ABSTRACT

TOLBERT, MARY KATHERINE. Mechanisms of Feline *Tritrichomonas foetus* Adherence to the Intestinal Epithelium and Novel Sites for Pharmacological Control. (Under the direction of Dr. Jody Gookin).

BACKGROUND AND AIMS: *Tritrichomonas foetus* (TF) is a mucosal protozoan that parasitizes the feline ileum and colon resulting in chronic diarrhea. TF has a worldwide distribution with no effective therapies to treat infection. Thus, defining the cellular mechanisms of disease and developing novel treatment strategies are of considerable interest. The biochemical mechanisms by which TF causes diarrhea are largely unknown, however adhesion to the epithelium and elaboration of cellular proteases are considered to be key events in venereal trichomonad pathogenicity. Using a developed *in vitro* model of feline TF adhesion to intestinal epithelium, these studies explore the hypothesis that adherence is a key initiating event in TF pathogenicity and that this event is linked to its pathogenic effects and is a potential pharmacological target for prevention or amelioration of clinical disease.

METHODS: The effect of multiplicity of infection, viability of TF, binding competition, formalin fixation, cytoskeletal inhibitors, sialic acid and protease inhibitors on adherence of feline TF to IPEC-J2 monolayers was quantified by liquid scintillation counting and immunofluorescence. To evaluate for adhesion-dependent cytotoxicity, light microscopy and western blotting for cleaved cytokeratin 18 was performed following co-culture with whole cell trichomonads or their secretory products. Gelatin-SDS-PAGE was used to characterize the proteolytic profile of feline TF isolates. To assess the role of proteases as virulence factors in adhesion-dependent cytotoxicity, adhesion and cytotoxicity studies were performed

in the presence or absence of protease inhibitors. **RESULTS:** ^{3}[H]thymidine and CFSE-labeled TF cytoadhered to IPEC-J2 monolayers in a dose and time-dependent manner with reproducible adhesion achieved by infection with 20×10^{6} Tf for 6hrs. Clinical isolates of feline TF (n=5) demonstrated significantly greater adhesion to intestinal epithelium that *Pentatrichomonas* hominis, the latter of which is a presumably nonpathogenic trichomonad of domestic animals and people. Adhesion of TF required viable trophozoites and was dependent on specific receptor-ligand interactions. Treatment of TF with *N*-acetylneuraminic acid and cysteine protease inhibitors significantly blocked adherence. Protein patterns from TF isolates (n=4) were similar and revealed numerous protease activities belonging to both the serine and cysteine protease class. In contrast, feline *Pentatrichomonas hominis*, produced little protease activity compared to TF and had no detectable cysteine protease activity. TF stimulated IPEC-J2 cells to undergo apoptosis, an effect that was found to be both dependent on adhesion of the parasite to the intestinal epithelium and exposure to trichomonad cysteine proteases. Inhibition of cysteine protease activity significantly reduced adhesion and ameliorated cytopathogenicity. **CONCLUSIONS:** Our studies have resulted in the development a unique model that can be used for further study of TF cytopathogenicity. We demonstrate for the first time that TF adhere to the intestinal epithelium via specific receptor ligand interactions. Further, cytotoxicity is dependent on direct interaction of the epithelium and exposure to TF cell-associated cysteine proteases. These studies support a central role for cysteine protease activity in TF adhesion-dependent cytotoxicity of the intestinal epithelium and may suggest a novel molecular target for therapeutic intervention of intestinal trichomonosis.

