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Identification of feline *Tritrichomonas foetus* surface epitopes: Putative targets for development of a novel diagnostic test for feline trichomonosis

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To the Graduate Council:

I am submitting herewith a thesis written by Emily Nissa Gould entitled "Identification of feline *Tritrichomonas foetus* surface epitopes: Putative targets for development of a novel diagnostic test for feline trichomonosis." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

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Identification of feline *Tritrichomonas foetus* surface epitopes: Putative targets for development of a novel diagnostic test for feline trichomonosis

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ABSTRACT

BACKGROUND: *Tritrichomonas foetus* (*T. foetus*) is a flagellated protozoa that infects the distal ileum and proximal colon of domestic cats and also induces reproductive failure in cattle. Although feline trichomonosis is recognized to have a global prevalence of up to 30%, it still poses a diagnostic and therapeutic challenge to veterinarians; thus, there is a need for both improved diagnostics and therapeutics. Despite differing organ tropism between genotypes, evidence exists for conserved virulence factors between feline and bovine *T. foetus*. Two epitopes (1.15 and 1.17) of the bovine *T. foetus* glycosylated surface antigen 1.15-1.17 have been found to facilitate adhesion and cytotoxicity towards bovine urogenital epithelium. Although epitope 1.15 has been previously identified in feline isolates, conservation and function of epitope 1.17 has not yet been evaluated.

METHODS: Western blotting and indirect immunofluorescence were used to identify the presence of 1.15-1.17 in feline *T. foetus* isolates. One bovine *T. foetus* and one feline *Pentatrichomonas hominis* (a non-pathogenic feline trichomonad) isolate were used as positive and negative controls for all assays. Validated co-culture assays with in vitro porcine jejunal epithelial cell (IPEC-J2) cultures were used to determine the role this antigen plays in mediating adherence and cytotoxicity to the intestinal epithelium. Confluent epithelial cells were infected with feline *T. foetus* either treated with 1) isotype control or 2) 1:100 monoclonal antibody, and co-cultured for either 6 (i.e. adhesion assays) or 24 hours (i.e. cytotoxicity studies).

RESULTS: Presence and surface localization of both epitopes in feline *T. foetus* isolates was confirmed via western blotting, immunofluorescence and flow cytometry. Co-culture adhesion

and cytotoxicity assays showed that epitopes 1.15 and 1.17 were either found to decrease or have no role in adhesion of *T. foetus* to intestinal epithelial cells.

CONCLUSIONS: Discovery of this antigen in feline *T. foetus* provides continued evidence that similarities exist between the bovine and feline genotypes. Although the role of 1.15-1.17 differs in feline versus bovine *T. foetus in vitro*, the conservation of expression across isolates and surface location of these epitopes are ideal characteristics for engineering a novel diagnostic assay for detection of whole organism trichomonads in fecal samples.

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CHAPTER ONE: LITERATURE REVIEW

Introduction to Trichomonads

Members of the family Trichomonadidae and subfamily Tritrichomonadidae are eukaryotic protozoa that inhabit the urogenital, gastrointestinal and respiratory tracts of their respective hosts. All trichomonads belong to the class Parabasalia, and range in size from approximately 10-20 μm. The earliest classification systems for trichomonads identified organisms based on their morphological characteristics (i.e. a parabasal body, dorsal undulating membrane and axostyle) and differentiated between species based on their number of anterior flagella, which vary from 3 to 5 in number. (Cavalier-Smith, 1993; Schwebke and Burgess, 2004) Trichomonads are classified as microaerophilic organisms; they colonize organ systems with exposure to the outside environment, but require only low oxygen concentration. (<10%). (Mack and Muller, 1978) These organisms reproduce via binary fission, and unlike other protozoa (e.g. *Giardia* spp.), lack a cyst stage. Another hallmark of trichomonads is that, unlike many other eukaryotes, they lack mitochondria and instead possess a common organelle, called the hydrogenosome, which functions to degrade carbohydrate substrates to produce acid (i.e. hydrogen) as an end product. This structure is imperative for anaerobic pyruvate metabolism and subsequent energy production for the protozoa, making it necessary for trichomonad survival. (Benchimol et al., 1996; Lindmark and Muller, 1973). Therefore, these structures have been important targets for anti-protozoal therapies.

Trichomonads include *Trichomonas* spp., *Tritrichomonas* spp., *Tetratrichomonas* spp. and *Pentatrichomonas* spp., and consist of both pathogenic and non-pathogenic protozoa. Well-recognized and studied pathogenic species include the human and bovine venereal trichomonads,

Trichomonas vaginalis and *Tritrichomonas foetus*, respectively, as well as the avian trichomonad, *Trichomonas gallinae*. *Trichomonas vaginalis* is now recognized to be the most common non-viral sexually transmitted disease in humans worldwide. It is responsible for over 200 million cases of trichomoniasis annually, and the infection increases the risk of human immunodeficiency virus (HIV) contraction in comparison to non-infected individuals. (Kissinger and Adamski, 2013) The discovery and study of virulence factors integral to *T. vaginalis* cytopathogenicity (including adhesin proteins and proteases) have provided a foundation for studies on the pathogenicity of other trichomonad species, namely *Tritrichomonas foetus*.

Multiple Virulence Factors are Conserved Across Trichomonad Species

Both the human venereal pathogen, *Trichomonas vaginalis*, and bovine *T. foetus* have provided an important foundation in identification of virulence factors that have broad implications for most pathogenic trichomonad species. Several virulence factors have been identified as important mediators of cytopathogenicity in trichomonads infecting both humans and animals. These include glycoproteins (Hodgson et al., 1990; Petropolis et al., 2008; Shaia et al., 1998; Silva-Filho et al., 2002) and lipophosphoglycans (Fichorova et al., 2006; Singh, 1994; Singh et al., 2001; Singh et al., 1999), which have been shown as integral in facilitating adhesion to the host epithelium. Enzymes such as cysteine proteases have also been identified to play an important role in both mediating adhesion to, as well as exerting cytotoxicity towards epithelial cells. (Arroyo and Alderete, 1989; Lucas et al., 2008; Singh et al., 2005; Tolbert et al., 2014)

Identification of Bovine *Tritrichomonas foetus* as a Venereal Pathogen

     Tritrichomonas foetus (*T. foetus*) is a flagellated protozoal pathogen responsible for reproductive failure (i.e. endometritis, infertility, early embryonic death and abortions) in cattle.

Bovine *T. foetus* is reported to have first appeared as a venereal pathogen following culture of the protozoa from herds of dairy cattle in eastern Australia, with subsequent identification of the pathogen in beef herds throughout the country. (Dennett et al., 1974; Parsonson et al., 1974) The first cultures were harvested from the preputial cavity of bulls, which serve as asymptomatic carriers of the pathogen. The presumed route of transmission for both males and females is via coitus. The preputial cavity and urethral orifice of the penis have been identified as sites most likely to harbor bovine trichomonads in the bull, but trichomonads do not generally cause epithelial destruction in these locations. (Parsonson et al., 1974) This may explain why males fail to develop clinical signs and serve as silent carriers of bovine trichomonosis. Females also contract *T. foetus* via sexual intercourse and may go on to develop vaginitis, endometritis and suffer infertility from about 6-7 weeks on following infection. (Parsonson et al., 1976) If cows are able to conceive, loss of the fetus may result from early embryonic death or, less commonly, abortions. Loss of the embryo or fetus most often occurs within the first third to half of pregnancy. (Rhyan et al., 1988) Clinical signs of infection, as well as evidence of histopathologic lesions, do not develop until about 2 months post infection in cows with trichomonosis. (Parsonson et al., 1976) Pyometra has also been described as a rare side effect of venereal trichomoniasis.

Identification of *T. foetus* throughout cattle in the United States, (BonDurant et al., 1990; Goodger and Skirrow, 1986; Skirrow, 1987) Spain (Martin-Gomez et al., 1998; Mendoza-Ibarra et al., 2012) and Argentina (Mancebo et al., 1995) re-classified bovine trichomonosis as a globally emerging reproductive pathogen with prevalence rates ranging from 4-30%. Given that bulls are often asymptomatic carriers, it is possible for one bull to infect many heifers, resulting in profound losses for the cattle industry. (Goodger and Skirrow, 1986) Some reports document

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that up to 42% of heifers became infected within a year following exposure to *T. foetus* positive bulls (Clark et al., 1983), and that up to 40% of cows lost calves to embryonic abortions. (Clark et al., 1986) Monetary losses due to trichomonosis in herds practicing natural breeding techniques were estimated to be as high as $70,000 per herd over an 18 month period from 1985 to 1986 in the United States. (Goodger and Skirrow, 1986)

Initial attempts at preventing transmission within herds included culling infected bulls and/or cows that failed to become pregnant following breeding (Mancebo et al., 1995), as well as strict use of younger bulls given that prevalence rates were lower in comparison to older males. (Christensen et al., 1977) As it became an established belief that cows failed to maintain active infections past 90 days due to self-limiting infection, some farms implemented a policy of at least 3 months "rest" in between the next breeding. (Mancebo et al., 1995) Artificial insemination became adopted as the method of choice to decrease transmission. As no efficacious preventatives (e.g. vaccines) existed, minimizing transmission by identifying infected animals was pivotal in minimizing disease spread and halting reproductive losses. Thus, the need for understanding cytopathogenicity and developing improved diagnostic and preventative techniques were imperative.

Surface Proteins Identified as Integral in Facilitating Adhesion in Trichomonad Species

The ability of trichomonads to successfully attach to host epithelial cells is one of the most important inciting events for the exertion of cytopathogenicity. This event requires surface proteins (e.g. glycoproteins, extra-cellular matrix [ECM] binding molecules and other surface adhesins) on both the pathogen and host epithelial cell (Bonilha et al., 1995; Silva-Filho et al., 2002), as well as motile, viable trichomonads capable of utilizing the posterior flagellum as an

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initial anchor. (Singh et al., 1999; Tolbert et al., 2013) Several molecules are crucial in mediating adhesion, including glycoproteins, which have been identified as important in facilitating adhesion of many protozoa species to the host epithelium. Multiple adhesins (i.e. AP65, AP51, AP33 and AP23) have been recognized as integral in mediating adhesion-dependent cytotoxicity exerted by *Trichomonas vaginalis* towards vaginal epithelial cells. (Alderete and Garza, 1988; Arroyo et al., 1995) Many factors seem to play a role in the mobilization of these adhesion proteins to the surface of the protozoa, including the presence of an iron-rich environment. It has been shown that in the absence of iron, *T. vaginalis* isolates were unable to mobilize these adhesins to the surface of the protozoa, and were subsequently unable to attach to vaginal epithelial cells. (Lehker et al., 1991) Additionally, differing phenotypic expression of *T. vaginalis* surface adhesins has been shown to occur between isolates and differing passage numbers. (Alderete and Garza, 1988) The underlying etiology of these phenotypic differences or shifts has not been fully elucidated, but could explain why some hosts are not as susceptible to clinical infections as others.

Binding to glycoproteins and proteoglycans in the host ECM complex have been shown to facilitate both adhesion to host cells as well as initiate a morphologic shape change of the parasite. (Petropolis et al., 2008) This change transforms the protozoa from its natural "teardrop" shape to an elongated, amoeboid form, and has been noted in both *T. vaginalis* (Crouch and Alderete, 1999) and *T. foetus (*Petropolis et al., 2008) following interaction with ECM binding proteins. It has been hypothesized that this morphological change may also upregulate cytotoxic mediators (e.g. cysteine proteases), but the full significance of this transformation has not been completely elucidated.

Another surface complex that has been identified in many protozoa, but may vary greatly between species, is the lipophosphogylcan (LPG) complex. The LPG complex has been identified to be composed of a rich matrix of carbohydrates and phospholipid anchors, and is recognized as the most abundant surface complex in both *T. vaginalis* and bovine *T. foetus*. (Singh, 1993, 1994; Singh et al., 2001) The LPG complex in *T. vaginalis*, but not bovine *T. foetus,* has also been identified as an important molecule mediating not only adhesion to, but upregulation of proteases that induce cytotoxicity to host epithelial cells. (Fichorova et al., 2006) While we know that the LPG complex is present in high numbers in bovine *T. foetus*, ongoing work is needed to further classify the role of this complex and how it both resembles and differs from that of the *T. vaginalis* LPG.

Lastly, some molecules that act to facilitate adhesion in trichomonad species may actually hamper or diminish adherence to host cells in others. Sialic acid (i.e. carbohydrate) residues on the surface of host epithelial cells are an example of such a molecule. Presence of such residues have been shown to facilitate adhesion following interaction with lectin molecules in bovine *T. foetus*, while abrogating adhesion in *T. vaginalis*. (Bonilha et al., 1995) Differing roles of surface complexes reinforces that ongoing research is necessary to fully understand the significance of different molecules between species and genotypes.

Diagnostic Tests for Bovine Trichomonosis

Microscopic identification of motile trichomonads served as the first assay for diagnosis of bovine trichomonosis and was used to confirm the presence of protozoa in herds of beef cattle in Australia. (Clark et al., 1974; Dennett et al., 1974; Parsonson et al., 1974) Following development of culture methods found to support *in vitro* isolation and propagation of several trichomonad species (Diamond, 1957), *in vitro* culture of preputial swabs with microscopic (i.e.

