed again in distilled water for 2-3 minutes. After a dehydration series of increasing ethanol concentrations (30%, 50%, 70%, 80%, 90%, 95%, 100%) for 10 minutes each,

slides were critical point dried with CO_2 (1150-1200 psi at 33^oC), coated twice with gold and viewed using a Zeiss DSM 940 scanning electron microscope (Carl Zeiss Inc., Oberkochen, Germany).

Transmission Electron Microscopy (TEM)

Suspensions of AUTf-1 in TYM Diamond's medium without agar were concentrated from 15ml to 1ml by gentle centrifugation and removal of excess medium. Concentrated organisms were resuspended and fixed in 2.5% glutaraldehyde, 4% paraformaldehyde, and 5mM $CaCl_2$ in 0.1M cacodylate buffer (pH 7.4) overnight at 4C. The organisms were washed in 0.1 M cacodylate buffer for 5 minutes, embedded in 1% SeaPlaque (FMC Marine Colloids, Rockland, ME), and washed 3 additional times, 5 minutes each. The parasites were then post-fixed in 1% $OsO_{4,}$ 0.1M cacodylate buffer, pH 7.4 at room temperature for 2 hours, and dehydrated in increasing concentrations of ethanol (50%, 70%, 95%, 100%) 3 changes, for 20 minutes each at 4^oC. Propylene oxide was used as a transient solvent. After embedding in Epon 812 epoxy resin (Ladd Research Industries, Inc., Burlington, VT), thin sections (70nm) were made and stained with uranyl acetate/lead citrate then viewed with a Philips 301 transmission electron microscope (FEI Co., Hillsboro, OR) operated at 60kV.

Molecular characterization of *Tritrichomonas foetus*

Trichomonad DNA was extracted from 12 feline trichomonad isolates using a previously described protocol (Billeter, et al., 2007) and identified using PCR with primers targeting the ITS-1 and ITS-2 regions, the 5.8S rRNA gene and partial 18S and 28S rRNA genes using previously described protocols (Felleisen, et al., 1998, Gookin, et al., 2002, Grahn, et al., 2005). The PCR products were directly sequenced using the

TFR-3 and TFR-4 primer sets, giving sequences of both strands (Appendix C). Other trichomonad sequences were obtained from GenBank and included trichomonads isolated from feline (AF466750, AF466751 and EF165538) (Levy, et al., 2003, Dahlgren, et al., 2007) and canine hosts (AY758392) (Gookin, et al., 2005), and additional bovine (U85967 and AF339736) (Felleisen, 1997, Walker, et al., 2003), porcine (U85966) (Felleisen, 1997), and reptilian trichomonads (AY886845) (Cepicka, et al., 2006).

Random amplification of polymorphic DNA (RAPD)

Nine previously described 12 mer oligonucleotide primers (Table 5.1) were used to amplify random DNA markers (Fraga, et al., 2002, Rojas, et al., 2004). Template DNA totaling 15 samples was extracted from 6 feline trichomonad isolates, 5 bovine *T. foetus* isolates, trichomonads isolated from the peritoneal cavity of a human, *T. mobilensis* from cotton rats*, Trichomonas vaginalis* from humans and *Giardia* sp. from humans as an outgroup. The DNA amplification method was adapted from Rojas et al., 2004 and performed in a final volume of 25µl. The reaction conditions were in 1X Thermopol II PCR buffer (includes 2 mM $MgSO_4$, New England Biolabs), 200 µM dNTPs (US Biochemicals), 25pM 12-mer primer, 2U StandardTaq DNA polymerase (New England Biolabs) and 100ng of template DNA. The PCR amplification conditions were an initial denaturation at 94°C for 5 min and 40 cycles of 94°C for 1 min, 35°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The PCR products were analyzed by electrophoresis on in 1.5% agarose gels in TBE buffer and stained with ethidium bromide, and photographed with a digital camera. The RAPD analysis was performed three times on different days by two separate investigators to ensure reproducibility.

Table 5.1. Oligonucleotide primers used for amplification of random DNA markers of various trichomonads (adapted from Rojas et al., 2004).

Primer	Sequence (5'-3')
1	CGC ACT CGG AGT
2	TCG GCC GCT ATC
3	CCG TGA CAC GCA
4	GGG ACA CTC TGG
5	CCG CTG TAC TCC
6	GGG ACC TAC TGC
7	GAG TCG CAC AGG
8	TCC TCA CCG ACC
9	CCC CAG TAC CAG

Phylogenetic analysis

Internal transcribed spacer regions and ribosomal RNA genes

Nucleotide sequence data was collected using Chromas Lite version 2.01 (Technelyium PTY Ltd, Australia) and sequence alignment was performed using the AlignX® option in Vector NTI™ Advance 10 (Invitrogen, Carlsbad, CA) followed by manual manipulation. Sequence comparisons were performed using the Maximum Likelihood method (dnaml) found in the Phylogeny Inference Package (PHYLIP) version 3.67 (Joseph Felsenstein, University of Washington, Seattle, WA). The best of tree from 100 replicates was created for each comparison using Phylodraw ver. 0.8 (Graphics Application Lab, Pusan National University).

RAPD analysis

The bands visualized in the RAPD gels were scored as present (1) or absent (0) for each isolate, with each compared against the other. The inverse of Jaccard's similarity coefficient (Sj) was used in the following formula: $Sj = 1 – a / (a + b + c)$ where a = number of bands present in both isolates, b = number of bands present in isolate 1 and absent in isolate 2, and c = number of bands present in isolate 2 and absent in isolate 1. Results were compiled into a 15x15 matrix and analyzed using three methods, Fitch-Margoliash, Fitch-Margoliash with molecular clock (Kitsch) (this method is shown in the results) and Neighbor-Joining distance matrix programs included in the PHYLIP version 3.67.

Results

Microscopic analysis

Measurements of feline trichomonad trophozoites using SEM and TEM (Figs 5.1-5.2) photomicrographs resulted in a mean cell body length of 10µm (range 5.7-13µm) and mean cell body width of 4.4µm (range 3.9-5.5µm). The axostyle extended from the posterior end of the cell body approximately 2µm and tapers to a thin tip. There were three anterior flagella each averaging 15µm in length, and a posterior flagellum following along and extending from the undulating membrane for approximately 12µm. The undulating membrane was approximately ¾ the length of the cell body. Internal organelles and cytoskeletal structures were also observed by transmission electron microscopy. Feline trichomonad trophozoites contained a single nucleus and Golgi apparatus. The axostyle ran the length of the cell body and a densely banded costa extending from the basal bodies followed the undulating membrane. Both structures were closely associated with spherical hydrogenosomes.

Molecular comparisons

DNA sequences containing the ITS-1 and ITS-2 regions, 5.8S rRNA gene and partial 18S and 28S rRNA genes of 12 feline trichomonad isolates were submitted to GenBank and given accession numbers listed in Table 5.2. When compared to the bovine (AF339736) and feline (AF466750) isolates registered in GenBank, all 12 feline isolates demonstrated differences in DNA sequences, including deletions, insertions and nucleotide changes, and point mutations (Table 5.3). We also compared the feline isolates with DNA sequences of trichomonads isolated from additional feline, canine, bovine, porcine and reptilian hosts found in GenBank (Appendix B).

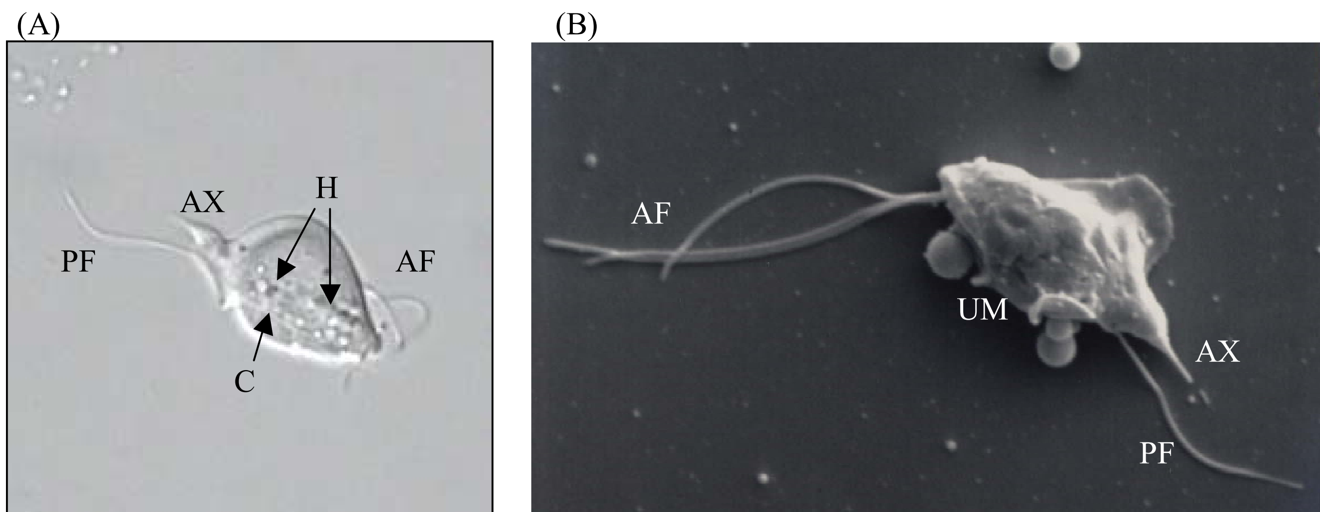


Figure 5.1. Photomicrographs taken of the feline AUTf-1 isolate of *Tritrichomonas foetus*. (A) The differential interference contrast micrograph (DIC) details the axostyle (AX), posterior and anterior flagella (PF and AF, respectively), costa (C), and hydrogenosomes (H). (B) The scanning electron micrograph (SEM) details the axostyle, undulating membrane (UM), posterior and anterior flagella.