Mechanisms of Feline *Tritrichomonas foetus* Adherence to the Intestinal Epithelium and Novel Sites for Pharmacological Control

by
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BIOGRAPHY

Mary Katherine (Katie) Tolbert was born on November 25th, 1979 in Lexington, KY. She moved to Marietta, GA in 1985 and graduated from Pope High School in 1998. From there, she attended Berry College to study Animal Science with a goal of attending veterinary school upon graduation. During this time she studied the epidemiology of feline heartworm and gastrointestinal parasites in Northwest Georgia, which furthered her desire to pursue a career in translational veterinary medicine. After receiving her B.S. in Animal Science in 2001, she attended the University of Georgia (UGA) College of Veterinary Medicine. While a veterinary student, Katie spent several summers in research laboratories to continue to develop the skills necessary for a career as a clinician scientist. Following veterinary school, Katie stayed on at UGA and completed a rotating small animal surgery and medicine internship. After the internship, Katie accepted a clinical investigator position at North Carolina State University, which combines a small animal medicine residency with graduate training. In 2010, Katie completed her residency and became board certified by the American College of Veterinary Internal Medicine in small animal internal medicine. From there, she became a full time graduate student under the direction of Dr. Jody Gookin in the Comparative Biomedical Sciences department where Katie pursued a degree in cell biology. The focus of her graduate work has been in the development of an in vitro model of feline *T. foetus* infection using co-cultures of *T. foetus* and intestinal epithelial cells. She used this model to characterize the pathogenic mechanisms of *T. foetus* infection and to explore novel therapies to prevent and/or ameliorate *T. foetus*-induced colitis.

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From a very early age, I was nurtured into a career in science. My parents both had jobs in science-related fields and fostered my inquisitive nature into a passion for science. At both UGA and NCSU, I benefited greatly from supportive mentorship of internists and basic science researchers with a variety of focus areas including urology, pulmonology, endocrinology, gastroenterology, immunology and infectious disease. Following completion of my internship and residency, I was privileged to be welcomed into the laboratory of Dr. Jody Gookin, which afforded me the opportunity to develop the skills to investigate in the lab questions that arose in the clinic simultaneously allowing me to grow in my clinical proficiency and to develop my research skills. During this time, Jody has become a transformative teacher, relentless mentor, and unyielding friend. My committee of Drs. Sam Jones, Adam Moeser and Scott Magness has played a key role in my development as a clinician scientist. I would also like to acknowledge Stephen Stauffer who served as a patient teacher and an invaluble friend throughout my time in the lab. I sincerely thank my former fellow graduate student, Dr. Derek Foster, who provided both research input and PhD survival skills. I owe a debt of gratitude to my incredibly supportive family and friends who have cheered me on every step of the way. I have to acknowledge my two furry kids, Pugsly and Dante, who faithfully slept by my side for every typed word of this dissertation. Finally, I want to sincerely thank the Morris Animal Foundation, the Waltham Foundation, and the Center for Comparative Medicine and Translational Research Program, which provided significant monetary funding towards this research.

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Molecular Mechanisms of Trichomonad Cytopathogenicity

Abbreviations used in this paper: CHO, Chinese hamster ovary; CP, cysteine protease; LPG, lipophosphoglycan; PLA_2, Phospholipase A_2; TF, *Tritrichomonas foetus*; TV, *Trichomonas vaginalis*; VEC, vaginal epithelial cell

Introduction

Trichomonads belong to the parabasalid class of protozoans. They are found as inhabitants of oxygen-poor mucosal environments such as the lumen of the urogenital and gastrointestinal tract of many vertebrates (Schwebke et al, 2008). Unlike most eukaryotes, trichomonads lack mitochondria and instead have primitive organelles called hydrogenosomes that are responsible for anaerobic energy production (Schwebke et al, 2004). This characteristic finding explains the protozoans' adaptation towards living in microaerophillic environments (Lopez et al, 2000). Trichomonads possess variable numbers of anterior flagella and a noncontractile costa that adjoins an undulating membrane to the cell body, a prominent nucleus, and a rod-like endoskeletal structure known as the axostyle, which bisects the cell body longitudinally to provide structural support, and a posterior projection of the axostyle, which can feed into a posterior flagellum (Ovcinnikov et al, 1975). Trichomonads reproduce by binary fission. They live as trophozoites with a one-stage life cycle and no true cyst form hence they have limited ability to survive outside of their hosts (Schwebke et al, 2004). Several species of trichomonads have been identified. Only a handful of these are recognized to be pathogenic. Parasitic trichomonads include *Trichomonas vaginalis*, *Tritrichomonas mobilensis*, *Trichomonas gallinae*, *Tetratrichomonas gallinarum* and *Tritrichomonas foetus* (Schwebke et al, 2004). Among these, most research has focused