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morphologic) identification became the preferred method for diagnosis of bovine *T. foetus*. (BonDurant et al., 1990) A significant problem with this technique was a lack of specificity for *T. foetus*, as the media also supported growth of other protozoa. An important diagnostic breakthrough for bovine trichomonosis was the development of a commercially available pouch culture system (In-Pouch™ TF Culture, Bio Med Diagnostics), which subsequently became recognized as the most sensitive and specific method for diagnosis of bovine trichomonosis. (Schonmann et al., 1994) It was found that six serial weekly cultures from preputial samples yielded a sensitivity of 80-96% and specificity of up to 96% (Hoevers et al., 2003; Parker et al., 2003a; Parker et al., 2003b), with these percentages fluctuating depending on the exact method of sample collection and culture conditions (i.e. collection media, transport media, temperature and storage conditions). (Parker et al., 2003b; Skirrow et al., 1985) The In-Pouch™ culture soon became the gold standard for diagnosis of bovine trichomonosis.

Unfortunately, this system still relied on visual identification based on morphological characteristics and the incorrect assumption that the media would result in only *T. foetus* growth. The In-Pouch™ culture was subsequently found to also support the growth of other trichomonad species (i.e. *Tetratrichomonas* spp., *Pentatrichomonas hominis* and *Pseudotrichomonas* spp.) from the urogenital tract of cattle. (Dufernez et al., 2007) Several studies have demonstrated lack of specificity of the In-Pouch™ culture, including the growth of presumable gastrointestinal trichomonads from virgin bulls. (Campero et al., 2003) These findings revealed a lower specificity of the In-Pouch™ TF culture than originally thought, which in turn spurred development of more sensitive and specific diagnostics.

Polymerase chain reaction (PCR), using either single or pooled preputial samples from bulls, has now surpassed the In-Pouch™ TF culture system as a gold standard diagnostic and has

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been used either alone or in conjunction with culture. (Cobo et al., 2007; Felleisen, 1997; Garcia Guerra et al., 2014; Garcia Guerra et al., 2013; Ho et al., 1994) One study found that three serial weekly cultures with PCR, performed in parallel, had equivalent sensitivity and specificity to that of six weeks of culture alone. (Cobo et al., 2007) In a study evaluating the specificity of a *T. foetus* PCR, only *T. foetus*, but not other trichomonad species, was found to amplify (Dufernez et al., 2007), confirming a specificity of 100% for this technique. With proper sample handing and performance by experience diagnosticians, the excellent specificity of PCR has been found to be repeatable across laboratories (Garcia Guerra et al., 2014). Several PCR assays have been developed for reliable detection of bovine *T. foetus*, and detect DNA from as little as 1 organism per 1 mL sample from cell lysate (McMillen and Lew, 2006) or clinical specimens (i.e. smegma from infected bulls). (Felleisen et al., 1998) Furthermore, assays with an internal validity control have helped to eliminate false negatives due to insufficient sample size or failed amplification. (Grahn et al., 2005) As a result of these diagnostic advancements, PCR is now recognized as the gold standard assay for diagnosis for bovine trichomonosis, either alone or in conjunction with culture. Repeat serial assays are recommended to increase both sensitivity and specificity. Discovery of such specific diagnostic testing subsequently shaped the development of feline *T. foetus*-specific assays as well.

Discovery of Feline Trichomonosis

Years following the recognition of bovine trichomonosis as an established venereal pathogen of cattle, another protozoa was identified in the distal intestine of domestic cats suffering from diarrhea of large bowel origin. Although feline trichomonosis was initially recognized in Europe, the first published reports of *T. foetus* as an emerging feline enteric pathogen followed culture of trichomonads from the distal ileum, cecum and colon of clinically

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affected domestic cats in the United States. (Gookin et al., 1999; Gookin et al., 2001; Levy et al., 2003) These protozoa were harvested from the feces of cats suffering from foul, waxing and waning diarrhea. Many of these cats also suffered a similar constellation of clinical signs, including tenesmus, hematochezia, dyschezia, fecal incontinence, flatulence, and a prominent, swollen anus. (Gookin et al., 1999) Infection of specific pathogen-free cats with *T. foetus* produced similar, albeit less severe, clinical and histopathologic disease of that seen in naturally infected cats, (Gookin et al., 2001), confirming *T. foetus* as a feline enteric pathogen. Cats suffering from trichomonosis are often mis-diagnosed with giardiasis, (Gookin et al., 1999), and while diarrhea may improve with paromomycin or metronidazole, these treatments often fail to completely clear infection. Moreover, time to resolution of diarrhea for cats with failed anti-protozoal therapy is longer than for cats who naturally clear infection. (Foster et al., 2004) Although infected cats often present with *Giardia* spp. or *Pentatrichomonas hominis* (*P. hominis*) co-infections, cats from which *P. hominis* alone is cultred do not have the same diarrhea. Standard anti-protozoal therapy (e.g. metronidazole and fenbendazole) is also usually recognized to clear diarrhea associated with giardiasis (Levy et al., 2003), but as mentioned, fails to permanently clear diarrhea associated with *T. foetus*. Younger, purebred cats and animals in densely crowded environments (e.g. shelters, catteries and cat shows) have been identified as more commonly affected. (Burgener et al., 2009; Gookin et al., 1999; Gookin et al., 2004) The observation of an increased disease prevalence in cats that are in close contact with each other suggests that feline *T. foetus* is transmitted via fecal-oral spread. In reports documenting experimental infection of cats with T. foetus via an orogastric route, cats developed clinical signs and their feces were PCR positive within 7-14 days of inoculation. (Gookin et al., 1999; Gookin

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et al., 2006) Collectively, this information provided evidence that *T. foetus* is an enteric pathogen of domestic cats and should be differentiated from other feline intestinal protozoa.

Lack of a Sensitive and Specific, Point of Care Diagnostic Test for Feline *T. foetus*

Similar to bovine trichomonosis, feline *T. foetus* was first identified based on morphological characteristics via wet mount direct microscopy. This technique is neither sensitive nor specific given feline *T. foetus* microscopically resembles both *Giardia* spp. and *Pentatrichomonas hominis*. The In-Pouch™ TF culture (Bio-Med Diagnostics) was the first method to reliably culture feline *T. foetus* from fecal samples of diarrheic cats. The commercially available culture system has an improved sensitivity when compared to wet mount cytologic diagnosis (i.e. 55% versus 14%, respectively), but false negatives and positives are still possible. False negatives often occur following inoculation of too much fecal material into the pouch, resulting in bacterial overgrowth that hampers growth of trichomonads. Although rare, the In-Pouch™ TF culture has also been reported to support positive growth of other trichomonad species. (Ceplecha et al., 2013) Additionally, growth of the organisms can take days to even weeks and must be performed in a laboratory setting. Therefore, culture techniques are not useful for clinic use for practicing veterinarians to rapidly differentiate cats infected with *T. foetus* from those infected with other protozoal pathogens (i.e. *Giardia* or *Pentatrichomonas hominis*). For this reason, more specific, sensitive, and rapid diagnostic tests are recommended for the diagnosis of feline trichomonosis

Similar to bovine trichomonosis, PCR subsequently became the most sensitive and specific test for identification of feline *T. foetus*, and is currently considered the gold standard for diagnosis of feline trichomonosis. One study demonstrated that nested PCR required the presence of only 50-500 *T. foetus* organisms/gram of feces for 100% sensitivity when samples

were handled and processed appropriately. (Gookin et al., 2002) This same study showed that when performed following *in vitro* culture, PCR increased the chance of a positive diagnosis by 20% compared to either technique alone. Here, fresh feces were cultured at the time of sample acquisition and deemed as a positive or negative based on *in vitro* culture in modified Diamond's medium. Remaining feces were stored at -70°C until subsequent PCR processing, with samples for PCR all taken from the original feces rather than fecal culture.

Although PCR has greatly improved the ability of veterinarians to correctly diagnose feline *T. foetus*, the method is not without limitations. One such limitation is that this assay should only be performed by experienced diagnosticians at a properly equipped laboratory, and can take days for the return of definitive results. Given that PCR is not a patient-side assay, it hampers the ability of veterinarians to rapidly and accurately diagnose a cat with *T. foetus* infection on site. While waiting for the return of results, some cats are given oral therapies that are inappropriate for the treatment of trichomonosis (i.e. metronidazole) that may also put them at additional unnecessary risk for drug side-effects. As feline *T. foetus* is presumably spread via fecal-oral transmission, identification and isolation of affected cats is recommended. This underscores the importance of rapidly and accurately identifying infected animals and removing them from the general population. An in-clinic diagnostic assay would allow veterinarians to not only identify infected cats, but to also avoid misdiagnosis with non-*T. foetus* species and to diminish initiation of inappropriate or unwarranted therapies.

Lack of Safe, Efficacious Therapeutics for Feline *T. foetus* Infection

As both awareness of and diagnostics for feline trichomonosis began to improve, feline *T. foetus* was identified in diarrheic cats throughout Europe, and is now recognized worldwide as a feline enteric pathogen. (Bissett et al., 2008; Bissett et al., 2009; Burgener et al., 2009;

Dabrowska et al., 2015; Miro et al., 2011) The combination of an increased prevalence rate (i.e. up to 31% in densely crowded environments) (Gookin et al., 2004; Paris et al., 2014), lack of preventatives and worldwide recognition of feline trichomonosis has led to investigation of anti-protozoal agents that may eradicate infection.

One of the earliest drugs utilized for attempted therapy for feline trichomonosis was paromomycin, an aminoglycoside used for the treatment of trichomonosis in humans (i.e. *T. vaginalis*), but reported to have poor gastrointestinal absorption. (Foster et al., 2004; Gookin et al., 1999; Poppe, 2001; Tayal et al., 2010) Paromomycin has mixed efficacy in eradicating diarrhea and clearing feline *T. foetus* infections, evidenced by positive cultures following therapy. (Gookin et al., 1999) Although some cats do have a clinical response to this drug, the time to resolution of diarrhea was actually extended in comparison to cats who naturally clear infection (Foster et al., 2004) and the drug carries risk of producing acute renal failure and deafness. (Gookin et al., 1999)

Drugs of the 5-nitroimidazole class were next to undergo investigation as potential therapies, given their precedential use in human trichomonosis and success in eradicating other protozoal pathogens. (Upcroft et al., 2006) Their proposed mechanism of action is to create DNA damage secondary to production of free radical, toxic anions due to a competitive inhibition of hydrogenase by nitro groups in the hydrogenosome. (Kulda, 1999) Metronidazole (MDZ) is a 5-nitroimidazole drug that is recognized as an accepted therapeutic agent for giardiasis in companion animals. (Scorza and Lappin, 2004; Zimmer, 1987) Unfortunately, feline *T. foetus* isolates have been documented as resistant to MDZ *in vitro*, (Gookin et al 2006) and many infected cats have no resolution of clinical signs following MDZ therapy. The mechanism of action of metronidazole resistance is thought to be a mutation in or absence of a normally

functioning pyruvate: ferredoxin oxioreductase pathway found in resistant trichomonads (Cerkasovova et al., 1984), as well as utilization of oxygen in microaerophilic environments to outcompete the drug for bound electrons. (Kulda, 1999) Tinidazole (TDZ), another 5-nitroimidazole drug, was initially shown to be effective at preventing replication of *T. foetus* isolates *in vitro*, as well as successfully producing negative cultures during treatment of experimentally-infected specific pathogen free kittens. (Gookin et al., 2007) However, TDZ is not recommended as a treatment for feline trichomonosis as many cats relapsed following the withdrawal of therapy. (Gookin et al., 2007)

To date, there are no approved drugs shown to effectively clear feline *T. foetus* in all infected cats. Ronidazole, also of the 5-nitroimidazole class, is the only therapeutic agent now utilized for off-label treatment of feline *T. foetus* infection. Ronidazole, also of the 5-nitroimidazole class, is the only therapeutic now utilized for off-label treatment of feline *T. foetus* infection. (Gookin et al., 2006) Unfortunately, multiple problems have been identified with the use of ronidazole as a therapeutic agent. Firstly, development of neurologic abnormalities (e.g. nystagmus, ataxia and mentation changes) have been documented following oral administration to infected cats. (Rosado et al., 2007) The risk of neurotoxicoses is thought to increase with twice daily dosing of $\geq$ 30 mg/kg (Rosado et al., 2007), and twice-daily dosing is no longer recommended due to concern for a long half-life and plasma accumulation of the drug following twice daily administration. (LeVine et al., 2014; LeVine et al., 2011) Although lower doses (i.e. 10 mg/kg twice daily) have not been documented to initiate adverse effects, they are ineffective at clearing infection. (Gookin et al., 2006) Secondly, in addition to the risk of unacceptable adverse effects, both *in vitro* and *in vivo* aerobic resistance of feline *T. foetus* isolates to ronidazole has been recognized. (Gookin et al., 2010) Though the true prevalence of

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T. foetus resistance to ronidazole is unknown, documentation of *in vitro* resistance and failure to resolve clinical signs in naturally infected cats is evidence that ronidazole is less than ideal for treatment of this disease in cats. Increasing resistance, risk of adverse effects and the lack of suitable alternatives to ronidazole makes it so that no safe, completely efficacious therapeutics exist for treatment of feline trichomonosis. Therefore, there is a need for improved diagnostic, therapeutic and/or preventative strategies for feline *T. foetus* infection.