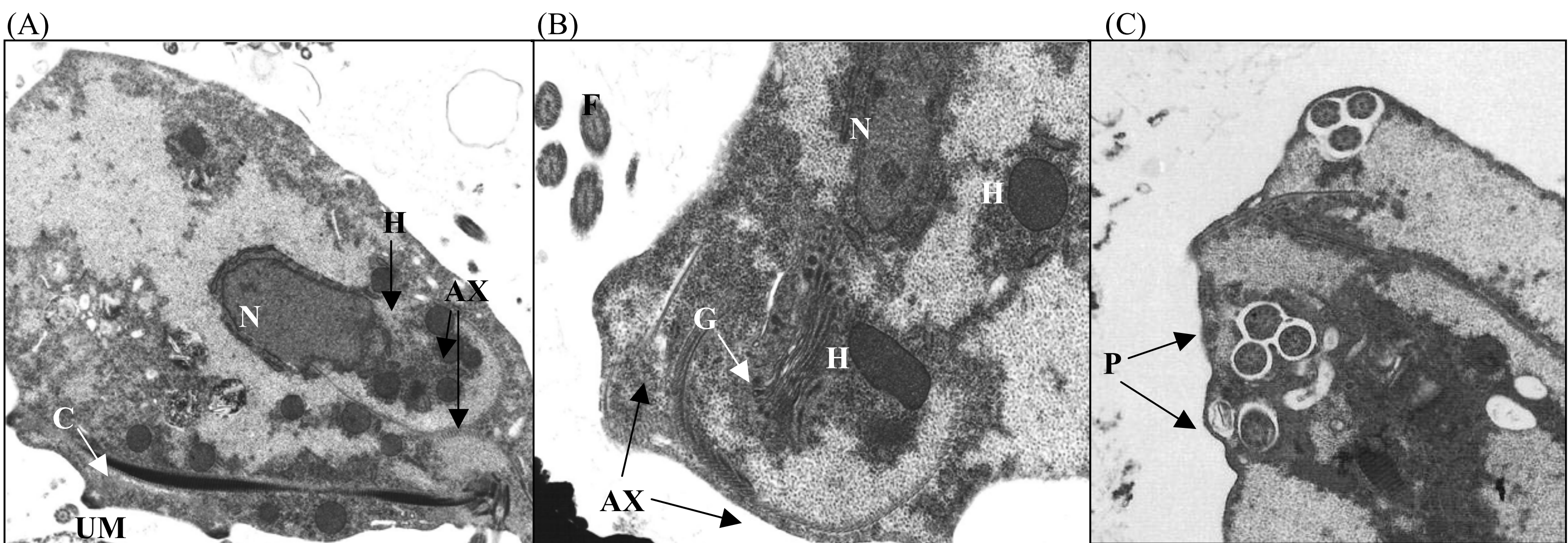


Figure 5.2. Transmission electron micrographs (TEM) taken of the feline AUTf-1 isolate of *Tritrichomonas foetus*. (A) Several internal organelles are visible, including the costa (C), nucleus (N), axostyle (AX) and hydrogenosomes (H). (B) The anterior end of the cell body detailing the axostyle, nucleus, hydrogenosomes and Golgi apparatus (G). Flagella (F) are also visible in the top left corner. (C) This detailed view of the anterior cell body shows the pelta (P).

Table 5.2. Accession numbers given to feline (AUTf) trichomonad isolates registered in GenBank. The gene regions used for comparisons and corresponding base pairs are also listed.

Accession number	**AUTf isolate**	**18S**	**ITS-1**	**5.8S**	**ITS-2**	**28S**
EU569301	1	1-3	4-83	84-243	244-308	>309
EU569302	2	1-4	5-84	85-244	245-309	>310
EU569303	3	1-3	4-83	84-243	244-308	>309
EU569304	4	1-4	5-84	85-244	245-309	>310
EU569305	5	0	1-80	81-240	241-305	>305
EU569306	6	1-3	4-83	84-243	244-308	>309
EU569307	7	1	2-81	82-241	242-301	>302
EU569308	9	1	2-81	82-241	242-301	>302
EU569309	10	1-2	3-82	83-242	243-308	>309
EU569310	11	1-5	6-85	86-245	246-310	>311
EU569311	12	1-5	6-85	86-245	246-310	>311
EU569312	13	1	2-81	83-241	242-301	>302

Table 5.3. Genetic sequences differences between 12 feline trichomonad isolates and the bovine (AF339736) isolate of *T. foetus* found in GenBank. Compared sequences are divided into three regions, ITS-1, ITS-2 and 5.8SrRNA. The number of insertions, deletions and single point mutants are given for each feline isolate.

Isolates	Gene Regions	Changes in Genetic Sequences		
	ITS-1, ITS-2, 5.8SrRNA	Insertions	Deletions	Point mutant changes
1		3	3	4
2		4	3	19
3		2	2	1
4		3	5	2
5		7	34	15
6		2	1	2
7		0	2	6
9		6	2	6
10		2	1	0
11		2	3	1
12		2	4	7
13		3	5	7
	ITS-1	Insertions	Deletions	Point mutant changes
1		1	3	4
2		4	3	19
3		2	2	1
4		2	5	2
5		7	34	15
6		2	1	2
7		0	2	5
9		5	2	6
10		1	1	0
11		2	3	1
12		2	4	6
13		2	4	7
	ITS-2	Insertions	Deletions	Point mutant changes
1		2	0	0
2		0	0	0
3		0	0	0
4		1	0	0
5		0	0	0
6		0	0	0
7		0	0	1
9		1	0	0
10		1	0	0
11		0	0	0
12		0	0	1
13		1	1	0

Nucleotide sequence identities between the 12 AUTf isolates and those from GenBank ranged from 96-100% (Table 5.4).

A phylogenetic tree constructed using the maximum likelihood method (PHYLIP, dnaml) indicates different results depending on the gene(s) compared. When sequences for the partial 28S, 18S and complete 5.8S rRNA genes, along with the ITS-1 and ITS-2 regions, were analyzed, the resulting tree indicates similarities between feline isolates and the isolates evaluated from GenBank (Fig 5.3). These results were also similar to those comparing the ITS-2 region of each trichomonad sequence (Fig 5.4). However, when DNA sequences from the ITS-1 region were compared between feline isolate sequences and sequences from GenBank, the feline isolate sequences clustered separately from the other either as their own group or with the chosen outlier, *P. hominis.* This clustering was statistically significant ($P<0.005$) (Fig 5.5).

The RAPD results generated from PCR amplification of trichomonad DNA using 9 random primer sequences further support the data obtained by the ITS-1 DNA sequence analysis. The feline *Tritrichomonas* sp. isolates were more similar to each other than to the bovine *T. foetus* isolates. The bovine isolates were more similar to each other than to feline *Tritrichomonas* isolates (Fig 5.6). As expected, the *Trichomonas vaginalis* and *Giardia lamblia* DNA displayed different banding patterns than *Tritrichomonas* isolates of either feline or bovine origin. *Tritrichomonas mobilensis* and a trichomonad isolated from the peritoneal cavity of a human displayed banding patterns more similar to feline *T. foetus* isolates than those of bovine origin. The matrix of band differences is shown in Appendix D. The banding pattern differences in banding patterns are depicted in a

Table 5.4. Sequence identities (%) determined by BLASTn analysis between the 12 feline isolates (EU569301-EU569312, respectively) and 5 other trichomonads from GenBank.

Isolate	AF339736 (bovine)	AF466750 (feline)	AY349190 (*T. suis*)	AY886845 (*T. nonconforma*)	AY886824 (*T. mobilensis*)
1	97	98	98	97	97
2	99	100	99	98	98
3	98	99	98	98	98
4	97	97	98	97	97
5	97	97	97	100	100
6	98	99	98	98	98
7	97	97	97	96	96
9	97	97	97	96	96
10	99	99	99	99	99
11	98	99	98	98	98
12	98	98	98	97	97
13	97	98	98	97	97

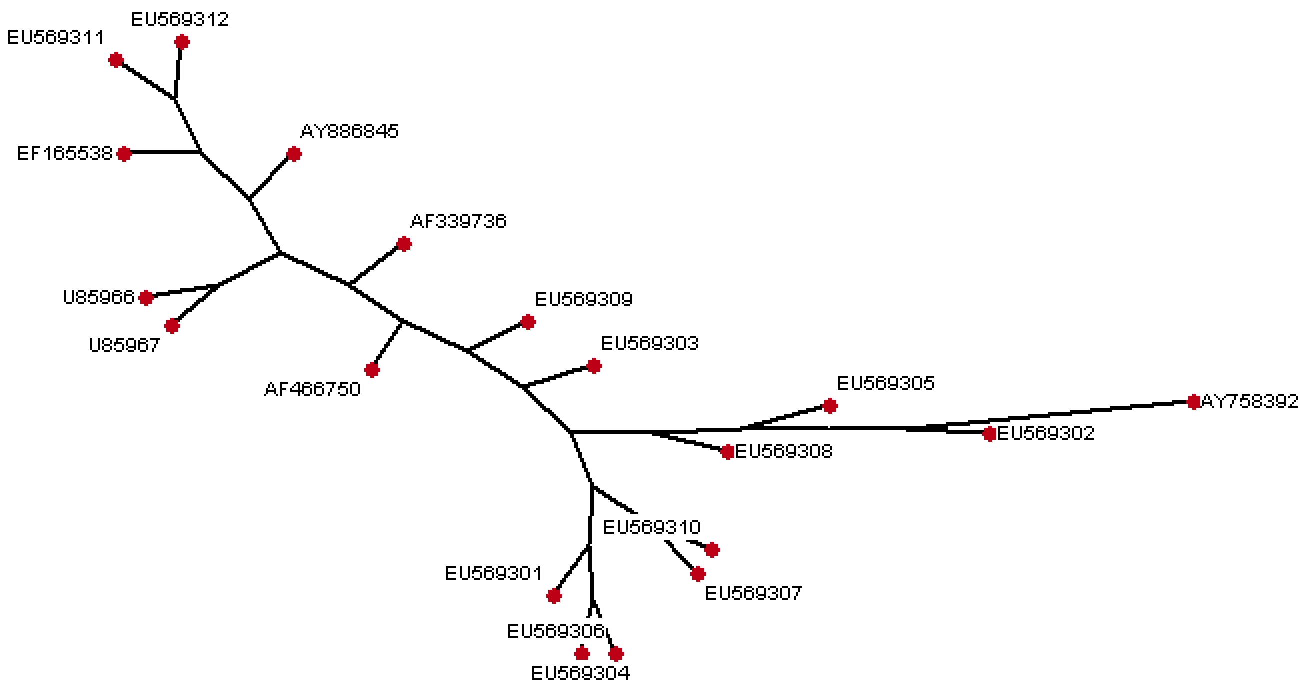


Figure 5.3. Phylogenetic tree depicting genetic sequence comparisons of the 5.8SrRNA gene and ITS-1 and ITS-2 regions between AUTf sequences and published GenBank sequences. AUTf isolates 1-7; 9-13 are listed as accession numbers EU569301-EU569312, respectively. *Pentatrichomonas hominis* (AY758392), bovine *T. foetus* isolates (AF339736 and U85967), feline *T. foetus* isolates AF466750 and EF165538), *T. suis* (U85966) and *T. nonconforma* (AY886845) are also used in the comparison.