on the venereal trichomonads, *T. vaginalis* and *T. foetus*. *T. vaginalis* (TV), the causative agent of human trichomonosis, is the most frequent non-viral sexually transmitted disease and affects over 248 million people worldwide (World Health Organization, 2005). Given its status as an important human pathogen, particular attention has been paid to studies of TV pathogenicity. TV infection induces a broad range of symptoms in women ranging from an asymptomatic carrier state to severe vaginitis, cervicitis, endometritis, transient and/or permanent infertility, and premature labor. TV also increases the risk for HIV transmission and has been linked to an increased risk of cervical and prostate cancer (McClelland et al, 2007, Gander et al, 2008, Stark et al, 2009). The majority of men infected with TV are asymptomatic carriers or are able to rapidly clear the infection. This lack of symptomatology in most men may be explained by the differences between the urogenital microenvironments of men and women. The urogenital tract of men is often oxidative and rich in zinc which is unfavorable to trichomonad survival whereas the urogenital tract of women is a reducing environment with low zinc and higher concentrations of iron which favors trichomonad growth and colonization (Krieger et al, 1982). *Tritrichomonas foetus*, is a venereal pathogen of cattle that causes similar pathology as that observed in TV-infected women and can result in considerable economic losses in infected herds. Strict regulations and artificial insemination practices have been used to successfully control the disease in many areas, however the infection is still prevalent where natural breeding is practiced (Rae et al, 2004).

Mechanisms of Trichomonad Pathogenicity as Learned by Studies of Venereal Trichomonosis in People and Cattle

The interaction of venereal trichomonads with epithelial cells is believed to be an inciting event of host pathogenicity that results in disruption of the host mucosal epithelial barrier. Several virulence factors have been investigated as contributing factors in instigating the pathogenic effect between venereal trichomonads and their mammalian hosts.

Adhesion of Trichomonads to Host Epithelial Cells

Trichomonads are obligate lumen-dwelling parasites, a characteristic that benefits from their ability to adhere to host epithelium and enables them to establish and maintain a chronic infection (Petrin et al, 1998). In venereal infection, trichomonads adhere to vaginal epithelium before invading the uterus and oviducts (Singh et al, 2004). The mucous epithelium of the vagina is rich in mucin glycoproteins and antimicrobial agents such as immunoglobulins and lactoferrin that normally serves as a barrier against pathogen invasion. In vitro studies demonstrate the ability of TV to adhere to and penetrate bovine submaxillary and porcine stomach mucin (Lehker et al, 1999). In vivo mucinase activity may allow trichomonads to solubilize the mucous matrix and gain access to the underlying vaginal epithelium. An ability to evade the immune system and acquire nutrients through proteolytic degradation of immunoglobulins and lactoferrin by trichomonads has also been demonstrated (Talbot et al, 1991). Following depletion and/or invasion of the mucous layer, trichomonads adhere to the vaginal epithelium. Upon initial binding to vaginal epithelial cells, signal transduction events upregulate the transcription of TV virulence genes and stimulate

trichomonads to undergo a rapid morphological transformation from a pear-like shape to a flattened amoeboid organism (Kucknoor et al, 2005, Arroyo et al, 1993). During this transformation, trichomonads extend short filopodia towards epithelial cells and numerous membrane-membrane contact points can be observed (Arroyo et al, 1993). Amoeboid transformation likely maximizes host cell contact, which is essential for effective adherence in the challenging, secretory environment of the vagina. Host cell recognition and signaling is both a host cell and species-specific event. Amoeboid transformation does not take place when trichomonads are in contact with fibroblasts or other cell types and TV organisms adhere with a weaker affinity to bovine VECs compared to bovine TF (Arroyo et al, 1993, Singh et al, 1999, Singh et al, 2000). Trichomonads possess multiple cell surface molecules that can be used to adhere specifically to uterovaginal epithelial cells. These include adhesin proteins, glycoconjugates and lectins, and cellular proteases (Arroyo et al, 1989, Flichorova et al, 2006, Moreno-Brito et al, 2005, Singh et al, 1999).