Classification of Bovine and Feline *T. foetus* in Regards to Organ Tropism

Before development of a novel diagnostic or therapeutic, an initial marker must be identified that is present in all feline *T. foetus* isolates and either easily identifiable for the purposes of an assay or accessible to any drugs targeting it. In an attempt to understand more about shared pathogenic mechanisms between *T. foetus* genotypes, multiple comparative analysis studies have been performed to classify the similarities and differences between bovine and feline *T. foetus*. (Slapeta et al., 2010; Slapeta et al., 2012; Sun et al., 2012) From these analyses, two main theories have been proposed as to the most appropriate classification system. One current belief is that bovine and feline *T. foetus* are of the same trichomonad species, but represent two distinct genotypes. This is based on work that has identified groups of feline and bovine isolates as 100% similar to each other when comparing multiple isolates from the same host species (i.e. all genes of feline isolates are 100% similar and all genes of bovine isolates are 100% similar), but consistently found differences in transcriber regions between species. (Slapeta et al., 2010; Slapeta et al., 2012) These differences in transcriber regions include a repeatable polymorphism in the ITS-2 region (Slapeta 2010), as well as several nucleotide mutations resulting in amino acid differences between bovine and feline *T .foetus* cysteine protease (CP) genes (i.e. CP2, CP4, CP5 and CP8). (Slapeta et al., 2012) The significance of

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these differences remains unknown. A second theory proposes that feline *T. foetus* is an entirely different species than bovine *T. foetus* and should be recognized as a separate entity (i.e. *Tritrichomonas blagburni* spp.). This is based on work investigating similarities and differences between bovine and feline tritrichomonas isolates when comparing highly conserved ribosomal DNA gene sequences, which are 97-100% similar between not only bovine and feline isolates, but also between *T. foetus*, *T. suis* and *T. mobilensis*. The argument for this latter theory is that if bovine *T. foetus* is classified as a separate species from porcine and/or simian trichomonads, feline tritrichomonads should also be recognized as a new species given there is the same amount of genetic diversity between these protozoa. (Walden et al., 2013)

Several studies have shown that there are also discrepant degrees of cytotoxicity towards host epithelial cells following trans-species infections (e.g. infection of cattle with feline *T. foetus* isolates, and vice versa). Although cytotoxicity to vaginal epithelial cells is histologically evident following infection of cattle with feline *T. foetus*, the bovine urogenital epithelium does not display the same degree of inflammatory infiltrates or destruction of the uterine glands as is seen when cattle are infected with their natural host genotype. (Stockdale et al., 2007) Similarly, when cats were experimentally infected with bovine *T. foetus*, the protozoa did not achieve the same degree of colonization or induce equivalent cytotoxicity to host intestinal epithelium as is seen following experimental infection of cattle. In this same study, only one cat cultured positive for the protozoa weeks after infection and the bovine isolates failed to colonize the distal ileum and colon as is seen with natural infection of felines. (Stockdale et al., 2008) It was also found that bovine *T. foetus* was cleared much more rapidly from the feline alimentary tract than cats infected with feline isolates. (Stockdale et al 2007) This suggests that the genotypes may have specific tolerances or adaptations to their host environments regardless of similarities in genomes

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or transcriptomes. Although some feel these differences in trans-species infections support the theory that feline and bovine genotypes are two different species, experimental infection of cats with feline *T. foetus* isolates fails to result in the same degree of inflammatory damage to the epithelium seen with natural infection. (Gookin et al., 2001; Yaeger and Gookin, 2005) Therefore, any differences in histopathology from trans-species infection may result from experimental infection alone, rather than indicating a need for delineation into separate species.

Interestingly, earlier studies investigating the transmission of bovine trichomonosis demonstrated that when *T. foetus* cultures were applied directly to the vulva instead of the vagina, infection was not established. (Clark et al., 1977) This may suggest that bovine *T. foetus* lacks de-adhesins necessary to migrate from the vulva up into the vagina for colonization. Although a fecal-oral route of transmission for feline trichomonosis has only been hypothesized and not proven, it makes sense feline *T. foetus* would require a mechanism of de-adhesion or de-attachment from host epithelium, whereas bovine *T. foetus* may only require mechanisms facilitating attachment to epithelial cells following direct deposition into the vaginal vault.

Some of the proposed phenotypic differences between bovine and feline trichomonads include not only possible functional differences between similar adhesins or surface markers, but differences in pH tolerance as well. Recent work has looked at viability of both bovine and feline trichomonads in response to a gradient of buffered pH between 6 and 8. (Morin- Adeline et al 2015). Feline trichomonads displayed a higher tolerance for acid buffering/resistance than bovine trichomonads in this study, suggesting differences in phenotypes depending on host niche. Collectively, these studies support hypotheses that there is host specificity in regards to degree of infectivity, which is evidenced by distinct organ tropism.

Bovine and Feline *T. foetus* Share Virulence Factors

Although bovine and feline *T. foetus* are classified as two distinct genotypes, conserved virulence mechanisms have been identified. This may be unsurprising given the large amount of homology seen between bovine and feline sequences, with only a 1% total nucleotide divergence found in the available genome. (Reinmann et al., 2012; Slapeta et al., 2012) Cysteine proteases (CPs) have been identified as integral in not only directly promoting adhesion of the protozoa to epithelial cells, but upregulating the release of substances cytotoxic to the epithelium. Following analysis of 10 different genetic loci representing cysteine proteases, only a 1% difference was found between feline and bovine *T. foetus* for all 10 genes. (Slapeta et al., 2012) Of particular interest is the homology found between genotypes in CP8 (also called CP30), which has already been identified as highly expressed (Mallinson et al., 1995) and an integral virulence factor in bovine *T. foetus* cytopathogenicity. (Lucas et al., 2008) It has not only been shown that bovine trichomonads utilize CP30 in order to exert adhesion-dependent cytotoxicity towards epithelial cells, but that the use of cysteine protease inhibitors abolishes *T. foetus*-induced destruction of urogenital epithelium. (Singh et al., 2005) Because CP8/CP30 was first identified as important in the pathogenesis of bovine *T. foetus*, the next logical step was to look for conservation of this protease feline isolates. Two single nucleotide polymorphisms have been identified in CP8 between *T. foetus* species, which are not thought to be in the functional region of the gene. (Sun et al., 2012) The polymorphisms are of unknown significance at this time, but perhaps have a role in phenotypic differences seen between genotypes in terms of organ tropism between cattle and domestic cats. Other recent analyses comparing bovine and feline genotypes have revealed a large amount of homology between their functional transcriptomes. (Morin-Adeline et al., 2014) Moreover, recent work has also established that cysteine proteases are as important in mediating

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adhesion-dependent cytopathogenicity in feline *T. foetus* as they are in bovines. This same study also demonstrated that the use of broad spectrum cysteine protease inhibitors had an ameliorative effect on destruction of host intestinal epithelial cells by feline *T. foetus*. (Tolbert et al., 2014) Thus, it is reasonable to conclude that cysteine proteases represent major virulence factors for both bovine and feline *T. foetus* by facilitating both adhesion and cytotoxicity to respective host epithelial cells.

As already mentioned, the lipophosphoglycan complex has been identified in most protozoa and thought to help mediate adhesion to the host epithelium. Further studies are needed to determine if feline *T. foetus* also possess an LPG complex, but conservation of this complex seems likely given its identity has already been determined to be conserved across multiple protozoa (i.e. bovine *T. foetus*, *T. vaginalis* and *Leishmania* spp. (Singh, 1993; Turco, 1988)).

Further work is necessary to determine exactly which CPs are utilized by feline *T. foetus*, as well as if other surface proteins important to adhesion are conserved between genotypes. Given the similarities seen thus far between *T. foetus* genomes and transcriptomes, bovine trichomonosis represents a reasonable model in searching for novel diagnostic or therapeutic targets for feline *T. foetus*.

Surface Antigen 1.15-1.17 Found to Participate in Bovine *T. foetus* Adhesion and Complement Mediated Killing

As previously mentioned, several groups have shown that bovine *T. foetus* utilizes surface glycoproteins to mediate attachment to mammalian host cells. One of these is the bovine *T. foetus* 190 (Tf190) glycoprotein, which has been classified as part of the *T. foetus* lipophosphoglycan (LPG) group. Inhibition of Tf190 with monoclonal antibodies has been

shown to inhibit the ability of bovine trichomonads to adhere to bovine urogenital epithelial cells. (Burgess and McDonald, 1992) Similarly, a distinct bovine *T. foetus* surface glycoprotein was also found to mediate adhesion of the protozoa to bovine epithelial cells. The antigen was originally classified as a glycoprotein based on thymol staining and diffusely migrating nature of the band via immunoblotting, and named 1.15-1.17. (Hodgson et al., 1990) It was proposed that two epitopes of the surface antigen existed (i.e. 1.15 and 1.17) and demonstrated that treatment of bovine trichomonads with anti-1.17 and anti-1.15 inhibited *T. foetus* adhesion to epithelial cells by up to 73%. (Hodgson et al., 1990) Application of mAb 1.15 also killed bovine trichomonads in a complement-independent manner, suggesting that these epitopes may have a role in induction of protective immunity. Importantly, the presence of 1.15-1.17 was found to be conserved across 37 bovine isolates tested via indirect enzyme-linked immunosorbent assay (ELISA) (JS et al., 1993), making this antigen an attractive target for development of a novel diagnostic test or therapeutic agent.

To further test the hypothesis that this antigen has a role in protective immunity, surface epitope 1.17 was purified and administered as an immunogen to both bulls and heifers which were subsequently experimentally infected with *T. foetus*. As hypothesized, vaccination of heifers with purified epitope 1.17 was found to significantly increase the percentage of cows that were able to successfully clear vaginal *T. foetus* infection (80%) when compared to equal numbers of cows vaccinated with control that had negative cultures at 7 weeks of infection (37%). (Bondurant et al., 1993) This same study also showed that purified 1.17 stimulated an immune response via indirect enzyme-linked immunosorbent assay (ELISA) quantification of IgG in both serum and vaginal secretions. Later work revealed that a local IgA response persisted in vaginal secretions for up to 24 weeks (Ikeda et al., 1995), and vaccinated heifers had

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significantly less histologic evidence of endometritis compared to non-vaccinated animals who either failed to clear or took longer to clear the infection. (Anderson et al., 1996) Thus, immunization with purified 1.17 was shown to improve clearance of *T. foetus*, diminish endometritis and reduce rates of fetal loss and infertility.

The relationship between 1.15-1.17, Tf190 and the previously discovered LPG complex was also examined via ELISA and western blotting. Monoclonal antibodies 1.15 and 1.17 were tested for reactivity with purified Tf190, epitope 1.17 and LPG. All three antigens were positive for reactivity with both mAbs, lending evidence to the fact that 1.15-1.17 likely belongs to this larger family of lipophosphoglycans with an integral role in mediating adhesion of bovine trichomonads to host urogenital epithelium. (Singh et al., 2001)

Lastly, the presence of epitope 1.15 in feline *T. foetus* isolates has been evaluated by several different groups. Combining the results of several different studies, a total of 8 feline isolates from experimentally infected cats (Gookin et al., 2001) and 10 feline isolates from naturally infected cats (Gookin et al., 2010; Gray et al., 2010; Yaeger and Gookin, 2005) were shown as positive for the surface epitope 1.15. An additional 3 feline isolates were found positive for epitope 1.15, although the source of these isolates (i.e. from experimentally or naturally infected cats) was not specified. (Corbeil et al., 2008) All studies utilized the same ascitic fluid mAb 1.15 from the original studies with cattle (Hodgson et al., 1990), except for one which utilized a polyclonal anti-TF1.15 antibody. (Gray et al., 2010) No studies to date have investigated for the presence of epitope 1.17 in feline *T. foetus*, nor evaluated for antigen 1.15-1.17 in other trichomonads that infect cats (e.g. *Pentatrichomonas hominis* [*P. hominis*]). Evaluation of feline intestinal trichomonads for continued conservation of 1.15, the presence of epitope 1.17 and any role this antigen may play in mediating feline *T. foetus* cytopathogenicity is

warranted. If 1.15-1.17 is not present in feline *P. hominis* and/or aides in cytopathogenicity, it may represent an excellent novel diagnostic or therapeutic target for feline trichomonosis.

Although multiple protective surface proteins and/or epitopes have been identified as potential targets for a key role in initiating a protective immune response, epitope expression may differ between isolates and even between different *in vitro* passage numbers of the same isolate. (Hodgson et al., 1990; JS et al., 1993) Further investigation of factors mediating heterogeneous levels of expression are necessary in order to understand how best to utilize these molecules as preventative, diagnostic and/or therapeutic targets.