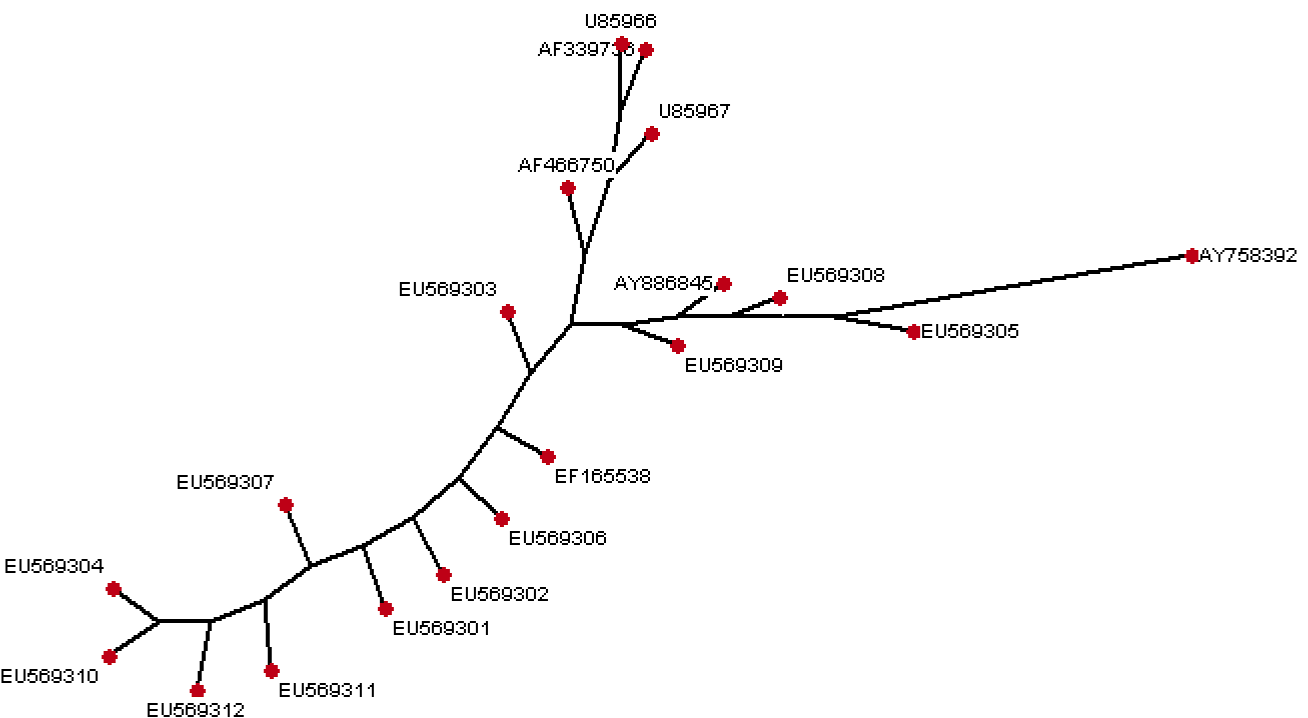


Figure 5.4. Phylogenetic tree depicting genetic sequence comparisons of the ITS-2 region between AUTf sequences and published GenBank sequences. AUTf isolates 1-7; 9-13 are listed as accession numbers EU569301-EU569312, respectively. *Pentatrichomonas hominis* (AY758392), bovine *T. foetus* isolates (AF339736 and U85967), feline *T. foetus* isolates (AF466750 and EF165538), *T. suis* (U85966) and *T. nonconforma* (AY886845) are also used in the comparison.

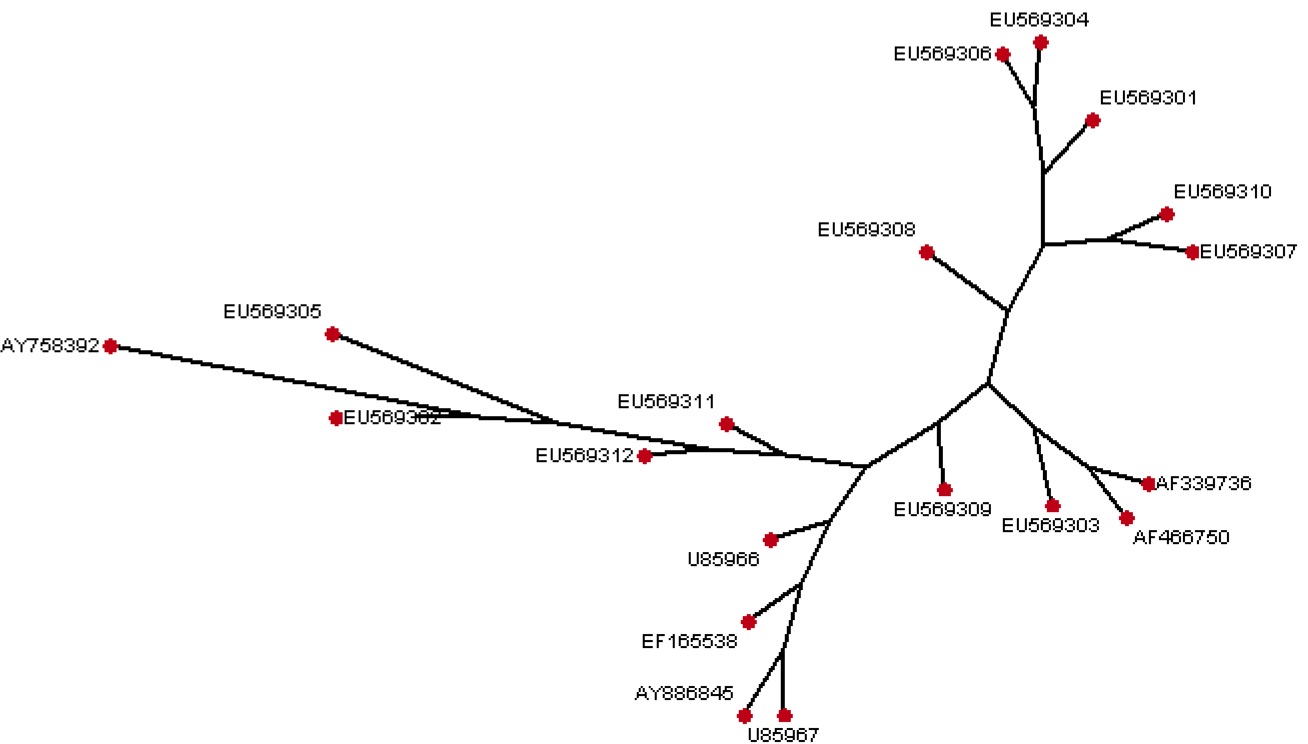


Figure 5.5. Phylogenetic tree depicting genetic sequence comparisons of the ITS-1 region between AUTf sequences and published GenBank sequences. AUTf isolates 1-7; 9-13 are listed as accession numbers EU569301-EU569312, respectively. *Pentatrichomonas hominis* (AY758392), bovine *T. foetus* isolates (AF339736 and U85967), feline *T. foetus* isolates (AF466750 and EF165538), *T. suis* (U85966) and *T. nonconforma* (AY886845) are also used in the comparison.

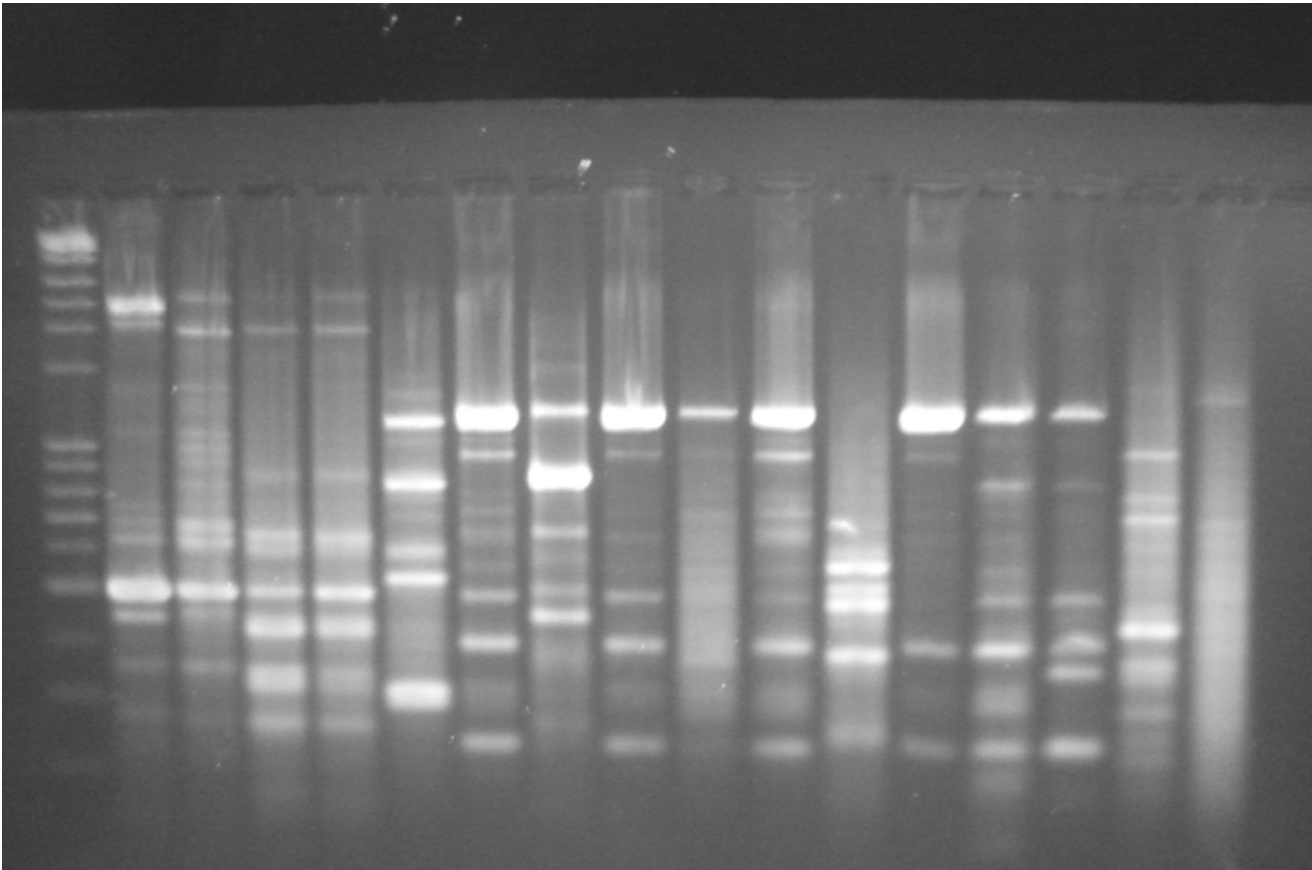


Figure 5.6. RAPD PCR results using 9 oligonucleotide 12-mer primers to compare genetic sequence banding patterns between AUTf feline trichomonad isolates and other trichomonad isolates. Far left: 1kb ladder, Lanes 1-5: bovine isolate (D-1), Florida bovine isolates no.25, 33, 34 and 36, respectively. Lanes 6-10, 12: AUTf isolates 1 (EU569301), 7 (EU569307), 9 (EU569308), 10 (EU569309), 4 (EU569304) and 12 (EU569311), respectively. Lane 11: *Trichomonas vaginalis*, Lanes 13-16: *Tritrichomonas mobilensis*, trichomonad from human peritoneal cavity, *Giardia* spp., and negative control, respective

dendrogram created using Phylodraw after using the Kitsch (PHYLIP) matrix analysis method (Fig. 5.7).

Discussion

The morphological characteristics of *T. foetus* recovered from the reproductive tract of cattle have been extensively described (Wenrich and Emmerson, 1933, Honigberg, et al., 1971, Mehlhorn, et al., 1988, Mattos, et al., 1997, Benchimol, 2004, 2005). The mean length of *T. foetus* isolated from cattle can range from 10-25µm and the width may be one-third the length, or an average of 6µm (Wenrich and Emmerson, 1933, Mattos, et al., 1997). There were no morphological differences between feline trichomonad isolates and *T. foetus* recovered from cattle.

A PCR protocol has been described that identifies trichomonads recovered from cattle using amplification of the ITS-1 region and differentiates between the trichomonads *T. foetus, P. hominis* and *Tetratrichomonas* sp. (Grahn, et al., 2005). The amplified sequence includes only 157 base pairs; however there is enough diversity in the ITS-1 region to differentiate between three separate genera of morphologically similar trichomonads. DNA sequence analysis of PCR products amplifying the ITS-1, 5.8SrRNA and ITS-2 genes (Felleisen, 1997, Gookin, et al., 2002, Grahn, et al., 2005) indicated a 97-100% sequence identity for feline (AF466750) and a 97-99% sequence identity for bovine (AF339736) isolates published in GenBank when compared against the 12 feline trichomonad isolate sequences (Table 5.4). Additionally, these feline sequences had a 97-99% sequence identity with *T. suis* (AY349190), 96-100% sequence identity with *T. nonconforma* (AY886845) and 96-100% sequence identity to *T. mobilensis* (AY886842).