Sialic acid-binding lectins

Sialic acid is a generic name that describes a vast family of nine carbon ketosugars that are commonly expressed as the terminal monosaccharide on cell surface glycoconjugates. Because of their position, sialic acid performs a number of host cell functions including cellular recognition, immune cell communication and maintenance of mucosal barrier integrity (Varki et al, 2012). Mucosal membranes are particularly rich in sialic acid (Lewis et al, 2012). Thus, it is not surprising that many mucosal pathogens including *Tritrichomonas foetus* have developed mechanisms to disrupt and exploit host sialic acids (Lewis et al, 2012; Babál P et al, 1999). In vitro, bovine TF binds to Chinese hamster ovary

(CHO) epithelial cells and bovine cervical mucus using sialic acid-binding lectins. Adhesion of bovine TF can be inhibited by desialyation of CHO epithelial cells and mucus (Babál P et al, 1999). These findings suggest a role for sialic acid in bovine TF adhesion. However, sialidase activity, which catalyzes the hydrolytic cleavage of sialic acid residues from glycoconjugates in mucus and on cell surfaces, has been demonstrated in the plasma membrane of the cell body and flagella of bovine TF (Dias Filho et al, 1999). The mucus secretions of the female reproductive tract have high levels of sialoglycoproteins, which are thought to entrap and prevent access of microbial organisms to the underlying epithelium (Lewis et al, 2012). A possible explanation for the presence of both a sialic acid-binding lectin and sialidase activity is that bovine TF uses sialic acid as a binding site in the initial adherence event but later cleaves sialic acid residues following adhesion to other adhesins. Further investigation will be necessary to fully uncover the role of sialic acid in TF pathogenicity.

Adhesins

On initial contact with the epithelium, trichomonads increase the expression of epithelial binding proteins (adhesins) which mediate adhesion of trichomonads to vaginal epithelial cells (VECs) in vitro (Arroyo et al, 1993). At least one bovine TF and five TV adhesin proteins, named on the basis of their molecular weights (TF: Tf190; TV: AP120, AP65, AP51, AP33, AP23) have been identified (Arroyo et al, 1989; Moreno-Brito et al, 2005). TV adhesins are functionally diverse and can participate in trichomonad parasitism in a variety of ways as dictated by the surrounding microenvironment. Under iron-rich conditions and/or upon contact with VECs, adhesin proteins have been demonstrated to be

synthesized and mobilized to the trichomonad cell surface. A direct relationship was demonstrated between the levels of adhesion and amount of adhesins bound to VECs and HeLa cell surfaces (Moreno-Brito et al, 2005, Garcia et al, 2003, Vanacova et al, 2001). An isolate absent in adhesins was demonstrated to be non-adherent to VECs which further supports the role of adhesins in trichomonad adhesion (Garcia et al, 2003). When mobilization for adherence is not required, adhesin proteins can be utilized within the cytoplasm and hydrogenosome for nonenzymatic and enzymatic processes (Garcia et al, 2003, Alderete et al, 2001). Anti-adhesin antibodies inhibit the binding of TV to host epithelial cells and protect host cells from cytotoxicity (Arroyo et al, 1992). These results support both the role of adhesins in adhesion and the importance of contact-dependent cytotoxicity in host cell parasitism.