It is reasonable to propose that antigen 1.15-1.17 may represent a novel diagnostic or therapeutic target for feline trichomonosis if epitope 1.17 is conserved across feline *T. foetus* isolates and/or the function of this antigen in felines contributes to *T. foetus* adhesion-dependent cytopathogenicity. Given the similarities identified between virulence factors shared by genotypes, we propose that surface antigen 1.15-1.17 is also conserved between bovine and feline *T. foetus*.

CHAPTER TWO: IDENTIFICATION AND INVESTIGATION OF BOVINE *TRITRICHOMONAS FOETUS* SURFACE EPITOPES CONSERVED IN FELINE *TRITRICHOMONAS FOETUS*

ABSTRACT

Tritrichomonas foetus (T. foetus) is a flagellated protozoa that infects the distal ileum and proximal colon of domestic cats and the urogenital epithelium of cattle. Feline trichomonosis is recognized as a prevalent cause of large bowel diarrhea in cats worldwide. The suspected route of transmission is fecal-oral, with cats in densely crowded environments at highest risk for infection. Thus, the recommended strategy for minimizing spread of infection is to identify and isolate *T. foetus*-positive cats from the general population. Rapid and accurate identification of infected cats can be challenging with currently inadequate ability to accurately detect the organism in samples at point of care facilities. Thus, identification of targets for use in development of a novel diagnostic test, preventative, or therapy for feline *T. foetus* infection is a significant area of research. Despite a difference in organ tropism between *T. foetus* genotypes, evidence exists for conserved virulence factors between feline and bovine *T. foetus*. The bovine *T. foetus* surface antigen, 1.15-1.17, has been identified as an adhesin that is conserved across isolates. Vaccination with the purified antigen results in amelioration of cytopathogenicity and more rapid clearance of infection in cattle. We hypothesized that antigen 1.15-1.17 is conserved across feline *T. foetus* isolates and that this antigen would represent an attractive target for development of a novel diagnostic test or therapy for feline trichomonosis. In these studies, we used monoclonal antibodies previously generated against epitopes of the bovine *T. foetus* 1.15-1.17 antigen, to evaluate for the presence and role of antigen 1.15-1.17 in the cytopathogenicity of feline *T. foetus*. A previously validated *in vitro* co-culture approach was used to model feline

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T. foetus infection. Immunoblotting, immunofluorescence assays, and flow cytometric analysis demonstrated the presence and surface localization of antigen 1.15-1.17 across all feline *T. foetus* isolates tested. Antigen 1.15-1.17 was notably absent in the presumably nonpathogenic intestinal trichomonad, *Pentatrichomonas hominis*, a parasite that can be confused microscopically with *T. foetus*. In contrast to bovine trichomonosis, antigen 1.15-1.17 did not promote *T. foetus* adhesion to or cytopathogenicity towards the intestinal epithelium in an *in vitro* model. Although a porcine, rather than feline, *in vitro* intestinal epithelial cell line was utilized for all co-culture assays, these results support further investigation of protein 1.15-1.17 as a target for the diagnosis of feline *T. foetus* infection.

Introduction

Tritrichomonas foetus (*T. foetus*) is a protozoal pathogen responsible for chronic diarrhea in domestic cats as well as reproductive failure (i.e. infertility, abortions and early embryonic death) in cattle. Feline *T. foetus* is a global enteric pathogen with infections recognized in North America, Europe and Australia, among others. (Bissett et al., 2008; Gookin et al., 2004; Gunn-Moore et al., 2007; Hosein et al., 2013; Miro et al., 2011; Xenoulis, 2011) The suspected route of transmission is fecal-oral, with cats in densely crowded environments (e.g. shelters, catteries and cat shows) at highest risk for infection. Thus, the recommended strategy for minimizing spread of infection is to identify and isolate *T. foetus*-positive cats from the general population. Rapid and accurate identification of infected cats can be challenging, particularly in point of care facilities, with detection currently limited to fecal smear and In-Pouch™ culture. The sensitivities of these tests are low, 14 and 55%, respectively (Foster et al., 2004; Gookin et al., 2003; Gookin et al., 2002) and may be associated with misdiagnosis as a result of the presence of other intestinal protozoal pathogens that infect cats (e.g. *Giardia* spp and *Pentatrichomonas hominis*). The best available diagnostic test, polymerase chain reaction (PCR), is not an in-clinic assay at present. Although PCR carries moderately good sensitivity, as long as performed by experienced diagnosticians (Gookin et al., 2002), results may take up to 2 weeks. Therefore, PCR may not be a viable option for shelters needing to frequently test suspect cats.

Safe and effective therapies for the treatment of feline trichomonosis are also limited. Only one drug, ronidazole, is available to treat feline *T. foetus* infection, but this drug is used off-label for treatment of cats, has been associated with increasing resistance, and has a narrow margin of safety, potentially resulting in neurotoxicity. (Gookin et al., 2010; LeVine et al., 2011; Rosado et al., 2007) Thus, identification of targets for use in development of a novel

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diagnostic test, preventative, or therapy for feline *T. foetus* infection is a significant area of research. Despite a difference in organ tropism, bovine and feline *T. foetus* are genetically similar and evidence exists for conserved virulence factors between genotypes. (Morin-Adeline et al., 2014; Slapeta et al., 2012; Sun et al., 2012; Tolbert et al., 2014)

The bovine *T. foetus* surface antigen, 1.15-1.17, has been identified as an adhesin that promotes parasite attachment to the urogenital epithelium (Hodgson et al., 1990). This surface antigen, later identified to be part of the *T. foetus* lipophosphoglycan (LPG) complex, was found to be conserved across 37 bovine isolates tested. (Ikeda et al., 1993; Singh et al., 1994) Treatment of bovine *T. foetus* with monoclonal antibodies directed against epitopes of the 1.15-1.17 antigen significantly reduced adhesion of bovine *T. foetus* to bovine vaginal epithelial cells. (Hodgson et al., 1990) Given the similarities previously identified between the two genotypes, we hypothesized that feline *T. foetus* also express antigen 1.15-1.17, which, based on the findings for bovine *T. foetus*, may represent a novel diagnostic and therapeutic target for feline trichomonosis.

In these studies, we used monoclonal antibodies previously generated against epitopes of the bovine *T. foetus* 1.15-1.17 antigen, to evaluate for the presence and role of antigen 1.15-1.17 in feline *T. foetus*. Our results suggest that antigen 1.15-1.17 is present in all feline *T. foetus* isolates tested (n=12). Moreover, antigen 1.15-1.17 is notably absent in *P. hominis*, a nonpathogenic intestinal trichomonad that can be confused for *T. foetus*. In contrast to bovine trichomonosis, 1.15-1.17 does not promote *T. foetus* adhesion to or cytopathogenicity towards intestinal epithelium in this *in vitro* model. While these results suggest this antigen may not be a useful target for treatment of this infection in cats, our data does support further investigation of antigen 1.15-1.17 as a target for the diagnosis of feline *T. foetus* infection.

Materials and Methods

Feline T. foetus isolates: Twelve axenized feline *T. foetus* isolates (Isolates A, B, C, D, F, Ja, JT, K, Ma, Mo, Sta and Sti) were used for all assays to account for variability amongst trichomonads. Isolates were harvested at the University of Tennessee and North Carolina State University from fecal samples obtained from naturally infected cats geographically distributed throughout the USA. Passage numbers 3-10 were used for all assays. An isolate of the nonpathogenic feline intestinal trichomonad, *Pentatrichomonas hominis*, harvested from the feces of a naturally infected cat, and two bovine *T. foetus* isolates, one from industry (American Type Culture Collection [ATCC®], Manassas, KS) and one isolated from a naturally infected bull from Auburn University (Aub), were used in all assays. *P. hominis* and bovine *T. foetus* isolates served as negative and positive controls, respectively. As only one *P. hominis* isolate has ever been harvested from a naturally infected cat, this was the only negative control isolate utilized. All isolates were cultivated as previously described. (Gookin et al., 2001; Tolbert et al., 2012) Bovine *T. foetus* isolates were donated from Dr. Rebecca Wilkes (University of Tennessee) and Dr. Chance Armstrong (Auburn University).

IPEC-J2 cells: The porcine jejunal epithelial cell line (IPEC-J2), a non-transformed primary cell line originally isolated from neonatal piglet jejunum, was cultured as previously described. (Tolbert et al., 2014; Tolbert et al., 2013) This line was obtained as a gift from Helen M. Berschneider. Briefly, IPEC-J2 monolayers were cultivated in culture media that included Advanced Dulbecco's Minimal Essential Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 5 μg/ml each of insulin, transferrin, and selenium, EGF (5 ng/ml), penicillin (50,000 IU/ml), streptomycin (50,000 mg/ml) and 5% fetal bovine serum and incubated at 37°C

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in 5% CO_2. All cell culture reagents were supplied by Thermo Fisher Scientific (Thermo Scientific, Rockford, IL). Prior to co-culture studies with feline *T. foetus* isolates, IPEC-J2 were seeded on 24-well polystyrene plates and allowed to reach confluence as previously described. (Tolbert et al., 2014; Tolbert et al., 2013) Immediately prior to infection studies, culture media was replaced with co-culture media, which contained the same constituents as culture media but was devoid of serum to prevent replication of trichomonads and epithelial cells during infection studies. Uninfected IPEC-J2 monolayers and monolayers infected with isotype control (unlabeled mouse IgG; Southern Biotech, Birmingham, AL)-treated *T. foetus* were used as negative and positive controls, respectively, for all co-culture assays using mAb-treated *T. foetus*. IPEC-J2 cells were used at passage numbers 45-61.

Monoclonal antibodies: Monoclonal antibodies (mAbs) targeting bovine *T. foetus* surface epitopes 1.15 and 1.17, kindly provided by Dr. Bibhuti Singh (Upstate Medical University), were used for all assays. Both mAbs had been prepared via standard methods using hybridomas prepared with splenocytes from BALB/c mice immunized with 0.8×10^6 live bovine trichomonads. (Hodgson et al., 1990) All antibodies were stored as aliquots at -20°C to prevent antibody degradation from repetitive freezing and thawing.

Western blot: Western blot analysis was used to evaluate for the presence of bovine *T. foetus* epitopes 1.15 and 1.17 in feline *T. foetus* isolates. Trichomonads at mid-logarithmic growth phase were washed twice with 1X Dulbecco's Phosphate Buffered Saline (DPBS), lysed in 400 µL radioimmunoprecipitation assay (RIPA) buffer containing 1X phosphate buffered saline (PBS) (pH 7.4), 1% IGEPAL, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS), then sonicated twice for 15 seconds each prior to incubation for 30 min at 4°C.

Lysates were centrifuged at 13,793 x g for 10 min at 4°C, with supernatant recovered and diluted in lithium dodecyl sulfate (LDS) buffer (Novex® by Life Technologies, Carlsbad, CA). Protein concentrations were determined with a bicinchoninic acid (BCA) assay according to manufacturer's instructions (Thermo Scientific, Rockford, IL). Lysates were reduced in SDS reducing buffer (Novex® by Life Technologies, Carlsbad, CA) and heated at 70°C for 10 minutes prior to SDS-PAGE analysis. Proteins were electrophoretically separated at a voltage of 200V for 45 minutes using 4-12% Bis-Tris gels (Novex® by Life Technologies, Carlsbad, CA), and transferred to a nitrocellulose membrane at 30V for 60 minutes. Nitrocellulose membranes were blocked overnight in blocking buffer (Starting Block T20 buffer; Thermo Fisher Scientific, Rockford, IL) at 4°C. Immunoblotting was performed using the anti-1.15 and 1.17 primary antibodies diluted 1:500 in Tris-Buffered Saline Tween-20 (TBST) for 4 hours at room temperature (RT) under gentle agitation. Membranes were washed three times with TBST for 15 minutes each under gentle agitation prior to addition of horseradish peroxidase-conjugated F(ab')$_2$ goat anti-mouse IgG and IgM secondary antibody (Jackson Immuno, West Grove, PA) diluted at 1:500 in blocking buffer for 30 minutes at RT. All membranes were washed in TBST six times for 15 minutes each prior to developing. Immunoblots were exposed to a chemiluminescent agent for 5 minutes (Thermo Fisher Scientific, Rockford, IL) and evaluated with ImageQuant™ LAS 4000 software.