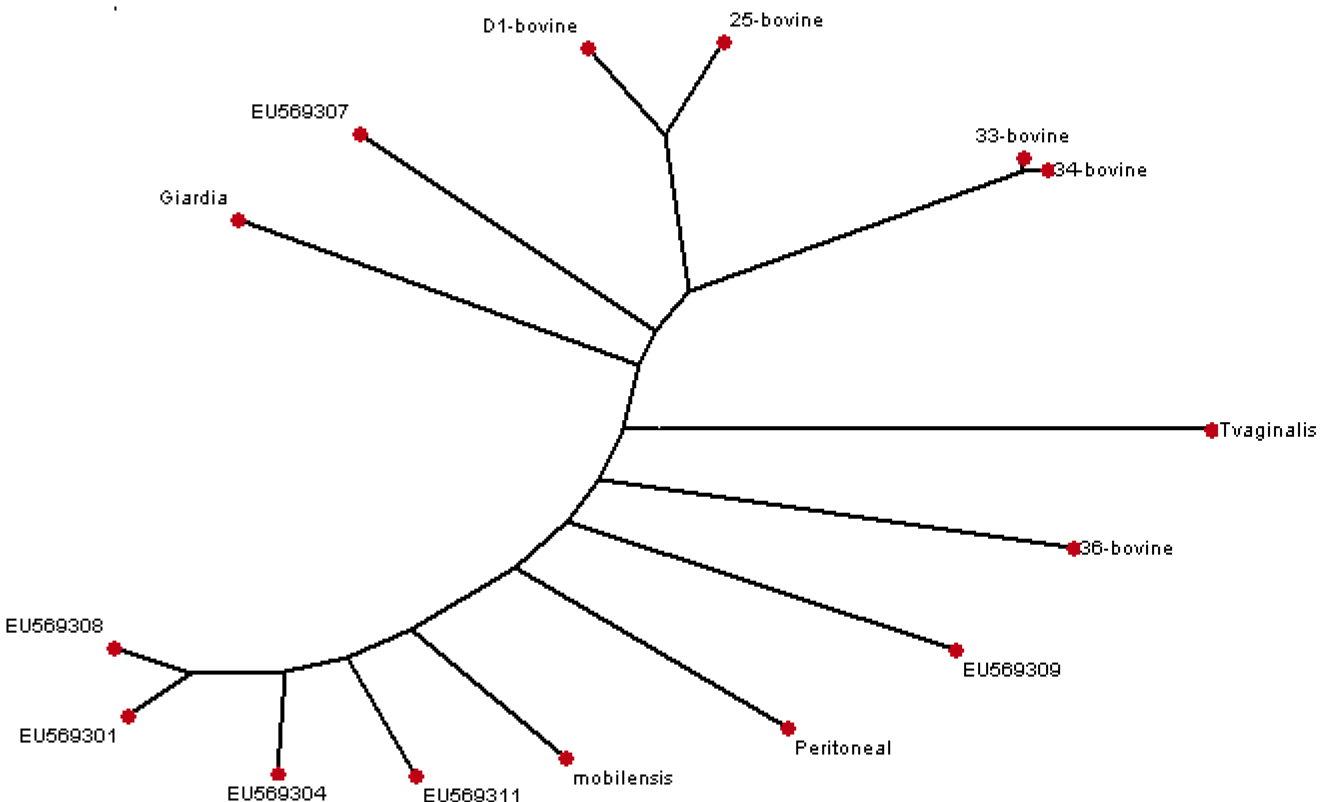


Figure 5.7. Phylogenetic tree depicting genetic sequence comparisons of the RAPD PCR results between AUTf sequences and published GenBank sequences. AUTf isolates 1,4,7, 9-10, 12 are listed as accession numbers EU569301, -304, -307, -308, -309, and -311, respectively. Bovine *T. foetus* isolates included (D-1) and those isolated from naturally infected cows in Florida (25-, 33-, 34-, and 36-bovine). Additional sequences from *T. mobilensis*, *Giardia* spp., *Trichomonas vaginalis* and trichomonads isolated from a human peritoneal cavity (peritoneal) were also used for comparison.

DNA sequence analysis at the ITS-1 and ITS-2 regions along with rRNA genes of feline trichomonad isolates are similar to those of *T. foetus* species recovered from naturally infected cats and cows. However, this is only true when the DNA sequences including both ITS regions and rRNA gene are compared. When looking at this region as a whole, the feline trichomonad isolates grouped with *T. foetus* isolates of both bovine and feline origin (Fig 5.3). This was also true when the ITS-2 region was used for comparison (Fig 5.4). These results are to be expected since the rRNA genes are highly conserved between species. When the nucleotide sequences are evaluated more closely, there are clear sequence differences at the ITS-1 region, demonstrated in both the phylogenetic tree (Fig. 5.5) and DNA sequences (Table 5.3). If only the ITS-1 gene region is used for comparison between the 12 feline trichomonad isolates and the feline (AF466750) and bovine (AF339736) isolates published in GenBank, the number of nucleotide insertions range from 0-7, nucleotide deletions range from 1-34 and nucleotide point mutations range from 1-19 (Table 5.3). The feline trichomonad isolates grouped separately or closer to *P. hominis* in a statistically significant manner (Fig 5.5).
This shift between two genera may suggest a species in transition with more than intra-specific genetic differences. Additionally, *T. suis* (U85966) grouped closely with *T. foetus* of bovine origin (AF339736, U85967) in each separate ITS sequence analysis (Figs. 5.3-5.5) supporting the proposed synonymy between *T. suis* and *T. foetus* (Tachezy, et al., 2002).

The RAPD results further support the differences between the feline and bovine trichomonad isolates at the ITS-1 region (Figs 5.6-5.7). Five of six feline trichomonad isolates grouped together, along with *T. mobilensis* and trichomonads isolated from the

human peritoneal cavity. Additionally, four of the five bovine isolates grouped together, branching separately from the feline isolates. The phylogenetic trees depicted the differences visualized in the banding patterns on the gel (Fig 5.6). Although there were differences among members of the bovine and feline isolates within each group, there were similarities between banding patterns of isolates within each group and between the two groups. The banding patterns of *T. mobilensis* and the additional trichomonad found in the human peritoneal cavity are more similar to those of the feline rather than bovine isolates. This difference is displayed in the phylogenetic tree (Fig 5.7).

These differences observed at the molecular level are further supported by the cross-infection studies that demonstrated significant differences in the host specificity of a trichomonad isolated from the feces a cat and the reproductive tract of a cow. *Tritrichomonas* sp. isolated from felines did not induce the same level pathogenesis in infected heifers when compared heifers infected with the bovine *T. foetus* isolate (Stockdale, et al., 2007). Of 8 sampled heifers infected with a feline trichomonad isolate, only 1 had lost the surface epithelial layer of the uterus, while all 6 sampled heifers infected with a bovine isolate of *T. foetus* has lost the surface epithelial layer. Additionally, of six cats infected with the bovine D-1 *T. foetus* isolate fecal culture samples from only one cat was culture positive for trichomonads and at PI day 32, the last day of sampling. The cat infected with the feline *Tritrichomonas* sp. was fecal culture positive by PI day 16 (Stockdale, et al., 2008).

DNA sequence analysis using highly conserved genes should not be the sole basis of species differentiation. There are numerous examples of parasite species within the same genus that can be differentiated based on host specificity and/or pathogenicity that

possess similar gene sequences. The *Plasmodium* gene *PgSES* contains 3 highly conserved regions within various protein homologs (LaCrue, et al., 2006). These conserved regions are found within 7 *Plasmodium* species and are expressed in multiple parasite stages. The ITS and rRNA genetic sequence data of the feline trichomonad isolates and published sequences of *T. foetus* of bovine and feline origin were nearly identical (98-99%) to *T. nonconforma* and *T. mobilensis*, two established and valid species (data not shown).

I feel that there are inconsistencies in host specificity and differences in pathogenicity of the feline and bovine trichomonad isolates, along with the DNA sequence analysis that exceed what would be expected as intra-specific variation. When ITS-1 gene regions are isolated for comparison between feline and bovine isolates of *T. foetus* there are clear sequence differences that may account for species differences in addition to genera differentiation as previously described (Grahn, et al., 2005). Results from the RAPD PCR analysis suggest additional genetic differences between bovine and feline trichomonad isolates and suggest species differences and that the causative agent of feline trichomoniasis is not *Tritrichomonas foetus*, in light of past molecular and morphological data.

CHAPTER VI

OVERALL CONCLUSIONS

Results of my research indicate that *Tritrichomonas foetus* is prevalent in the pet population and that its presence appears to correlate with the presence of diarrhea. Of the 173 cats surveyed, 17 were fecal culture positive for *T. foetus* and all suffered from chronic diarrhea. Our survey offers limited insight into the prevalence of *T. foetus* in the pet cat population and produces additional questions. One question that remains is whether *T. foetus* is the only cause of large-bowel disease in cats or if it is an opportunist working in conjunction with other pathogens to exacerbate the symptoms of enteritis. Many reported cases of feline trichomoniasis also report concurrent infections with *Giardia* spp., *Cryptosporidium* spp., coccidia or the cat also suffered from FIP. Another question is whether pure bred cats are at a greater risk of *T. foetus* infection than domestic mixed breeds or if likelihood of infection can be attributed more to living environments. Many of the infected cats in this survey live in multi-cat households or catteries where cats are densely populated. There have also been no reports linking *T. foetus* infection in the feline intestine to *T. foetus* infection in the bovine reproductive tract. The possibility of cross-transmission of *T. foetus* between the two species seems unlikely.

The combined results of the experimental infections in bovines and felines indicate that the diseases caused by the feline and bovine isolates of *T. foetus* in cattle are not identical and the susceptibility of cats to the feline and bovine isolates of *T. foetus*

also appears demonstrably different. Biopsy samples taken from the 8 heifers experimentally infected with *T. foetus* collected from a naturally infected cat demonstrated that a single heifer lost the surface epithelial layer of the uterus. When the same procedure was performed using heifers infected with *T. foetus* collected from a naturally infected, pyometritic cow all 6 available samples showed a loss of surface epithelium. Lesions scores between two groups were significantly different (P = 0.005). Additionally, there was a significant difference in the time required to clear the trichomonad infection between the two groups. Those heifers infected with the bovine isolate cleared the infection by post-inoculation (PI) week 19, while 2 heifers infected with the feline isolate were still culture positive by PI week 30.

In the second experimental infection study, the cat infected with a feline isolate of *T. foetus* became fecal culture positive by PI day 16, while only 1 of 6 cats infected with the bovine isolate was fecal culture positive by PI day 32. Additionally, when intestinal contents of each cat were analyzed it was found that the cat infected with the feline isolate was culture positive in the ileum, cecum, middle and distal colon. Of the 6 cats infected with the bovine isolate of *T. foetus,* 2 were culture positive in the cecum only. These results suggest that feline and bovine trichomonad isolates are different species with different host specificities. These data also suggest that direct transmission from bovines to felines is not the primary means of trichomonad infection in cats.