Lipophosphoglycan

Carbohydrates and glycolipids form a dense glycocalyx on the surface of trichomonads (Singh et al, 1999, Singh, 1994). Removal of surface carbohydrates through periodate oxidation of trichomonad surfaces abrogates adhesion, which suggests that trichomonad surface carbohydrates are multifunctional providing both protection to the parasite as well as serving a role in adhesion (Singh et al, 1999). Lipophosphoglycan (LPG) is the most abundantly expressed glycosylated molecule found on the surface of bovine TF and TV (Singh, 1994, Singh et al, 1993). LPG is anchored to the cell membrane by inositol-phosphoceramide moieties and contains mostly carbohydrate and lipid and is devoid of peptide (Singh et al, 2009, Singh et al, 1994). Several studies implicate LPG in trichomonad adhesion and contact-dependent cytotoxicity of epithelial cells (Bastida-Corcuera et al,

2005). Purified exogenous LPG significantly inhibited adhesion of bovine TF and TV to host vaginal epithelial cells (Fichorova et al, 2006, Singh et al, 1999). Further, TV LGP-truncated mutants adhered sevenfold less and induced significantly less cytotoxicity to ectocervical cells compared to parent strains (Bastida-Corcuera et al, 2005). Trichomonad LPG is structurally unique compared to other protozoan pathogens and may also contribute to differences in species-specific pathogenicity between the trichomonads (Singh et al, 2009, Singh, 1994). For example, LPG isolated from TV but not TF has the ability to stimulate release of pro-inflammatory cytokines IL-8, MIP-3α, and IL-6 from vaginal epithelial cells (Singh et al, 2009). Bovine TF infection does induce an inflammatory response in vivo, hence it is possible that TF contains an as yet unidentified pro-inflammatory virulence factor similar to LPG (Anderson et al, 1996).

Cysteine Proteases

Cysteine proteases play numerous indispensable roles in the cytopathogenicity of trichomonads. Like adhesins, CPs are multifunctional proteins that can participate in adhesion but also participate in a number of other cellular functions that contribute to the parasite's cytopathogenicity. They have been demonstrated to mediate efficient adhesion of TV to HeLa cells and VECs in vitro. Their role as adherence proteins in bovine TF infection has been hypothesized but remains to be determined. At least 23 and 10 different CPs have been identified in TV and bovine TF, respectively (Mallinson et al, 1995, Lockwood et al, 1984, 1987, Neale et al, 1990). Two TV surface expressed cysteine proteases, CP30 and CP65, play a key role in cytoadhesion (Solano-González et al, 2006, Mendoza-López et al, 2000, Alvarez-Sánchez et al, 2000, Arroyo et al, 1995, Arroyo et al, 1989) and adhesion-

dependent cytotoxicity to cervical and vaginal epithelial cells. Anti-CP antibodies significantly inhibited adhesion of TV to HeLa cell monolayers by 50% and reduced HeLa cell toxicity (Solano-González et al, 2006, Mendoza-López et al, 2000). Similarly, anti-adhesin antibodies also inhibited adhesion by a maximum of 50%, which further demonstrates the multifactorial nature of trichomonad adhesion and adhesion-dependent cytotoxicity (Arroyo et al, 1992).

Cytotoxicity is Contact-Dependent and Independent

Adhesion to the host cell is a prerequisite for contact-dependent cytoxicity. Following adhesion, detachment, lysis and apoptosis of VECs and HeLa cell monolayers can be observed. Much of the evidence for the role of adhesion in trichomonad cytotoxicity has come from in vitro experiments where inhibition of TV adhesion was observed to significantly reduce cytotoxic effects on VECs and HeLa cells (Alderete, Garza et al, 1988, Alderete, Demes et al, 1988, Alderete et al, 1992). Contact-independent mechanisms also contribute to cytopathogenicity and several secreted virulence factors have been identified including phospholipases, porins and cysteine proteases.