Indirect immunofluorescence: Immunofluorescence (IF) was used as an additional qualitative assay to evaluate for the presence of 1.15 and 1.17 in feline *T. foetus* isolates. Mid-log phase whole organism trichomonads were pelleted at 1500 x g for 5 minutes at RT, washed once with 1X PBS and re-suspended to 1 x 10^7 trichomonads/mL in 1XPBS. A hydrophobic barrier pen (Vector Laboratories, Burlingame, CA) was used to create barriers on slides pre-

treated for electrostatic adherence (Thermo Fisher Scientific, Rockford, IL) in order to ensure trichomonads would remain contained to specified areas of the slide. 3×10^6 whole organism trichomonads were applied to slides pre-treated for electrostatic adherence (ThermoFisher Scientific) via direct pipetting to areas already denoted by the barrier pen and allowed to adhere at RT for 1 hour. All samples were fixed in acetone at -20°C for 15 min and then allowed to air dry for up to 3 minutes. Both adherence and fixation steps were optimized from an original protocol that utilized a cytospin to adhere trichomonads to slides without a permeabilizing agent, thus allowing surface localization of the antigen via IF. At centrifugation speeds required to successfully adhere whole *T. foetus* to slides, damage to protozoal membranes was observed. Thus, hand application with fixation was necessary to preserve the shape of trichomonads. As acetone acts as a simultaneous permeabilizing agent, results of these assays confirmed presence of antigen but did not distinguish between intracellular and surface localization. Slides were rinsed briefly in 1X PBS for 5 minutes and incubated for 1 hr at RT in blocking buffer (1X PBS, 5% goat serum and 2% BSA). Slides were then incubated with mAb (1.15 or 1.17) diluted 1:100 in blocking buffer for 3 hours in a humidified chamber at RT. Following incubation with primary antibody, slides were rinsed three times in 1X PBS for 5 minutes each followed by incubation in fluorescein isothiocyanate (FITC)-conjugated F(ab')$_2$ goat anti-mouse IgG and IgM (Jackson Immuno Research, West Grove, PA) diluted 1:50 in block buffer for 1 hour at RT. Following three rinses with 1X PBS for 5 minutes each at RT, the nuclear counterstain, DAPI (i.e. 4'6-diamidino-2phenylindole) (Vectashield, Vector Laboratories, Burlingame, CA) was applied for visualization of epithelial and trichomonad nuclei. A mercury epifluorescence microscope (Nikon® Digital Sight DS, Melville, NY) was used for detection of fluorescence.

Flow cytometry: Flow cytometry was performed for semi-quantitative analysis and confirmation of surface localization of 1.15-1.17. Trichomonads, harvested at mid-logarithmic phase of growth, were pelleted at 1500 x g for 5 minutes, washed once in 1X PBS and re-suspended to 5×10^6 trichomonads/mL in flow buffer containing 60 mL of 0.5% sodium azide solution, 87 mL of 1X PBS and 3 mL of fetal bovine serum. This flow buffer preserved trichomonad membrane structure while preventing permeabilization of the cells, thus allowing for the detection of epitopes 1.15 and 1.17 on the surface of feline isolates of *T. foetus*. The use of microcentrifuge tubes was avoided for all flow cytometric analyses as shear forces from a microcentrifuge lysed the protozoa, rendering it impossible to evaluate for the presence of surface antigens. Conical tubes (15 mL) preserved the shape of mid-log phase trichomonads and were used for all flow assays. Three isolates (A, Sti, Ja) were evaluated for the presence of both epitopes with flow cytometric analysis. Each isolate was divided into 1 mL aliquots for the following treatment groups: (1) *T. foetus* receiving both primary and secondary antibody, (2) *T. foetus* receiving only secondary antibody (negative control), and, (3) *T. foetus* receiving no antibodies (auto-fluorescence). Isolates were pelleted, and 10 µL of primary mAb, either 1.15 or 1.17, was added to the pellet for a final dilution of 1:100 per 1 mL of re-suspended trichomonads. All samples were incubated on ice for 30 minutes and washed once with 1 mL of 1X PBS. Fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-mouse IgG and IgM antibody was applied at the same concentration of 1:100 directly to the pellet, incubated for 30 min on ice, washed once more and re-suspended to a final volume of 1 mL in 1XPBS for analysis. A total of 10,000 events (i.e. trichomonads) were analyzed for each treatment population, and each isolate was analyzed in triplicate. All data were acquired on an Attune®

acoustic focusing flow cytometer (Applied Biosystems, Thermo Fisher Scientific, Rockford, IL).

Adhesion assays: To quantitatively evaluate the effect of epitopes corresponding to antibodies 1.15 and 1.17 on the adhesion of feline *T. foetus* to the intestinal epithelium, adhesion assays were performed using uninfected monolayers and monolayers infected with isotype control-treated (unlabeled mouse IgG, Southern Biotech, Birmingham, AL) or mAb (i.e. anti-1.15 or 1.17)-treated *T. foetus*. Carboxyfluorescein succinimidyl ester (CFSE) (CellTrace™ CFSE cell proliferation kit, Thermo Fisher Scientific, Rockford, IL) was used to fluorescently label viable trichomonads prior to treatment with antibody or isotype control groups. IPEC-J2 monolayers were cultivated to confluence at a previously established concentration of approximately 5×10^4 cells/well in 24-well polystyrene plates prior to infection with *T. foetus* as previously described. (Tolbert et al., 2014; Tolbert et al., 2013) Immediately prior to infection, culture media was replaced with co-culture media, which contained the same added ingredients except serum, to prevent replication of trichomonads and epithelial cells during infection studies. IPEC-J2 monolayers were inoculated with 10×10^6 *T. foetus* per well as previously described (Tolbert et al., 2014; Tolbert et al., 2013), with all trichomonad groups pre-treated with either 1:100 mAb (anti-1.15 or 1.17) or a volume equivalent to 1 µg unlabeled mouse IgG per 1×10^6 trichomonads, according to the manufacturers recommendations for a validated isotype control, prior to infection. IPEC-J2 monolayers were infected with trichomonads immediately following application of the respective treatment (i.e. antibody or isotype control) to each group. Any volume differences between the isotype control and mAb-treated groups were accounted for with addition of DPBS. A third group containing only 1:100 primary antibody (mAb 1.15 or 1.17) but lacking *T. foetus* was used as additional control to evaluate for

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non-specific binding of antibody to the IPEC-J2 cells. Infected and uninfected groups of IPEC-J2 cells were maintained at 37°C in 5% CO_2. After 6 hours of co-culture, an established time for adhesion based on a previously validated co-culture model (Tolbert et al., 2013), the cells were washed twice gently with 37°C DPBS and counterstained with DAPI (Vectashield, Vector Laboratories, Burlingame, CA). Adherent trichomonads in individual wells were counted in six high power fields (HPFs) using an epifluorescence microscope (Nikon® Digital Sight DS, Melville, NY), with the average of six HPFs representative of one replicate. Two feline *T. foetus* isolates (A, Sti) were utilized for adhesion experiments. Assays were performed with a minimum of 4 replicate cultures per treatment group and repeated twice for a total of three experiments per isolate. Mean or median adherence were compared among all groups with either parametric or nonparametric distributions, respectively.

Crystal violet cytotoxicity assays: To provide a quantitative analysis of the effect of epitopes 1.15 and 1.17 on *T. foetus*-induced epithelial cytotoxicity, crystal violet (CV) assays were performed, as previously described, (Tolbert et al., 2014) using uninfected monolayers and monolayers infected with isotype control-treated (unlabeled mouse IgG) or 1:100 mAb (i.e. anti-1.15 or 1.17)-treated *T. foetus*. Monolayers were infected as described above for adhesion assays, with the exclusion of labeling with CFSE. After 24 hours of co-culture (time based on previously established cytotoxicity model), wells were washed twice with DPBS and stained with crystal violet as previously described. (Tolbert et al., 2014) IPEC-J2 monolayers were gently washed twice with dH_2O and allowed to air dry. Monolayers were immediately observed by light microscopy using a Nikon inverted light phase-contrast microscope (Nikon® Digital Sight DS-U3, Melville, NY), then were solubilized in 100 μL 1% SDS in 50% ethanol for spectrophotometric analysis. Solubilized cells were transferred to 96 well plates and the

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intensity of staining was quantified using a spectrophotometer at a wavelength of 570 nM. Two feline *T. foetus* isolates (A, Sti) were used for cytotoxicity experiments. Assays were performed in a minimum of 6 replicate cultures per treatment group and repeated twice for a total of three experiments per isolate. Spectrometric means or medians were compared among all groups (*T. foetus* with or without mAb treatment and uninfected monolayers) for either parametric or nonparametric distributions, respectively.

Statistical Analysis: Data were analyzed for normality (Shapiro-Wilk) and variance (Brown-Forsythe) using a statistical software package and tested for significance using parametric or non-parametric tests as appropriate (Sigma Plot13, Systat Software, Inc.). Parametric data were analyzed using either a Student's t-test or one-way ANOVA. Non-parametric data were analyzed using either a Mann-Whitney rank sum test or Kruskal-Wallis one-way ANOVA on Ranks. When a significant treatment effect was observed, a posthoc Tukey, Holm-Sidak or Dunn's test was performed to determine which groups were significantly different from each other. n = number of replicates. Results are reported as mean ± standard deviation. For all analyses, $P \leq 0.05$ was considered significant.

Results

Both epitopes of bovine T. foetus antigen 1.15-1.17 are present in feline T. foetus isolates: Western blotting was performed to evaluate for the presence of epitopes 1.15 and 1.17 across multiple feline *T. foetus* isolates. Protein lysates from all twelve feline and two bovine *T. foetus* isolates were found to possess a diffusely migrating band, characteristic of a glycosylated protein (e.g. lipophosphoglycan complex or glycoprotein), ranging from approximately 36 to 64 kDa following incubation with mAb 1.15 (**Fig. 1A**). Diffuse bands of approximately the same

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molecular weight were also seen in the same isolates following application of mAb 1.17 **(Fig. 1B)**. Both epitopes were identified to be absent in *P. hominis* (**Fig. 1A and B**). Although equivalent concentrations of protein lysate, based on the previously performed BCA assay, were evaluated in SDS-PAGE analysis, no acceptable protein loading control is available for trichomonads. Thus, densitometric quantification of antigen presence was not performed.

Indirect immunofluorescence assays were used as an additional qualitative methodology to confirm presence of epitopes 1.15 and 1.17 in feline *T. foetus*. Immunofluorescence was first confirmed using a bovine *T. foetus* isolate obtained from a naturally infected bull (Auburn University) for both 1.15 (**Fig. 2A**) and 1.17 (**Fig. 2B**). Both feline *T. foetus* isolates displayed positive fluorescence (**Fig. 3**) following application of mAbs 1.15 (**Fig. 3 A, B**) and 1.17 (**Fig. 3 C, D**). No fluorescence was observed following application of either mAb to *P. hominis* (**Fig. 4A, B**). All trichomonad populations were negative for fluorescence following omission of all antibodies (i.e. auto-fluorescent controls) and after application of secondary antibody alone (i.e. negative controls).

Epitopes 1.15 and 1.17 are expressed on the surface of feline T. foetus: Flow cytometry was used to confirm surface localization of epitopes 1.15 and 1.17. Figure 5 graphically represents the positive fluorescence of one bovine and one feline *T. foetus* isolate following treatment with mAb 1.15 or 1.17, and the relative negative fluorescence of the respective auto and negative control populations. The use of a sodium azide without a permeabilizing agent such as formalin, prevented surface capping (i.e. inversion of the cell membrane) and confirmed positive fluorescence was due to the presence of a surface localized, rather than intracellular, antigen. Feline *T. foetus* isolates demonstrated surface expression of *T. foetus* mAb 1.15 (**5A**) and 1.17 (**5B**). The average mean fluorescence of all bovine *T. foetus* treatment groups for auto fluorescence, negative control and

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trichomonads treated with either mAb 1.15 or 1.17 were 1.7, 6.1, 15.7 and 22.9 mean fluorescent units (MFUs), respectively. Average mean fluorescence of bovine controls following addition of mAbs 1.15 and 1.17 were 2.9 and 3.8 times greater than the negative control, respectively. The average fluorescence of auto-fluorescent and negative control populations of feline *T. foetus* isolate A were 3.4 and 45.3 MFUs, while treatment with mAb 1.15 or 1.17 yielded 55.2 and 97.7 MFUs. Isolate A had average MFUs 1.2 and 2.2 times greater than that of negative control populations following addition of mAbs 1.15 and 1.17. Similarly, feline *T. foetus* isolate Sti had auto-fluorescent and negative control MFUs of 17.5 and 63.3, which increased to 82.7 and 128.3 MFUs with addition of mAbs 1.15 and 1.17, respectively. This resulted in average MFUs that were 1.3 and 2 times greater than negative control populations for isolate Sti. The single feline *P. hominis* isolate had auto-flourescent and negative MFUs that were equivalent, with the negative control population only 4% more fluorescent than the negative control. Addition of mAbs 1.15 and 1.17 to *P. hominis* resulted in MFUs also equivalent to those of the negative control populations, with populations that were only 9% and 17% less fluorescent, respectively. Mean fluorescence units of mAb-treated groups were compared to negative control populations (receiving only FITC-conjugated secondary antibody) to provide an accurate reflection of fluorescence secondary to the presence of antigen rather than background fluorescence and non-specific binding of secondary antibody.