Further biological characterization of 12 feline isolates of *T. foetus* presented to the parasitology laboratory at Auburn University demonstrated similar morphological characteristics when using published descriptions of both bovine and feline *T. foetus* isolates. Differences were found primarily in the internal transcribed spacer (ITS) 1

region rather than the ITS-2 or highly conserved 5.8SrRNA regions commonly used for comparisons of trichomonads. In some feline isolates, there were as many as 34 nucleotide changes that were either insertions, deletions or point mutations, when compared with bovine and some feline isolates published in GenBank. Phylogenetic analysis suggests variable sequence differences between the isolates depending on the genetic region analyzed (i.e. ITS-1, ITS-2, rRNA). Feline isolates tended to group with themselves or in many instances closer to *Pentatrichomonas hominis.* This could suggest a species in transition implying there are more than intra-specific differences between the isolates. Differences between feline and bovine isolates are also supported in data produced by random amplified polymorphic DNA (RAPD) analysis where banding patterns of feline isolates were more similar to each other than to bovine isolates. This was also depicted by the phylogenetic tree. Additionally, all *T. foetus* isolates used for DNA sequence comparisons were nearly identical (97-100%) to *T. nonconforma* and *T. mobilensis*, two established and valid species originating from an *Anolis* lizard and tree shrew, respectively. These studies provide compelling evidence that fundamental differences exist between the different isolates of *T. foetus* exceeding what one would expect to observe in normal intra-specific variation.

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APPENDIX A

COMPLETE DATA RESULTS OBTAINED FROM VETERINARIANS PARTICIPATING IN THE SURVEY OF THE PET CAT POPULATION

ID #	State of Origin	Date	Veterinarian	Age	Sex	Breed	Lifestyle	Origin	Geographic location	Fecal scale*	Diarrhea	Concurrent disease	Additional comments	Culture positive or negative
1	Alabama	02/24/05	West	2yo	M	Siamese	N/A	N/A	N/A	N/A	loose stool, bacterial overgrowth, soiling outside litterbox	ringworm	N/A	positive
2	Alabama	09/27/05	Hankes	2yo	M	Russian blue	N/A	N/A	N/A	N/A	chronic diarrhea	*Cryptosporidium*	N/A	positive
3	Alabama	10/01/05	McMillian	17 wo	F	Bengal	N/A	N/A	N/A	N/A	diarrhea w/blood, green	FIP	cat euthanized	positive
4	North Carolina	10/13/05	Whitley	2yo	F	Japanese bobtail	N/A	N/A	N/A	N/A	relapse from 6/04; chronic diarrhea	N/A	N/A	positive
5	Louisiana	02/06/06	Buzhardt	?/ adult	M	DLH	outdoor	stray	N/A	0	1st day noticed	no vaccines or deworm, FIV/FeLV neg	N/A	negative
6	Louisiana	02/06/06	Buzhardt	4yo	M	DLH	in/outdoor	stray	N/A	100	N/A	FIV/FeLV neg	N/A	negative
7	Louisiana	02/06/06	Buzhardt	7mo	M	DSH	indoor	stray	city	0	blood; duration 7mo, treated 5mo ago with Panacur 5days	none known, FIV/FeLV neg	N/A	negative
8	Louisiana	02/01/06	Buzhardt	6 yo	M	DSH	indoor	shelter	rural area	0	duration 30 days, no response to panacur, metronidazole, W/D	diabetes	presented for second opinion of weight loss and diarrhea	negative
9	Louisiana	02/01/06	Buzhardt	5.5mo	M	DSH	outdoor	stray	town	100	N/A	none known, periodontal disease, gingevitis	N/A	negative
10	Louisiana	02/01/06	Buzhardt	11mo	F	DSH	indoor	adopted	city	66	N/A	N/A	presented for recurring respiratory problem	negative
11	Louisiana	02/01/06	Buzhardt	7yo	M	DSH	outdoor	N/A	farm	100	N/A	FIV/FeLV neg, diabetes	N/A	negative
12	Louisiana	02/01/06	Buzhardt	5yo	F	DSH	indoor	adopted	city	N/A	N/A	FIV/FeLV neg, fecal neg	N/A	negative
13	Alabama	02/23/06	Houston	7yo	M	DSH	in/outdoor	owned since kitten	city	100	N/A	none	N/A	negative
14	Alabama	03/03/06	Houston	11yo	F	DSH	indoor	home	city	100	N/A	N/A	N/A	negative
15	Kentucky	03/04/06	Zink	8mo	F	DSH	outdoor	wild	farm	100	none	none	N/A	negative
16	Kentucky	03/03/06	Zink	5yo	F	DSH	indoor	pet	suburb	100	none	none	N/A	negative
17	Kentucky	02/28/06	Zink	1yo	F	DSH	outdoor	pet	suburb	33	unknown	N/A	N/A	negative
18	Kentucky	03/04/06	Zink	3yo	F	DSH	in/outdoor	pet	suburb	66/75	N/A	none	N/A	negative
19	Kentucky	03/06/06	Zink	6yo	F	DSH	indoor	pet	suburb	100	N/A	N/A	N/A	negative
20	Kentucky	03/06/06	Zink	1yo	F	DMH	outdoor	stray	suburb	66	N/A	unknown	N/A	negative
21	Alabama	03/10/06	Houston	8yo	M	DMH	indoor	owned since kitten	city	66	N/A	unknown	N/A	negative
22	Alabama	03/13/06	Houston	5yo	F	DSH	in/outdoor	stray	city	66	N/A	N/A	N/A	negative
23	Alabama	04/17/06	Houston	2yo	F	Ragdoll	indoor	breeder	city	66	N/A	N/A	N/A	negative
24	Alabama	05/01/06	Houston	5yo	F	DSH	in/outdoor	stray	city	100	N/A	N/A	N/A	negative
25	Alabama	05/08/06	Houston	3yo	F	DSH	indoor	shelter	city	100	N/A	N/A	N/A	negative
26	Alabama	05/08/06	Houston	9y	F	DMH	indoor	shelter	rural area	100	N/A	N/A	N/A	negative
27	Alabama	05/09/06	Houston	2yo	F	DSH	indoor	shelter	city	100	N/A	tapeworms	N/A	negative
28	Alabama	05/10/06	Houston	8yo	F	DSH	indoor	stray	city	N/A	N/A	N/A	ScienceDiet I/D dry	negative
29	Alabama	05/10/06	Houston	2yo	M	DSH	indoor	unknown	city	100	N/A	N/A	ScienceDiet I/D dry, stool formed/hard	negative
30	Alabama	05/11/06	Houston	2yo	M	DSH	in/outdoor	stray	city	66	N/A	N/A	N/A	negative

31	Alabama	05/11/06	Houston	1yo	F	DSH	indoor	stray	city	66	N/A	FeLV/FIV not checked	N/A	negative
32	Alabama	05/11/06	Houston	16yo	F	DSH	indoor	stray	city	N/A	N/A	heart murmur	ScienceDiet W/D	negative
33	Alabama	05/11/06	Houston	14yo	F	DSH	indoor	stray	city	100	N/A	allergies/steroid trt 3x yearly	N/A	negative
34	Alabama	05/11/06	Houston	6yo	F	Persian	indoor	breeder	city	100	N/A	N/A	N/A	negative
35	Alabama	05/11/06	Houston	6yo	F	Persian	indoor	breeder	city	100	N/A	N/A	ScienceDiet W/D	negative
36	Alabama	05/11/06	Houston	2yo	M	DSH	indoor	shelter	city	100	N/A	N/A	N/A	negative
37	Alabama	05/11/06	Houston	9yo	M	Siamese	indoor	breeder	city	100	N/A	N/A	N/A	negative
38	Alabama	05/16/06	Houston	12yo	M	DSH	outdoor	shelter	city	100	N/A	N/A	N/A	negative
39	Alabama	05/16/06	Houston	10yo	M	DSH	indoor	shelter	city	66	N/A	mast cell tumors	N/A	negative
40	Alabama	05/17/06	Houston	8yo	F	DSH	indoor	owned since kitten	city	100	N/A	N/A	N/A	negative
41	Alabama	05/18/06	Houston	13yo	M	DLH	indoor	owned since kitten	city	100	N/A	N/A	N/A	negative
42	Alabama	05/18/06	Houston	2yo	M	DSH	in/outdoor	shelter	city	100	N/A	N/A	N/A	negative
43	Alabama	05/17/06	Houston	6yo	M	DSH	indoor	owned since kitten	city	100	N/A	N/A	N/A	negative
44	Kentucky	05/19/06	Zink	2yo	F	DSH	in/outdoor	stray	farm	66	N/A	FeLV/FIV negative	N/A	negative
45	Kentucky	05/15/06	Zink	2yo	M	DSH	indoor	shelter	city	100	N/A	N/A	N/A	negative
46	Kentucky	05/16/06	Zink	4wo	F	DSH	outdoor	stray	farm	33	N/A	N/A	N/A	negative
47	Kentucky	05/16/06	Zink	8mo	F	DSH	indoor	unknown	suburb	66	N/A	N/A	N/A	negative
48	Alabama	05/23/06	Houston	6.5yo	F	DSH	outdoor	owned since kitten	city	66	N/A	N/A	N/A	negative
49	Alabama	06/02/06	Houston	N/A	F	DLH	outdoor	stray	city	66	N/A	combo test negative	possibly pregnant	negative
50	Alabama	06/02/06	Houston	14yo	F	DSH	indoor	owned since 2001	city	100	N/A	N/A	N/A	negative
51	Alabama	06/03/06	Houston	9yo	F	DSH	in/outdoor	owned since kitten	city	66	N/A	N/A	recently declawed, Science Diet Z/D	negative
52	Alabama	06/15/06	Houston	1yo	F	DMH	in/outdoor	stray	city	100	N/A	none known	N/A	negative
53	Alabama	06/15/06	Houston	6mo	F	DSH	indoor	stray	city	N/A	N/A	N/A	N/A	negative
54	Alabama	06/15/06	Houston	6mo	F	DSH	indoor	stray	city	N/A	N/A	N/A	N/A	negative
55	Kentucky	06/12/06	Zink	5yo	M	DSH	indoor	free	suburb	100	N/A	N/A	N/A	negative
56	Kentucky	06/14/06	Zink	3yo	F	DSH	in/outdoor	stray	farm	100	N/A	N/A	N/A	negative
57	Kentucky	06/19/06	Zink	1yo	M	DMH	in/outdoor	stray	suburb	100	N/A	N/A	N/A	negative
58	Kentucky	06/20/06	Zink	5wo	F	DSH	outdoor	stray	farm	33	N/A	nemex	N/A	negative
59	Hawaii	06/28/06	Gocke-Smith	6wo	M	DSH	N/A	cattery	N/A	N/A	chronic diarrhea	*Giardia*, ringworm	N/A	positive
60	Hawaii	06/28/06	Gocke-Smith	7wo	M	DSH	N/A	cattery	N/A	N/A	chronic diarrhea	*Giardia* , ringworm	N/A	positive
61	Kentucky	07/03/06	Zink	<3mo	F	DSH	indoor	stray	suburb	0	since birth ?	albon	N/A	negative
62	Kentucky	07/03/06	Zink	<3mo	M	DLH	indoor	stray	suburb	0	1 week	none	N/A	negative
63	Alabama	07/09/06	Houston	4mo	M	DSH	indoor	shelter	rural area	100	N/A	N/A	N/A	negative
64	Alabama	07/07/06	Houston	13yo	F	DLH	in/outdoor	shelter	rural area	100	N/A	N/A	N/A	negative
65	Alabama	08/01/06	Houston	1yo	M	DMH	N/A	N/A	city	N/A	N/A	previous roundworm infection	N/A	negative
66	Alabama	08/01/06	Houston	1yo	F	DMH	N/A	N/A	city	N/A	N/A	previous roundworm infection	N/A	negative
67	Alabama	08/03/06	Houston	3yo	F	DSH	indoor	unknown	city	100	N/A	N/A	N/A	negative
68	Alabama	08/03/06	Houston	3yo	F	DSH	indoor	unknown	city	100	N/A	N/A	N/A	negative
69	Hawaii	08/04/06	Goecke-Smith	7wo	M	DSH	N/A	cattery	N/A	N/A	bloody diarrhea	upper resp. infection, ringworm, *Giardia*	N/A	positive
70	California	08/07/06	Miller	12 wo	M	Scottish fold	N/A	cattery/ shelter	N/A	N/A	chronic diarrhea	N/A	N/A	positive
71	Alabama	08/08/06	Houston	6yo	F	DLH	indoor	unknown	city	100	N/A	N/A	N/A	negative