Surface protein 1.15-1.17 does not participate significantly in feline T. foetus adhesion to or cytotoxicity towards the intestinal epithelium: To investigate the role epitopes 1.15 and 1.17 play in facilitating adhesion and cytotoxicity of feline *T. foetus* to the intestinal epithelium, adhesion and cytotoxicity assays were performed. Figure 6 displays a representative image from an adhesion assay, illustrating a CFSE-labeled trichomonad adhering to an IPEC-J2 cell. No

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difference in adhesion was observed among groups at the 6 hour time point for mAb 1.15 (**Fig. 7**). However, there was a significantly increased number of 1:100 mAb 1.17 treated-feline *T. foetus* adhered to IPEC-J2 cells ($P < 0.05$) when compared to monolayers infected with isotype control-treated feline *T. foetus* at the 6 hour time point (**Fig. 8**). *T. foetus* uninfected IPEC-J2 monolayers had significantly less cytotoxicity ($P < 0.001$) as compared to treatment groups following 24 hours of co-culture for both mAbs 1.15 and 1.17 (**Fig. 9 A and B**). However, there was not a significant ameliorative effect of treatment with either mAb on feline *T. foetus*-infected monolayers compared to isotype control-treated monolayers ($P > 0.05$). No evidence of positive fluorescence was seen following application of mAbs 1.15 and 1.17 to IPEC-J2 cells, eliminating the possibility of non-specific binding to the epithelial cells themselves.

Discussion

Bovine *T. foetus* infects the reproductive tract in cattle. However, despite the difference in organ tropism, bovine and feline *T. foetus* are genetically similar and utilize analogous virulence factors in their pathogenesis. (Morin-Adeline et al., 2014; Slapeta et al., 2012; Tolbert et al., 2014) Given the role of the previously discovered bovine *T. foetus* surface antigen, 1.15-1.17, in mediating adhesion-dependent *T. foetus* induced cytotoxicity, it represented an appealing marker for exploration as a diagnostic or therapeutic target in feline trichomonosis.

Our first study objective was to determine if feline *T. foetus* also expressed antigen 1.15-1.17 and if this antigen was conserved across multiple feline *T. foetus* isolates. Using immunoblotting and immunofluorescence, we demonstrated that antigen 1.15-1.17 was expressed by all 12 feline *T. foetus* isolates tested. Moreover, an isolate of *Pentatrichomonas hominis*, a nonpathogenic feline intestinal trichomonad that can be confused for *T. foetus* using in-clinic

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diagnostics such as fecal smear and In-Pouch™ TF culture (Ceplecha et al., 2013; Gookin et al., 2003), did not express antigen 1.15-1.17. Flow cytometric evaluation demonstrated that, similar to the bovine genotype, this antigen was located on the surface of feline *T. foetus*, which would be important for the use of a drug targeting living trichomonads. The culmination of our initial results suggested that antigen 1.15-1.17 may represent a novel target for the diagnosis or therapy of feline *T. foetus* infection.

Antigen 1.15-1.17 is also recognized to be an important virulence factor for bovine *T. foetus* where it functions as an adhesin, facilitating attachment of the parasite to the urogenital epithelium. The antigen could be targeted to reduce bovine *T. foetus* cytopathogenicity both *in vitro* and *in vivo*. Treatment of bovine *T. foetus* with monoclonal antibodies directed against the two surface epitopes (i.e. 1.15 and 1.17) significantly reduced trichomonad adhesion to the bovine vaginal epithelium *in vitro*. Following vaccination with purified *T. foetus* 1.17, immunized heifers developed a robust local IgG response (Ikeda et al., 1995) and cleared infections more rapidly than non-vaccinated cattle, with 80% of immunized heifers clearing infections by week 7 post infection in comparison to only 37% of control heifers. (Anderson et al., 1996; Bondurant et al., 1993; Corbeil et al., 1998; Corbeil et al., 2001) Vaccinated animals also exhibited decreased severity of endometritis as assessed by histopathology than non-vaccinated cattle. (Anderson et al., 1996) Additionally, immunization of bulls with *T. foetus* 1.17 protected against trichomonad colonization of preputial and penile epithelium. (Cobo et al., 2009).

Given the precedential role established for antigen 1.15-1.17 in bovine cytopathogenicity, a second objective of the present study was to evaluate antigen 1.15-1.17 as a potential therapeutic target for feline trichomonosis. These studies were performed with a previously validated co-culture model system (Tolbert et al., 2014; Tolbert et al., 2013) using porcine jejunal epithelial

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(IPEC-J2) cells because no feline intestinal epithelial cell line is available. Feline *T. foetus* and porcine *T. foetus*, formerly known as *Tritrichomonas suis,* are genetically similar and both have a tropism for the gastrointestinal tract. (Mostegl et al., 2011; Slapeta et al., 2012; Tachezy et al., 2002) Given that no feline intestinal cell culture lines exist and it has previously been shown that feline *T. foetus* are capable of causing cytotoxicity to IPEC-J2 cells (Tolbert et al., 2014), these porcine epithelial cells are the most appropriate substitute. To characterize the role of antigen 1.15-1.17 in feline *T. foetus* infection, feline *T. foetus* were pre-treated with monoclonal antibodies (mAbs) directed against two epitopes of antigen 1.15-1.17 prior to co-culture with IPEC-J2 monolayers. In contrast to bovine *T. foetus*, neither inhibition of epitope 1.15 or 1.17 reduced adhesion of feline *T. foetus* to the intestinal epithelium. Rather, mAb treatment of feline *T. foetus* resulted in either no change or an increased ability to adhere to intestinal epithelial cells when compared to control-treated *T. foetus*. These findings suggest that antigen 1.15-1.17 is not critically important for promoting adhesion of feline *T. foetus* to the intestinal epithelium *in vitro*.

Previous studies have demonstrated the critical role of feline *T. foetus* adhesion to the parasite's induction of intestinal epithelial cell cytotoxicity. (Tolbert et al., 2013) Thus, it is unsurprising that, since 1.15-1.17 was not found to mediate adhesion, neither mAb 1.15 or 1.17 treatment ameliorated feline *T. foetus* destruction of IPEC-J2 cells. The reasons for the discrepancy in roles of the antigen between genotypes are unknown, but may be explained by differences in transmission (fecal-oral versus sexually transmitted) and/or organ tropism. Another possibility, considered less likely in the authors' opinion, is that the porcine nature of the intestinal epithelial cells used in this study may not reflect what would be seen with feline specific intestinal epithelial cells. As there are no commercially available feline intestinal epithelial cell lines with which to conduct these studies, porcine cells represent the next most suitable alternative, as feline and

porcine *T. foetus* (formely known as *Tritrichomonas suis*) are highly similar organisms that both demonstrate a unique tropism for the gastrointestinal tract and have recently been classified as the same species. (Slapeta et al., 2012) Although there are similarities between organisms, we cannot discount the possibility that a difference in *in vitro* epitope function would be seen with feline epithelial cells. Lastly, we cannot exclude the possibility of antibody degradation by *T. foetus*-secreted proteases (Kania et al., 2001), which would interfere with our ability to examine the effect of antibody treatment on the parasite. However, we think the latter is less likely as feline *T. foetus* have been demonstrated to secrete very little proteases compared to the bovine genotype. (Tolbert et al., 2014; Talbot et al., 1991; Thomford et al., 1996)

In conclusion, the present work demonstrates that feline *T. foetus* possess the previously identified bovine *T. foetus* antigen 1.15-1.17. The surface antigen was shared across all feline isolates tested. Documentation of this antigen in feline *T. foetus* isolates provides continued evidence of similarities between feline and bovine genotypes. Interestingly, unlike the bovine genotype, antigen 1.15-1.17 was not found to mediate adhesion to or exertion of cytotoxicity towards the intestinal epithelium in an *in vitro* model. We hypothesize that differences in pathogen transmission and host-specific organ tropism (i.e. direct deposition into the vaginal vault versus fecal-oral transmission and migration to the large intestines) may contribute to differences in the function of the antigen between genotypes. As antigen 1.15-1.17 was absent in the non-pathogenic feline intestinal trichomonad, *Pentatrichomonas hominis* andwas conserved across all feline *T. foetus* isolates evaluated, this antigen represents a candidate marker for development of a novel, on-site diagnostic assay to detect whole organism *T. foetus* in feline fecal samples.

Acknowledgments

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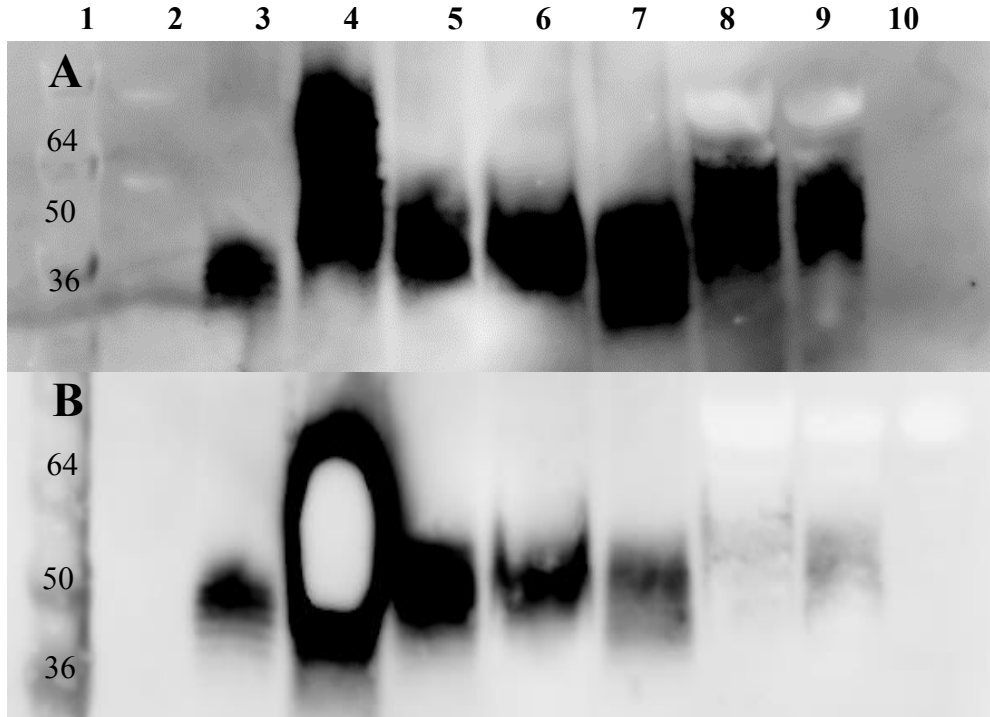
APPENDIX

Figure 1. Western blot confirms presence of antigen 1.15-1.17 in feline *T. foetus* (Tf) isolates and absence in *Pentatrichomonas hominis* (*P. hominis*). For both A and B, protein lysates were harvested from 2 bovine Tf isolates (lanes 3-4), 13 feline Tf isolates (5 depicted here are: A, Sti, Ja, Mo and F isolates; lanes 5-9) and 1 feline *P. hominis* isolate (lane 10). Immunoblotting confirms the presence of epitopes 1.15 (1A) and 1.17 (1B) following addition of anti-1.15 (1A) and 1.17 (1B) specific mAbs. *P. hominis* was negative for the presence of both epitopes. The diffusely migrating appearance of the band is characteristic for glycosylated proteins.
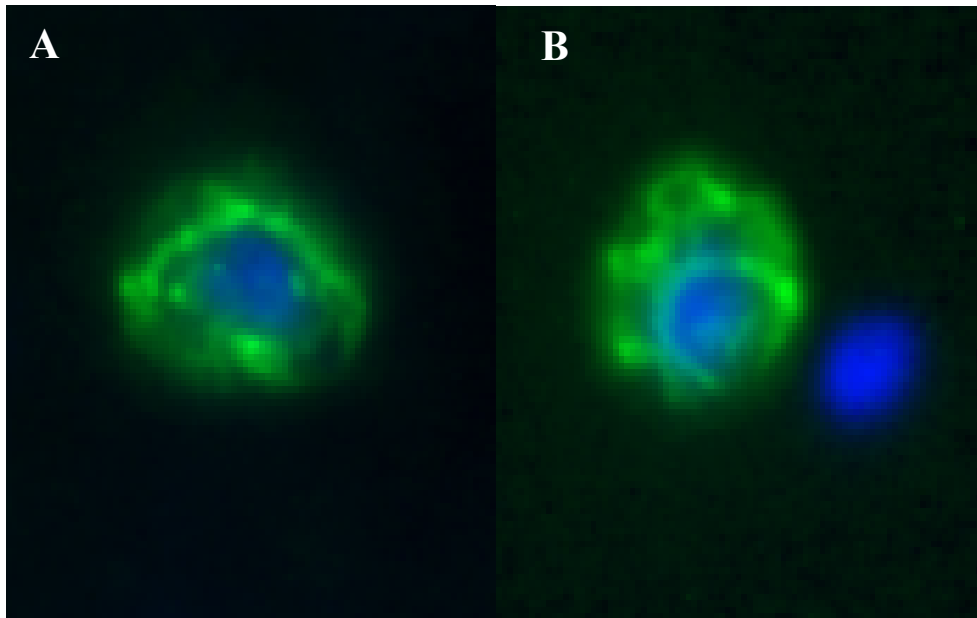
Figure 2. Indirect immunofluorescence confirms presence of surface antigen 1.15-1.17 in a bovine *T. foetus* (Tf) isolate (Aub). Fluorescence was noted following addition of both 1:100 mAb 1.15 (A) and mAb 1.17 (B). An auto-fluorescent (i.e. lacking application of all antibody), and negative (i.e. lacking addition of primary antibody only) controls were also evaluated when testing the bovine isolate. Neither control population displayed positive fluorescence. 1000x magnification.
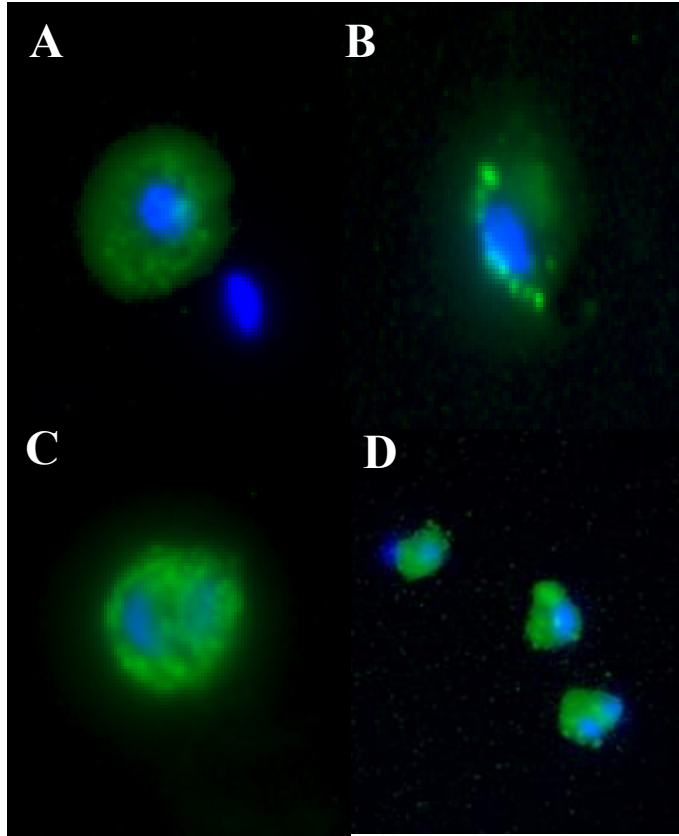
Figure 3. Indirect immunofluorescence confirms presence of surface antigen 1.15-1.17 in feline *T. foetus* (Tf) isolates. Positive fluorescence is observed following addition of 1:100 mAb 1.15 and 1.17 in feline Tf isolate A (A and C, respectively) and isolate S (B and D, respectively). An auto-fluorescent (i.e. lacking application of all antibody), and negative (i.e. lacking primary antibody only) control was performed for each isolate. Neither control population displayed positive fluorescence. All images were captured at either 400 or 1000x magnification.
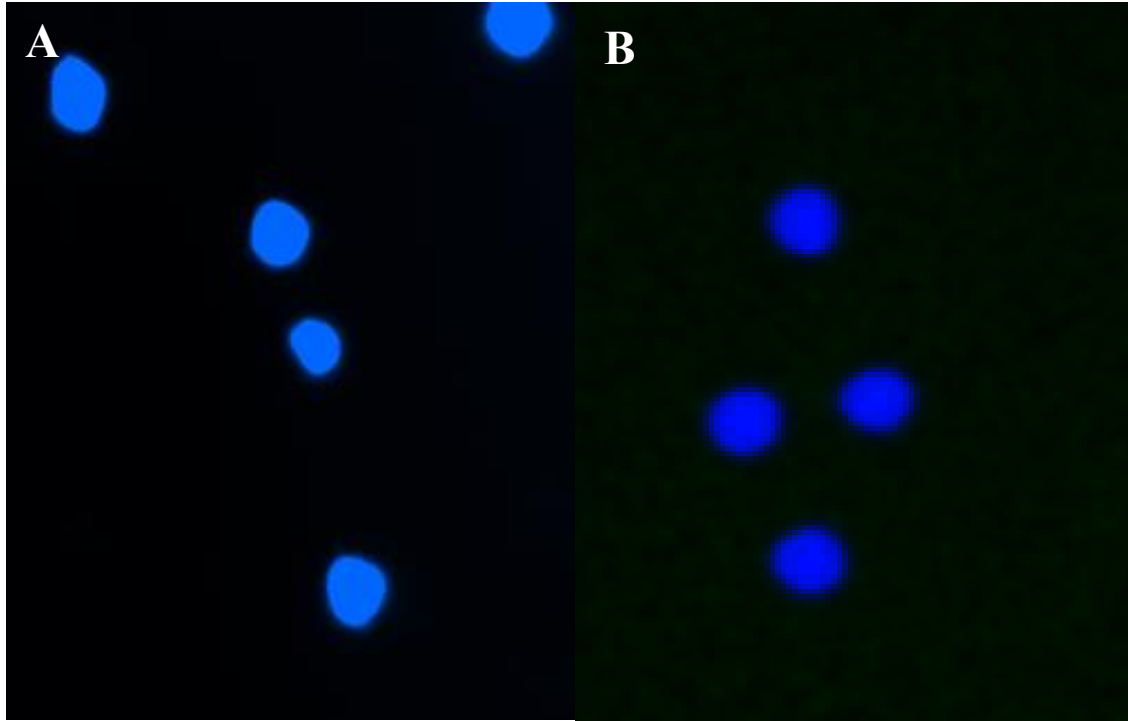
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Figure 4. Indirect immunofluorescence demonstrates absence of antigen 1.15-1.17 in a feline *Pentatrichomonas hominis* (*P. hominis*) isolate. No fluorescence was observed following addition of either 1:100 mAb 1.15 (A) or 1.17 (B) in a feline *P. hominis* isolate. An auto-fluorescent (i.e. lacking application of all antibody), and negative (i.e. lacking primary antibody only) control also performed. 1000X magnification.
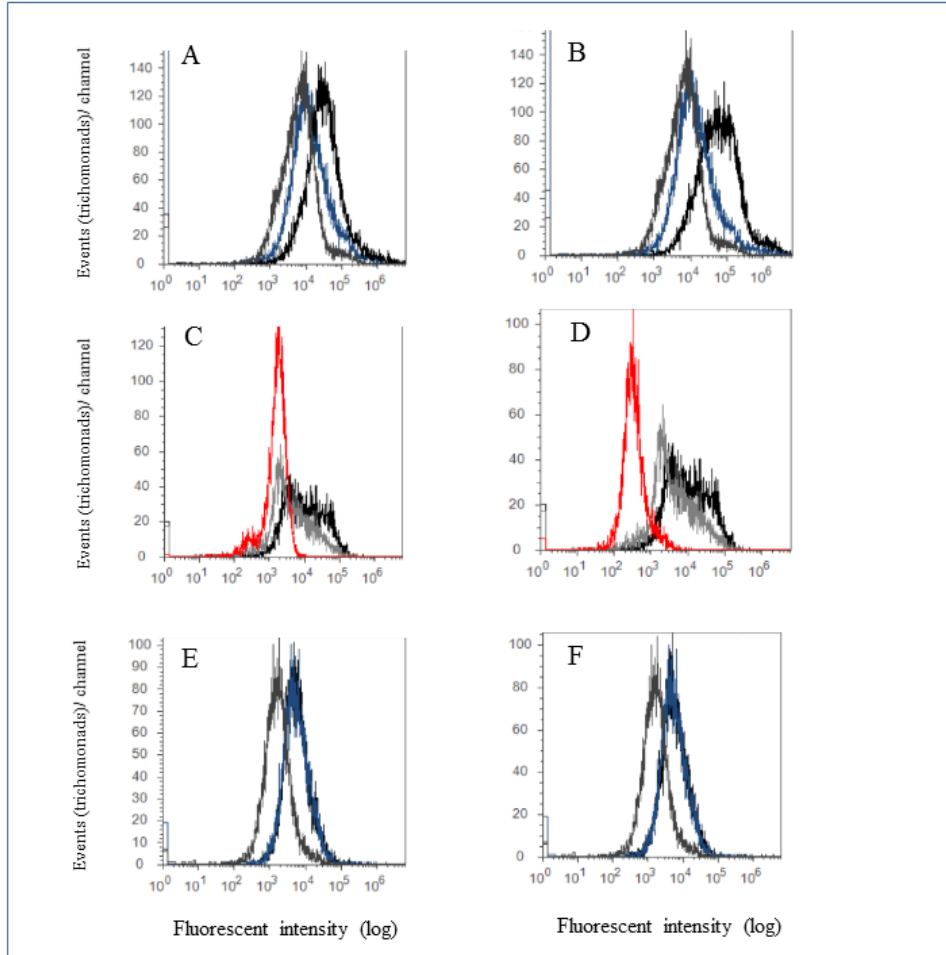
Figure 5. Flow cytometry confirms surface localization of antigen 1.15-1.17 in a feline *T. foetus* (Tf) isolate. Histograms denote positive fluorescence in one feline Tf isolate (Isolate Sti; A, B), one bovine Tf isolate (C, D), and negative fluorescence in a *P. hominis* isolate (E, F) following addition of mAb 1.15 or 1.17 and FITC-conjugated goat anti-mouse F(ab')2 IgG + IgM secondary antibody. For all histograms, the X axis represents the mean fluorescence intensity (FITC) on a logarithmic scale and the Y axis the cell count. Each peak, moving from left to right along the X axis, represents the mean fluorescence of the following groups: auto fluorescence (i.e. lacking addition of any antibody), negative control (i.e. lacking addition of primary monoclonal antibody but treated with 1:100 FITC-conjugated secondary antibody) and positive fluorescence following application of either mAb 1.15 (A, C, E) or mAb 1.17 (B, D, F) with FITC-conjugated secondary antibody. Assays were performed in triplicate with 10,000 events counted for each treatment population.
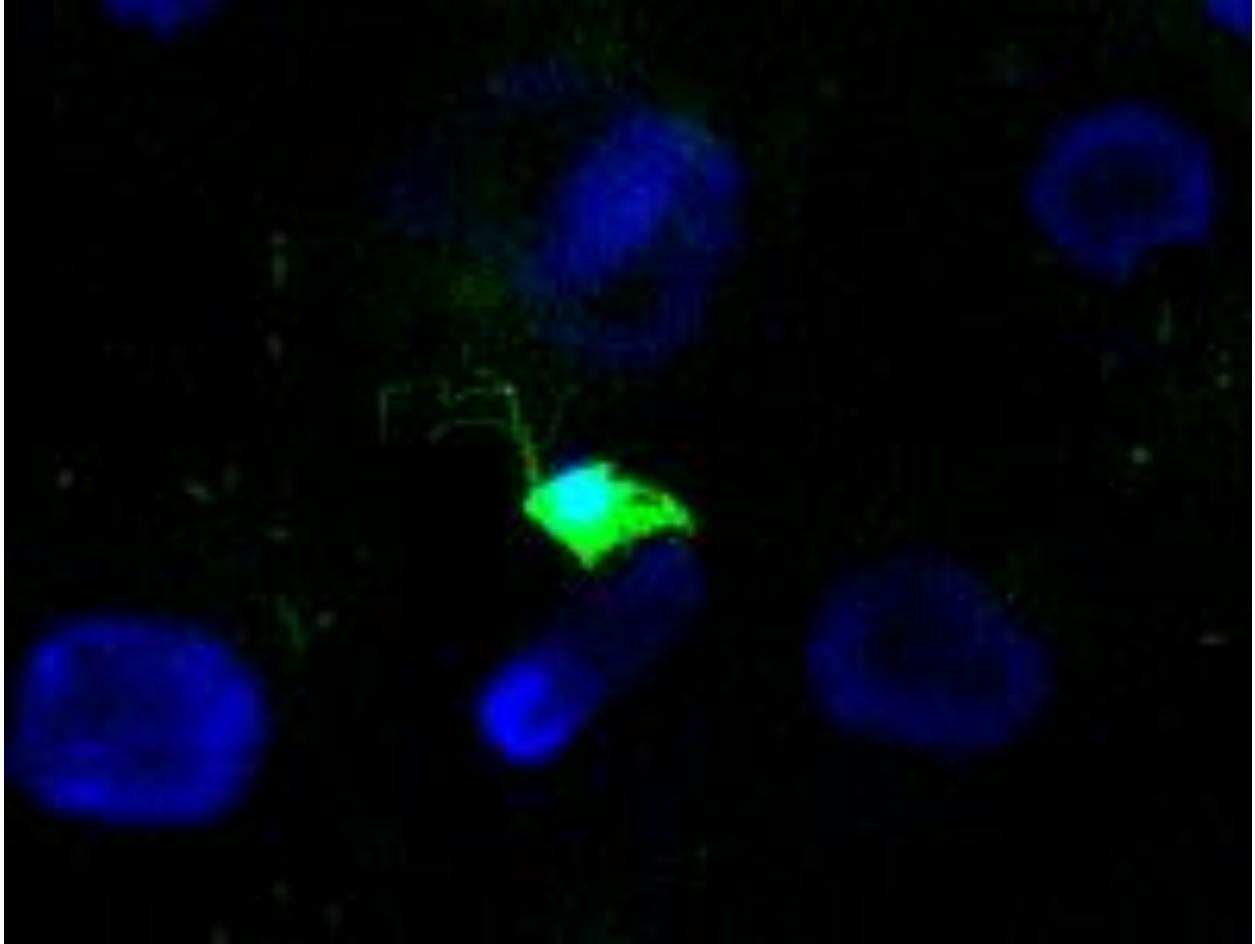
Figure 6. A Carboxyfluorescein diacetate, Succinimidyl Ester (CFSE) stained feline *T. foetus* (isolate A) adheres to an intestinal epithelial cell. Live trichomonads were labeled with CFSE, a viable stain, and co-cultured at 37°C in 5% CO2 with IPEC-J2 cells on 24-well polystyrene plates. Following co-culture for 6 hours, IPEC-J2 monolayers were washed twice with DPBS to remove unbound trichomonads and DAPI, a vital stain, was applied to stain the nucleus of living trichomonads and IPEC-J2 cells. 800X magnification.
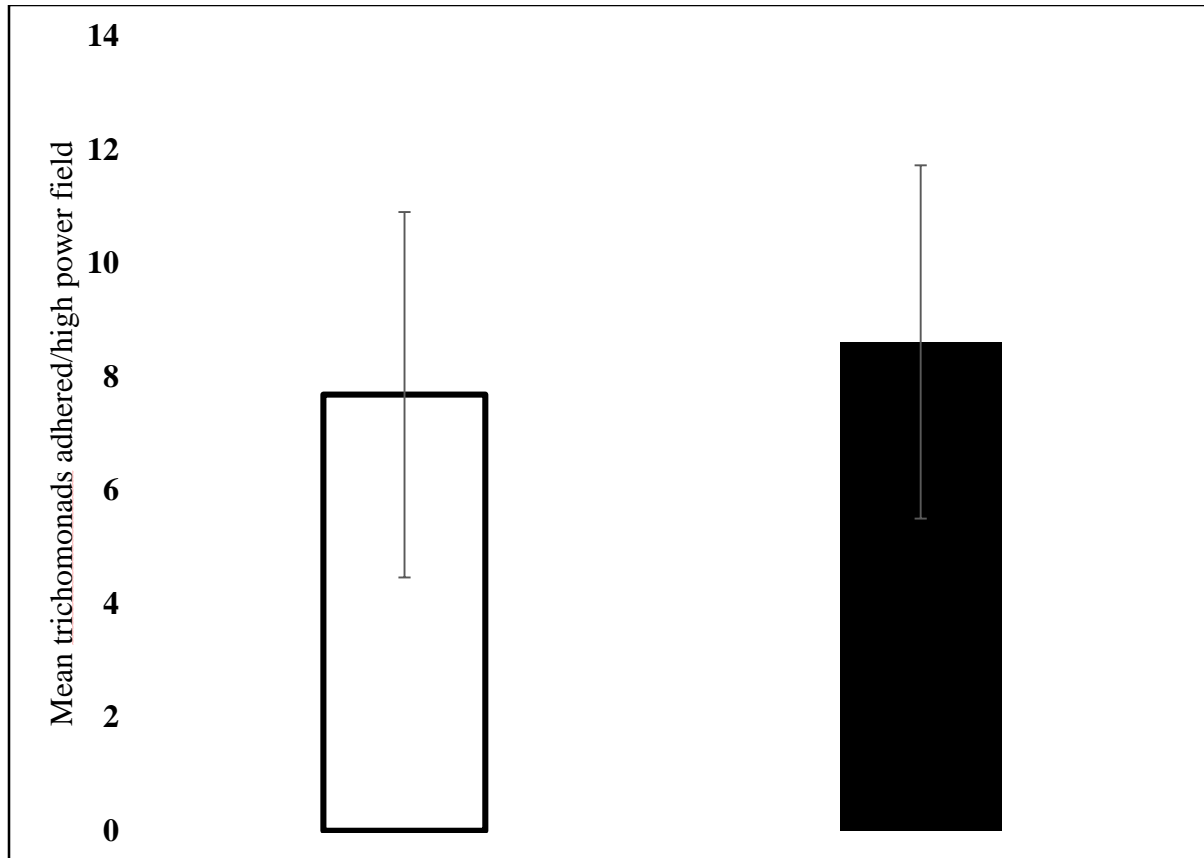
Figure 7. Epitope 1.15 does not facilitate adhesion of feline *T. foetus* to intestinal epithelial cells. The mean number of adhered trichomonads to IPEC-J2 monolayers after 6 hours of co-culture in presence of 10×10^6 feline *T. foetus* (isolate A) treated with either 1:100 isotype control (white bar; unlabeled mouse IgG) or mAb (black bar; 1.15). Data represent n=6 cultures per treatment and are reported as means $\pm$ SD. Determined by a Student's t-test. All assays were performed in triplicate.
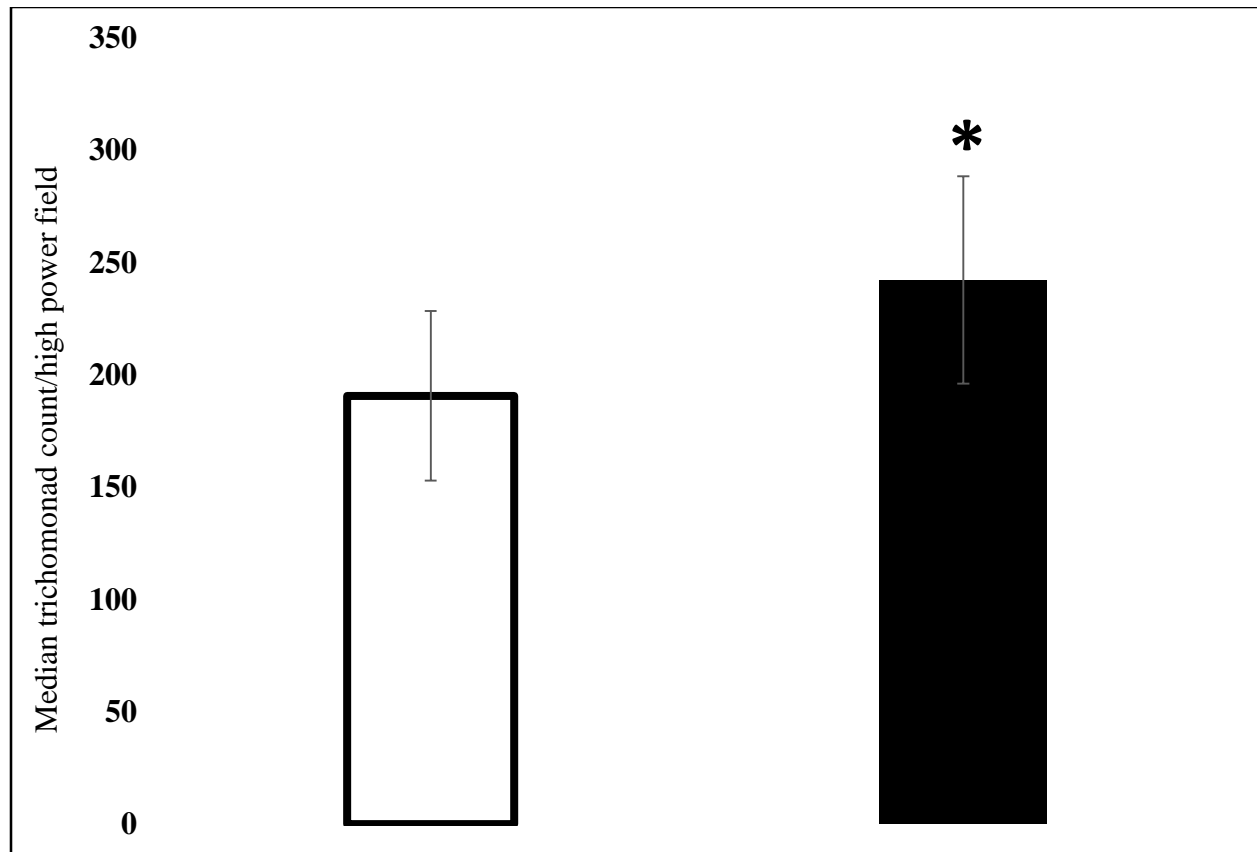
Figure 8. Epitope 1.17 does not promote adhesion of feline *T. foetus* to intestinal epithelial cells. The mean number of adhered trichomonads to IPEC-J2 monolayers after 6 hours of co-culture in the presence of 10 x 10^6 feline *T. foetus* (isolate A) treated with either 1:100 isotype control (white bar; unlabeled mouse IgG) or mAb (black bar; 1.17). Data represent n=6 cultures per treatment and are reported as median $\pm$ SE. *P < 0.05 between groups. Determined by a Mann-Whitney rank sum test. All assays were performed in triplicate.
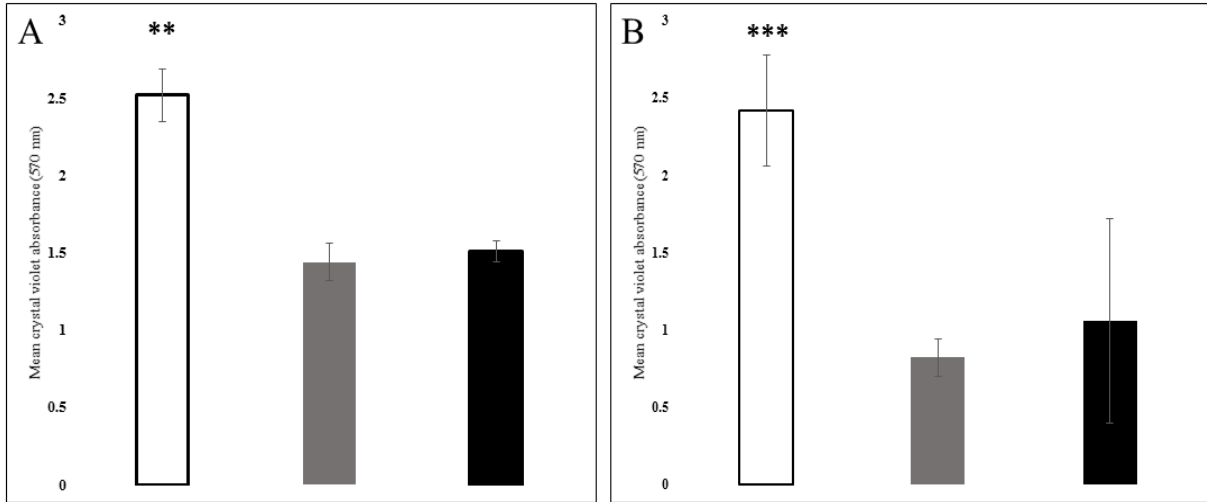
Figure 9. Antigen 1.15-1.17 does not promote *T. foetus*-induced intestinal epithelial cell cytotoxicity in vitro. Spectrophotometric analysis of crystal violet absorbance of IPEC-J2 monolayers following 24 h co-culture in the absence (white bars) or presence of 10 x 10^6 feline *T. foetus* (isolate A) pre-treated with either 1:100 mAb [black bars; 1.15 (A) or 1.17 (B)] or isotype control (gray bars; unlabeled mouse IgG). Data represent n=6 cultures per treatment and are reported as means ± SD. ***P < 0.0001 **P < 0.001 compared to *T. foetus*-infected monolayers. Determined by a one-way ANOVA and post-hoc Holm-Sidak. All assays were performed in triplicate.