72	Alabama	08/10/06	LCHS	<6mo	F	DSH	N/A	shelter	N/A	N/A	N/A	N/A	N/A	negative
73	Alabama	08/10/06	LCHS	<6mo	F	DSH	N/A	shelter	N/A	N/A	N/A	N/A	N/A	negative
74	Alabama	08/10/06	LCHS	<6mo	M	DSH	N/A	shelter	N/A	N/A	N/A	N/A	N/A	negative
75	California	08/13/06	Cooper	7mo	M	Toyger	indoor	cattery	N/A	N/A	chronic diarrhea	N/A	N/A	positive
76	California	08/13/06	Cooper	1.6 yo	F	Toyger	indoor	cattery	N/A	N/A	chronic diarrhea	N/A	N/A	positive
77	California	08/13/06	Cooper	7mo	F	Toyger	indoor	cattery	N/A	N/A	chronic diarrhea	N/A	N/A	positive
78	California	08/13/06	Cooper	2yo	F	Toyger	indoor	cattery	N/A	N/A	chronic diarrhea	N/A	N/A	negative
79	California	08/13/06	Cooper	1.1yo	F	Toyger	indoor	cattery	N/A	N/A	chronic diarrhea	N/A	N/A	negative
80	California	08/13/06	Cooper	3yo	F	Toyger	indoor	cattery	N/A	N/A	chronic diarrhea	N/A	N/A	negative
81	California	08/13/06	Cooper	8mo	M	Toyger	indoor	cattery	N/A	N/A	chronic diarrhea	N/A	N/A	positive
82	Hawaii	08/29/06	Goecke-Smith	21wo	M	DSH	indoor	shelter	city and suburb	100	N/A	FeLV neg	upper respiratory dx	negative
83	Hawaii	08/29/06	Goecke-Smith	17wo	M	DSH	indoor	shelter	city and suburb	100	N/A	FeLV neg	upper respiratory dx	negative
84	Hawaii	09/13/06	Goecke-Smith	14wo	M	DSH	indoor	stray	rural area	100	N/A	finished ronidazole trt 9.10.06	previously *T. foetus* and *Giardia* positive	negative
85	Hawaii	09/07/06	Goecke-Smith	2yo	M	DSH	indoor	rescue/breeder /shelter	city	N/A	had diarrhea at least 1 year	trt w/every anti-giardia med available	grew out of diarrhea	negative
86	Tennessee	10/02/06	Given	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	negative
87	Delaware	10/02/06	Stoltzfus	10yo	F	DSH	N/A	N/A	N/A	N/A	soft pasty stool	N/A	N/A	negative
88	Delaware	10/02/06	Stoltzfus	10yo	M	DSH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	negative
89	Hawaii	09/27/06	Goecke-Smith	1yo	F	DSH	in/outdoor	stray	suburb	N/A	soft w/ blood	N/A	N/A	negative
90	Hawaii	09/26/06	Goecke-Smith	8wo	F	DSH	outdoor	stray	rural area	N/A	soft	coccidia/ albon trt	N/A	negative
91	Hawaii	09/26/06	Goecke-Smith	10wo	F	DSH	outdoor	stray	rural area	N/A	firm/dehydrated	coccidia/ albon trt	N/A	negative
92	Texas	10/02/06	Cooper	10mo	M	Toyger	N/A	breeder	N/A	N/A	diarrhea	hx *T.foetus* infection, *Isospora*	N/A	positive
93	Kentucky	10/04/06	Wade	2yo	M	DSH	indoor	rescue	country	N/A	blood in stool, loose	FIV positive	N/A	negative
94	Kentucky	10/04/06	Wade	15yo	F	DSH	in/outdoor	rescue	city	N/A	pudding-like, blood, diarrhea on/off 5yrs	N/A	unthrifty coat	negative
95	Kentucky	10/04/06	Wade	13yo	F	DMH	indoor	rescue	city	N/A	pudding consistancy, bad odor, diarrhea 3/5 days for 4 months	N/A	green pea and duck diet	negative
96	Hawaii	10/05/06	Goecke-Smith	12wo	F	DSH	indoor	shelter/rescue	suburb	normal	initially had diarrhea when adopted	unknown	N/A	negative
97	Hawaii	10/05/06	Goecke-Smith	12wo	M	DSH	indoor	shelter/rescue	suburb	normal	initially had diarrhea when adopted	unknown	N/A	negative
98	Alabama	10/20/06	LCHS	1yo	M	DSH	N/A	shelter	N/A	N/A	N/A	not dewormed	N/A	negative
99	Alabama	10/20/06	LCHS	1yo	M	DSH	N/A	shelter	N/A	N/A	N/A	not dewormed	N/A	negative
100	Alabama	10/20/06	LCHS	5wo	F	DSH	N/A	shelter	N/A	N/A	N/A	not dewormed	littermate #201	negative
101	Alabama	10/20/06	LCHS	5wo	M	DSH	N/A	shelter	N/A	N/A	N/A	not dewormed	littermate #200	negative
102	Alabama	10/20/06	LCHS	1yo	F	DMH	N/A	shelter	N/A	N/A	N/A	dewormed (hooks, rounds, whips)	tapeworms	negative
103	Florida	11/01/06	Pitts	7mo	M	Bengal	N/A	breeder	N/A	diarrhea	N/A	N/A	N/A	negative
104	Louisiana	10/27/06	Buzhardt	14wo	M	DSH	indoor	stray	city	33	N/A	N/A	N/A	negative
105	Louisiana	10/27/06	Buzhardt	9yo	F	DSH	indoor	stray	city	100	N/A	N/A	N/A	negative
106	Alabama	11/03/06	LCHS	1yo	F	Persian	N/A	shelter	N/A	N/A	N/A	not dewormed	N/A	negative
107	Alabama	11/03/06	LCHS	5wo	M	Persian	N/A	shelter	N/A	N/A	N/A	not dewormed	N/A	negative
108	Alabama	11/03/06	LCHS	4wo	M	Persian	N/A	shelter	N/A	N/A	N/A	not dewormed	N/A	negative

109	Alabama	11/03/06	LCHS	5wo	F	Persian	N/A	shelter	N/A	N/A	N/A	not dewormed	N/A	negative
110	Alabama	11/03/06	LCHS	1yo	F	DSH	N/A	shelter	N/A	N/A	N/A	not dewormed	N/A	negative
111	Alabama	11/03/06	LCHS	5wo	F	DSH	N/A	shelter	N/A	N/A	N/A	not dewormed	N/A	negative
112	Hawaii	10/31/06	Goecke-Smith	6mo	M	DSH	indoor	shelter	N/A	6 or 7	constant diarrhea	N/A	dev. diarrhea 1 month after adoption, cowpie diarrhea 3 weeks	negative
113	Tennessee	11/06/06	Vanderpool	9yo	F	DSH	N/A	N/A	N/A	N/A	intermittent diarrhea, occ. blood, duration 1 year	prev. *Giardia* diagnosis, trt metronidazole	N/A	negative
114	Tennessee	11/08/06	Given	10yo	N/A	Himalayan	N/A	N/A	N/A	N/A	chronic diarrhea	N/A	N/A	negative
115	Tennessee	11/08/06	Given	11mo	N/A	Persian	N/A	N/A	N/A	N/A	chronic diarrhea	N/A	N/A	negative
116	Tennessee	11/08/06	Given	1yo	N/A	Himalayan	N/A	N/A	N/A	N/A	chronic diarrhea	N/A	N/A	negative
117	Alabama	11/10/06	LCHS	1yo	M	DLH	N/A	shelter	N/A	N/A	N/A	not dewormed	N/A	negative
118	Alabama	11/10/06	LCHS	1yo	M	DSH	N/A	shelter	N/A	N/A	N/A	not dewormed	N/A	negative
119	Alabama	11/10/06	LCHS	1yo	M	DSH	N/A	shelter	N/A	N/A	N/A	not dewormed	N/A	negative
120	Alabama	11/10/06	LCHS	1yo	F	DMH	N/A	shelter	N/A	N/A	N/A	not dewormed	N/A	negative
121	Alabama	11/10/06	LCHS	6mo	F	DLH	N/A	shelter	N/A	N/A	N/A	not dewormed	N/A	negative
122	Virginia	11/06/06	Maurs	5yo	M	DSH	indoor	adopted	suburb	cowpie	N/A	N/A	trt w/ Tyler powder, dewormed, metronidazole, hypoallergenic diet	negative
123	Alabama	11/17/06	LCHS	8wo	M	DLH	N/A	shelter	N/A	N/A	diarrhea at least 1 week	fleas, dehydrated	trt w/ doxycyclin	negative
124	Hawaii	11/21/06	Goecke-Smith	4yo	M	DSH	in/outdoor	unknown	suburb	6	diarrhea over 1 year	FIV, FeLV, *Giardia*--Negative	cysts observed in direct smear	negative
125	Tennessee	11/21/06	Graves	1y4mo	F	DSH	N/A	N/A	N/A	N/A	diarrhea for last couple months	N/A	N/A	negative
126	Tennessee	12/04/06	Prior	12yo	F	DSH	N/A	N/A	N/A	N/A	diarrhea for one month	N/A	N/A	negative
127	North Carolina	12/28/06	Carroll	10yo	M	DSH	indoor	stray	city	33	long term diarrhea, mucus, trt w/metronidazole, denosyl	pancreatic insuf.	N/A	negative
128	North Carolina	12/28/06	Carroll	12wo	F	DSH	outdoor	shelter	rural area	0	mucoid, no blood	N/A	nemex trt	negative
129	North Carolina	12/28/06	Carroll	4mo	F	DSH	outdoor	stray	unknown	0	frequent last 2-3 days	FeLV, FIV negative	nemex trt	negative
130	North Carolina	01/05/07	Carroll	5yo	F	DSH	outdoor	shelter	rural area	66	N/A	N/A	N/A	negative
131	North Carolina	01/05/07	Carroll	4mo	M	DLH	outdoor	shelter	rural area	66	N/A	N/A	N/A	negative
132	Alabama	01/09/07	Frederick	14yo	F	DSH	N/A	N/A	N/A	N/A	chronic diarrhea	N/A	trt metronidazole	negative
133	North Carolina	03/13/06	Carroll	11yo	M	DSH	outdoor	shelter/stray	farm	33	no blood; 3-4 months, clr. 2 weeks	poss. Hyperthyroid, never tested	stray	negative
134	Alabama	01/24/07	Amalsadvala	8mo	M	DSH	N/A	N/A	N/A	semi-solid	N/A	rectal prolapse, prev. coccidia infection	N/A	negative