VITA

Emily Nissa Gould was born in Grass Valley, California on September 17, 1984, with the majority of her childhood and adolescence was spent in Northern California. She attended high school at Casa Roble Fundamental High School in Orangevale, CA and was attended the University of California at Davis for both undergraduate and graduate degrees. During her time as an undergraduate at UC Davis, she was fortunate enough to work in the feline genetics laboratory of Dr. Leslie Lyons from 2004-2006. This project, which involved creating a linkage map for a craniofacial defect in Burmese cats, gave her initial exposure to molecular research techniques and facilitated bestowment of a Bachelors of Science Degree in Animal Biology, awarded in 2006. In between undergraduate and graduate school, Emily worked full time in Sacramento at a small animal general practice prior to attending the UC Davis School of Veterinary Medicine from 2008-2012.

After acquiring her Doctorate of Veterinary Medicine in 2012, she completed a rotating small animal private practice internship in Ventura, CA at the Veterinary Medicine and Surgical Group (VMSG). Her rotating internship continued to foster the desire to pursue a residency in Small Animal Internal Medicine. To this end, Emily then completed a private practice Internal Medicine specialty internship from 2013-2014 at the Animal Specialty and Emergency Center (ASEC) in West Los Angeles, CA prior to beginning her MS degree with the Comparative and Experimental Medicine (CEM) department at the University of Tennessee in Knoxville, TN.

Since joining the CEM department in 2014, the focus of Emily's thesis work has been the discovery of targets in feline *Tritrichomonas foetus* isolates for development of novel diagnostics or therapeutics for feline trichomonosis. The work detailed by this thesis confirms that a

previously discovered bovine *T. foetus* surface protein is conserved across and also surface localized in feline *T. foetus* isolates. These properties make it an ideal candidate for future development of a rapid, in-clinic assay to accurately differentiate cats with trichomonosis versus other protozoal causes of large bowel diarrhea.