135	North Carolina	03/14/06	Carroll	3yo	F	DSH	outdoor	shelter/stray	farm	100	N/A	unknown	stray	negative
136	Alabama	01/24/07	Amalsadvala	8mo	M	DSH	N/A	N/A	N/A	semi-solid	N/A	rectal prolapse, prev. coccidia infection	N/A	negative
137	North Carolina	03/14/06	Carroll	6yo	M	DSH	indoor	shelter/stray	rural area	100	N/A	FeLV/FIV not checked	stray	negative
138	North Carolina	03/14/06	Carroll	1yo	M	DSH	outdoor	shelter/stray	farm	100	N/A	N/A	stray	negative
139	Tennessee	01/26/07	Vanderpool	3yo	M	DSH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	negative
140	North Carolina	03/14/06	Carroll	5yo	M	DLH	outdoor	shelter/stray	farm	66	mild mucous	N/A	stray	negative
141	Georgia	N/A	Jones	12wo	M	Scottish fold	N/A	N/A	N/A	N/A	chronic diarrhea	N/A	N/A	negative
142	North Carolina	03/14/06	Carroll	10yo	F	DLH	outdoor	shelter/stray	farm	100	N/A	N/A	stray	negative
143	Georgia	N/A	Jones	12wo	M	Scottish fold	N/A	N/A	N/A	N/A	chronic diarrhea	N/A	N/A	negative
144	California	03/15/06	Barron	4mo	F	Himalayan X	N/A	N/A	N/A	N/A	chronic diarrhea	N/A	panacur/ metronidazole/ baytril	negative
145	North Carolina	03/14/06	Carroll	8yo	F	DSH	outdoor	shelter/stray	farm	100	N/A	N/A	stray	negative
146	Missouri	11/16/05	Perrin	N/A	N/A	DSH	N/A	N/A	N/A	N/A	chronic diarrhea	N/A	panacur and metronidazole	negative
147	North Carolina	03/14/06	Carroll	1yo	F	DSH	outdoor	shelter/stray	farm	100	N/A	N/A	stray	negative
148	Florida	08/08/05	Donofro	2yo	M	DSH	N/A	N/A	N/A	N/A	chronic diarrhea	N/A	N/A	negative
149	North Carolina	03/14/06	Carroll	1yo	F	DSH	outdoor	shelter/stray	farm	100	N/A	taeniid proglottid	stray	negative
150	Delaware	06/30/05	Patterson	11mo	M	DSH	N/A	N/A	N/A	N/A	chronic diarrhea	N/A	fenbendazole, metronidazole, w/d diet	negative
151	North Carolina	03/14/06	Carroll	2yo	M	DSH	outdoor	shelter/stray	farm	33	unknown	N/A	stray	negative
152	Delaware	05/10/06	Stoltzfus	4wo	F	DSH	N/A	N/A	N/A	N/A	chronic diarrhea	N/A	N/A	negative
153	North Carolina	03/14/06	Carroll	3yo	M	DSH	outdoor	shelter/stray	farm	100	N/A	roundworm egg	stray	negative
154	Alabama	05/10/06	Gamper	14mo	M	DSH	N/A	N/A	N/A	N/A	chronic diarrhea	coccida/*giardia* positive	N/A	negative
155	North Carolina	03/14/06	Carroll	8mo	F	DSH	outdoor	shelter/stray	farm	100	N/A	roundworm egg	stray	negative
156	Kentucky	08/23/05	Lacki	1yo	F	DLH	N/A	N/A	N/A	N/A	chronic diarrhea	N/A	N/A	negative
157	North Carolina	03/14/06	Carroll	3yo	F	DSH	outdoor	shelter/stray	farm	100	N/A	N/A	stray	negative
158	Alabama	03/05/07	McMillan	10yo	F	DSH	N/A	N/A	N/A	N/A	chronic diarrhea	N/A	clavamox, flagyl, albon, zeniquin, erythromycin	negative
159	North Carolina	03/14/06	Carroll	2yo	F	DSH	outdoor	shelter/stray	farm	66	unknown	N/A	stray	negative
160	California	05/17/07	Noah's Ark	9mo	M	Siamese	N/A	N/A	N/A	N/A	chronic diarrhea since 5mo	N/A	flagyl, panacur, ID	negative
161	North Carolina	N/A	Carroll	3yo	F	DLH	N/A	N/A	N/A	33	unknown	N/A	stray	negative

162	North Carolina	03/14/06	Carroll	2yo	M	DSH	outdoor	shelter/stray	farm	100	NIA	NIA	stray	negative
163	Alabama	05/21/07	Cobb	9mo	M	Himalayan x	indoor	NIA	NIA	NIA	chronic diarrhea since 7mo	lethargic, not eating,low fever, multiple bm, very smelly, motile bacteria, dx as giardia	panacur, metron., albendazole	positive
164	Tennessee	05/22/07	Cassetty	12yo	M	DSH	NIA	NIA	NIA	NIA	chronic diarrhea 1 year	NIA	metronidazole	negative
165	Alabama	06/13/07	Cobb	1.5yo	M	Bengal	NIA	NIA	NIA	NIA	diarrhea 1mo	found *Giardia* in ds, also coccidia	met, albon, panacur, baytril	negative
166	Tennessee	07/17/07	Prior	4yo	F	DSH	NIA	NIA	NIA	NIA	chronic diarrhea, otherwise healthy	*Giardia* snap neg.	NIA	negative
167	Tennessee	09/05/07	Bell	11mo	F	DSH	NIA	NIA	NIA	NIA	diarrhea in 4/07; dx giardia and severe diarrhea recently	*Giardia* snap pos	met, panacur	positive
168	Ohio	10/05/07	Burnett	2.6yo	M	DSH	NIA	NIA	NIA	NIA	chronic diarrhea, resolving	NIA	NIA	negative
169	Ohio	10/05/07	Burnett	4yo	M	DMH	NIA	stray; shelter	NIA	NIA	chronic diarrhea	coccidia; losing weight	unable to treat; euthanized	positive
170	Tennessee	10/20/07	Murphy road	17 wo	F	DSH	NIA	NIA	NIA	NIA	chronic diarrhea	*Giardia* snap pos	met, panacur	negative
171	Tennessee	11/14/07	Given	11yo	M	DSH	NIA	NIA	NIA	NIA	chronic diarrhea, highly motile bacteria, RBCs	*Giardia* possible on smear	met, panacur	negative
172	Indiana	11/14/07	Weisman	1yo	F	DSH	NIA	NIA	NIA	NIA	chronic diarrhea	NIA	NIA	negative
173	New Jersey	03/17/08	Hill	12yo	F	Abyssinian	NIA	cattery	NIA	NIA	chronic diarrhea	NIA	NIA	positive

APPENDIX B

ALIGNED SEQUENCES FOR ALL ISOLATES USED IN GENETIC COMPARISONS OF THE ITS-1, ITS-2 AND 5.8S rRNA GENES

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 20 samples (GenBank Accession numbers)  350 base pairs
AF339736  TCAGTTTCGTTAATAATTAC----AAACATAT-TTTTTTAATGTCTATAA
AF466750  TCAGTTTCGTTAATAATTAC----AAACATAT-TTTTTTAATGTCTATAA
DQ233455  -------------AAAATAC----AAACATTAATTTTTTTAAAATTTTAA
EU569301  -CAGTTTCGTTAATAATTAC-----AACATAT-TTTTTTAATGTCTATAT
EU569302  TCAGTTTCGTTAACTATGCC---GTTGTATCT-GTTCTCATAGCCCGGGC
EU569303  -CAGTTTCGTTAATAATTAC----AA-CATAT-TTTTTTAATGTCTATAC
EU569304  TCAGTTTCGTTAATTACAA-------ACATAT-TTTTTT-ATGTCTATAT
EU569305  -------------CCCTGCCC--GTTGGATCT-TTTCGT-TAACC--GGC
EU569306  -CAGTTTCGTTAATAATTAC---AAAACATAT-TTTTTTATTGTCTATAT
EU569307  ---GTTTCGTTAATA-TTAC---AAA-CATAT-TTTTTT-TTGTCTATAA
EU569308  ---GTTTCGTTAACTGAGTGT--ACAACATATATTTTTTATTGTCTATAA
EU569309  --AGTTTCGTTAATAATTAC----AAACATAT-TTTTTTAATGTCTATAA
EU569310  TCAGTTTCGTTAATAGTTAC-----AACATAT-TTTTTTATTGTCTATAA
EU569311  TCAGTTTCGTTAATAATTAC-----AACATAT-TTTTTT-ATGTCTATCT
EU569312  ---GTTTCGTTAACTATTAC-----AACATAT-TTTTTT-ATGTCTATCA
AY758392  TAACGGTAGGTGAACCTGCCGTTGGATCAGTTCTAGTTTAATACCAAATT
U85967    TCAGTTTCGTTAATAATTAC----AAACATAT-TTTTTTAATGTCTATAA
EF165538  TCAGTTTCGTTAATAATTAC----AAACATAT-TTTTTTAATGTCTATAA
AY886845  TCAGTTTCGTTAATAATTAC----AAACATAT-TTTTTTAATGTCTATAA
U85966    TCAGTTTCGTTAATAATTAC----AAACATAT-TTTTTTAATGTCTATAA

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CTATTTATAC--AAAATTAAA-CACATAATCTAAA---AAATTTAGACCT
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CTATTTATAC--AAAATTA-A-CACATAATCTAAC---AAATTTAGACCT
CTCTTTATAC--AAAATTAAA-CACATAATCTAAA---AAATTTAGACCT
CTATTTATACC-AAAATTAAA-CACATAATCTAAA---AAATTTAGACCT
CTATTTATAC--AAAATTAAA-CACATAATCTAAA---AAATTTAGACCT
CTCATGTATC--GATTTTATA-CACTGAATCTCAC---AA-TTTAGACCT
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AAAATA-AATATGTATTAACAAAAGGGTTCTGTCTCATATAG--------
AAAATA-AATATGTATTAACAAAAGGGTTCTGTCTCATATA---------
AAAACA-AATATGTATTAACAAAAGGGTTCTGTCTCATATAGG--AAGAC
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APPENDIX C

SEQUENCES OF FELINE ISOLATES REGISTERED IN GENBANK WITH CORRESPONDING ACCESSION NUMBERS

EU569301
1 CAGTTTCGTT AATAATTACA ACATATTTTT TTAATGTCTA TATCTATTTA
TACAAAATTA ACACATAATC TAACAAATTT AGACCTTAGG CAATGGATGT
CTTGGCTTCT TACACGATGA AGAACGTTGC ATAATGCGAT AAGCGGCTGG
ATTAGCTTTC TTTGCGACAA GTTCGATCTT TGAATGCACA TTGCGCGCCG
TTTTAGCTTG CTAGAACACG CATATATGTT ACAGTAACCC ATATTAATTT
AATACCAAAT TCTCTTTTTA AGCAAAAGAG CGAAAAATAA ATATGTATTA
ACAAAAGGGT TCTGTCTCAT ATAGGGGAAG ACCCGTACTT GCAGAAGAAG
TAGCATCGTC CACCTGTGTT TCAGGGGGGG GCCACCCCCC AGGAAAGG

EU569302
1 TCAGTTTCGT TAACTATGCC GTTGTATCTG TTCTCATAGC CCGGGCCTCT
TTATACAAAA TTAAACACAT AATCTAAAAA ATTTAGACCT TAGGCAATGG
ATGTCTTGGC TTCTTACACG ATGAAGAACG TTGCATAATG CGATAAGCGG
CTGGATTAGC TTTCTTTGCG ACAAGTTCGA TCTTTGAATG CACATTGCGC
GCCGTTTTAG CTTGCTAGAA CACGCATATA TGTTACAGTA ACCCATATTA
ATTTAATACC AAATTCTCTT TTTAAGCAAA AGAGCGAAAA ATAAATATGT
ATTAACAAAA GGGTTCTGTC TCATATAGGA AGACCCGACT TCCTGCTGAT
GAAGCAGGAC CAGCCGTCTT TTGTTTAAGG GGGGGGGGCC CCCCA

EU569303
1 CAGTTTCGTT AATAATTACA ACATATTTTT TTAATGTCTA TACCTATTTA
TACCAAAATT AAACACATAA TCTAAAAAAT TTAGACCTTA GGCAATGGAT
GTCTTGGCTT CTTACACGAT GAAGAACGTT GCATAATGCG ATAAGCGGCT
GGATTAGCTT TCTTTGCGAC AAGTTCGATC TTTGAATGCA CATTGCGCGC
CGTTTTAGCT TGCTAGAACA CGCATATATG TTACAGTAAC CCATATTAAT
TTAATACCAA ATTCTCTTTT TAAGCAAAAG AGCGAAAAAT AAATATGTAT
TAACAAAAGG GTTCTGTCTC ATATAGGAAG ACCCGTAGTT CCAGTCAAGG
ACCCGCGTTC TTCTTTCTAA TGGGGGTCGC GTGTAGGCCC CTTGTTGAGT
TAG

EU569304
1 TCAGTTTCGT TAATTACAAA CATATTTTTT TATGTCTATA TCTATTTATA
CAAAATTAAA CACATAATCT AAAAAATTTA GACCTTAGGC AATGGATGTC
TTGGCTTCTT ACACGATGAA GAACGTTGCA TAATGCGATA AGCGGCTGGA
TTAGCTTTCT TTGCGACAAG TTCGATCTTT GAATGCACAT TGCGCGCCGT
TTTAGCTTGC TAGAACACGC ATATATGTTA CAGTAACCCA TATTAATTTA
ATACCAAATT CTCTTTTTAA GCAAAAGAGC GAAAAATAAA TATGTATTAA
CAAAAGGGTT CTGTCTCATA TAGGGAAGAC CCGTGTTCTG AAGCATCACT
TGCTGCGGAG GCCATTTTTA CTACCTAGGA TACTCGGAGG AAGTGCCACC
AAAGAAAGTA GAGTTCGTCG ACCTAATCCC CCGAGAAATG TGGTTGAGAG
GCTTTTTGCC TTACGAAACT GTTCAACCGG C

EU569305
1 CCCTGCCCGT TGGATCTTTT CGTTAACCGG CCTCATGTAT CGATTTTATA
CACTGAATCT CACAATTTAG ACCTTAGGCA ATGGATGTCT TGGCTTCTTA
CACGATGAAG AACGTTGCAT AATGCGATAA GCGGCTGGAT TAGCTTTCTT
TGCGACAAGT TCGATCTTTG AATGCACATT GCGCGCCGTT TTAGCTTGCT
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TGTCTCATAT AGGAAGACCC GACCTGCTGT TGTTTCAGTT TCATCAATCA
GCAGCTGTTT CAATTCGATT AGAAGAGCGC TGCTCTATTT CTGTAACATA
ATGGCTATTA AAGTTTTAAA GAAGAAAGTG GGATTAGAAC AAATAAAATT
TTTGGACGAG TAAGATAAGT AAAATAAGAA AGATTAGGTT TGAGTATTAA
TTAAAAA

EU569306
1 CAGTTTCGTT AATAATTACA AAACATATTT TTTTATTGTC TATATCTATT
TATACAAAAT TAAACACATA ATCTAAAAAA TTTAGACCTT AGGCAATGGA
TGTCTTGGCT TCTTACACGA TGAAGAACGT TGCATAATGC GATAAGCGGC
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CCGTTTTAGC TTGCTAGAAC ACGCATATAT GTTACAGTAA CCCATATTAA
TTTAATACCA AATTCTCTTT TTAAGCAAAA GAGCGAAAAA TAAATATGTA
TTAACAAAAG GGTTCTGTCT CATATAGGAA GACCCGATCT TCTGTTGTAG
CAGCAGCTTC ATTAGGCTGT TGGTGATTTT TGGGAGAGGG CGCCCCCCCA
GCGACG

EU569307
1 GTTTCGTTAA TATTACAAAC ATATTTTTTT TTGTCTATAA CTATTTATAC
AAAATTAAAC ACATAATCTA TTCAAATTTA GACCTTAGGC AATGGATGTC
TTGGCTTCTT ACACGATGAA GAACGTTGCA TAATGCGATA AGCGGCTGGA
TTAGCTTTCT TTGCGACAAG TTCGATCTTT GAATGCACAT TGCGCGCCGT
TTTAGCTTGC TAGAACACGC ATATATGTTA CAGTAACCCA TATTAATTTA
ATACCAAATT CTCTTTTTAA GCAAAAGAGC GAAAAATAAA TATGTATTAA
CAAAAGGGTT CTGGCTCATA TAGGAAGACC CGA

EU569308
1 GTTTCGTTAA CTGAGTGTAC AACATATATT TTTTATTGTC TATAACTATT
TATACAAAAT TAAACACATA ATCTAAAAAA TTTAGACCTT AGGCAATGGA
TGTCTTGGCT TCTTACACGA TGAAGAACGT TGCATAATGC GATAAGCGGC
TGGATTAGCT TTCTTTGCGA CAAGTTCGAT CTTTGAATGC ACATTGCGCG
CCGTTTTAGC TTGCTAGAAC ACGCATATAT GTTACAGTAA CCCATATTAA
TTTAATACCA AATTCTCTTT TTAAGCAAAA GAGCGAAAAA TAAATATGTA
TTAACAAAAG GGTTCTGTCT CATATAGGGA AGACCCGACC TGCTGCTGGA
GCAGCTTCTT CATTACTGCT GCCGTTAATC AGGGGGGGGG GGGCCCCCAA
GAG

EU569309
1 AGTTTCGTTA ATAATTACAA ACATATTTTT TTAATGTCTA TAACTATTTA
TACAAAATTA AACACATAAT CTAAAAAATT TAGACCTTAG GCAATGGATG
TCTTGGCTTC TTACACGATG AAGAACGTTG CATAATGCGA TAAGCGGCTG
GATTAGCTTT CTTTGCGACA AGTTCGATCT TTGAATGCAC ATTGCGCGCC
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TAATACCAAA TTCTCTTTTT AAGCAAAAGA GCGAAAAATA AATATGTATT
AACAAAAGGG TTCTGTCTCA TATAGGGAAG ACCCGTATTT ATAAGAAGGG
GGGAAAAAAA AAAAATATAA AAAAAAGAGG GGGGGGGGGG CGCGCGCCCA
CCATAAAATA AATTTTTATT AATTTGAAAA GTAAAAAAAA A

EU569310
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CCGTTTTAGC TTGCTAGAAC ACGCATATAT GTTACAGTAA CCCATATTAA
TTTAATACCA AATTCTCTTT TTAAGCAAAA GAGCGAAAAA TAAATATGTA
TTAACAAAAG GGTTCTGTCT CATATAGGAA GACCCGA

EU569311

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GGATTAGCTT TCTTTGCGAC AAGTTCGATC TTTGAATGCA CATTGCGCGC
CGTTTTAGCT TGCTAGAACA CGCATATATG TTACAGTAAC CCATATTAAT
TTAATACCAA ATTCTCTTTT TAAGCAAAAG AGCGAAAAAT AAATATGTAT
TAACAAAAGG GTTCTGGCTC ATATAGGAAG ACCCGA

EU569312

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TGGCTTCTTA CACGATGAAG AACGTTGCAT AATGCGATAA GCGGCTGGAT
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TTAGCTTGCT AGAACACGCA TATATGTTAC AGTAACCCAT ATTAATTTAA
TACCAAATTC TCTTTTTAAG CAAAAGAGCG AAAAATAAAT ATGTATTAAC
AAAAGGGTTC TGTCTCATAT AGGGAAGACC C

APPENDIX D

MATRIX RESULTING FROM RAPD PCR BANDING PATTERN ANALYSIS

15 samples

D1-bovine	0.0000	0.2307	0.6875	0.6875	0.8000	0.8000	0.7500	0.8125	0.9230	0.7500	0.9375	1.0000	0.9444	0.8666	0.9411
25-bovine	0.2307	0.0000	0.5625	0.5625	0.8888	0.8947	0.8000	0.8888	0.8461	0.8947	0.9411	0.8823	0.8888	0.7142	0.6428
33-bovine	0.6875	0.5625	0.0000	0.0000	0.8750	0.8125	0.6363	0.8750	1.0000	1.0000	0.7692	0.9375	0.9411	0.8571	0.6923
34-bovine	0.6875	0.5625	0.0000	0.0000	0.8750	0.8125	0.6363	0.8750	1.0000	1.0000	0.7692	0.9375	0.9411	0.8571	0.6923
36-bovine	0.8000	0.8888	0.8750	0.8750	0.0000	0.7857	0.9166	0.9333	0.7777	0.7857	0.9230	0.6923	0.7692	0.8333	0.9285
EU569301	0.8000	0.8947	0.8125	0.8125	0.7857	0.0000	0.7272	0.1111	0.6666	0.2000	0.8461	0.5454	0.4545	0.6363	0.9333
EU569307	0.7500	0.8000	0.6363	0.6363	0.9166	0.7272	0.0000	0.9166	0.8571	0.9230	0.9000	0.9090	0.9166	0.9000	0.0000
EU569308	0.8125	0.8888	0.8750	0.8750	0.9333	0.1111	0.9166	0.0000	0.7777	0.3000	0.8333	0.5000	0.4000	0.6000	0.8461
EU569309	0.9230	0.8461	1.0000	1.0000	0.7777	0.6666	0.8571	0.7777	0.0000	0.6666	1.0000	0.5714	0.9000	0.8750	0.8888
EU569304	0.7500	0.8947	1.0000	1.0000	0.7857	0.2000	0.9230	0.3000	0.6666	0.0000	1.0000	0.2222	0.3000	0.5000	0.9333
T. vaginalis	0.9375	0.9411	0.7692	0.7692	0.9230	0.8461	0.9000	0.8333	1.0000	1.0000	0.0000	1.0000	0.9230	1.0000	1.0000
EU569311	1.0000	0.8823	0.9375	0.9375	0.6923	0.5454	0.9090	0.5000	0.5714	0.2222	1.0000	0.0000	0.6363	0.7000	0.9230
T. mobilensis	0.9444	0.8888	0.9411	0.9411	0.7692	0.4545	0.9166	0.4000	0.9000	0.3000	0.9230	0.6363	0.0000	0.6000	1.0000
Peritoneal	0.8666	0.7142	0.8571	0.8571	0.8333	0.6363	0.9000	0.6000	0.8750	0.5000	1.0000	0.7000	0.6000	0.0000	0.9166
Giardia	0.9411	0.6428	0.6923	0.6923	0.9285	0.9333	1.0000	0.8461	0.8888	0.9333	1.0000	0.9230	1.0000	0.9166	0.0000