Phospholipases and porins

TV produces phospholipases, which like many of the secreted virulence factors, appear to participate in both contact-dependent and independent cytolytic activities (Lubick et al, 2004, Vargas-Villarreal et al, 2005). Phospholipases hydrolyze ester groups formed from phospholipids. Hydrolysis of phospholipids yields free fatty acids which supports parasite pathogen survival by providing a source of lipid nutrients while also mediating lysis of host

cell membranes (Lubick et al, 2004). Phospholipase A_2 (PLA_2) activity has been demonstrated in the soluble fraction of TV protein extracts. Further, McGregor et al reported elevated PLA_2 concentrations in vaginal secretions of pregnant women infected with TV and suggested that this may contribute to the pathogenesis of preterm birth (McGregor et al, 1992). TV also secretes porins that exhibit a perforin-like activity and have been demonstrated to induce cellular cytotoxicity through creation of pores in erythrocyte membranes in vitro (Garber et al, 1989, Fiori et al, 1996). Bovine TF has been purported to possess endogenous phospholipase C activity however investigation into the role of phospholipases and porins in TF infection requires further study (Dias Filho et al, 1995).

Cysteine proteases

Among the arsenal of virulence factors available to trichomonads, cysteine proteases play the most numerous roles. Trichomonads possess a large number of surface expressed and secreted cysteine proteases that contribute to their cytopathogenicity. As mentioned, CPs play a vital role in adhesion but also serve a number of other cellular functions in cytopathogenicity including mucus and extracellular matrix degradation, complement and antibody destruction, cytotoxicity and nutrient acquisition (Mendoza-López et al, 2000, Alvarez-Sánchez et al, 2000, Provenzano et al, 1995, Talbot et al, 1991, Dailey et al, 1990). Expression of CPs can be dependent on many environmental factors including availability of polyamines and environmental zinc and iron concentrations (Alvarez-Sánchez et al, 2007, 2008). Many of the TV CPs have been identified in vaginal secretions from infected women (Hernández-Gutiérrez et al, 2004). Cysteine proteases can function as epithelial cell detaching factors through degradation of extracellular matrix proteins (fibronectin, several

types of collagen, laminin) (Hernández-Gutiérrez et al, 2004, Mendoza-López et al, 2000, Alvarez-Sánchez et al, 2000). They also are capable of degrading hemoglobin following hemolysis and can mediate direct cytotoxic effects on vaginal and uterine epithelial cells through induction of apoptosis (Sommer et al, 2005, Singh et al, 2005, Hernández-Gutiérrez et al, 2004).

Phagocytosis and Microbial Vaginosis

Bovine TF and TV are obligate protozoan parasites that must live in complex and frequently changing environmental conditions of the vagina including shifts in the presence of commensal bacteria, gametic and innate immune cells and factors, sex hormone induced epithelial changes, menorrhea and nutrient limiting conditions. The vaginal mucosa is inhabited by a diverse microbial population that serves an important role in barrier function and host cell defense. Lactobacilli are the predominant commensal bacteria of healthy vaginal mucosa and contribute to the maintenance of an acidic vaginal pH through the production of lactic acid (Antonio et al, 1999). TV has been reported to phagocytose bacteria including Lactobacilli in vivo and in vitro (Figueroa-Angulo et al, 2012, Midlej et al, 2010, Pereira-Neves et al, 2007, Rendón-Maldonado et al, 1998, Francioli et al, 1983). Phagocytosis of Lactobacilli is presumed to create a bacterial vaginosis and may promote a more basic vaginal environment, which favors the survival and colonization of TV (Rendón-Maldonado et al, 1998). Further, the ability of TV to phagocytose and degrade leukocytes may prevent the host from conducting an efficient immune response to eliminate the parasite (Rendón-Maldonado et al, 1998). TV also carries endosymbiont dsRNA viruses which have

unknown consequences for the human host (Goodman et al, 2011). Light and electron microscopy studies have revealed the ability of TV to phagocytose yeast and both TV and bovine TF to phagocytize spermatozoids although TF has historically been characterized by less phagocytic activity compared to TV (Benchimol et al, 2008, Pereira-Neves et al, 2007, Chen et al, 2004). Phagocytosis of erythrocytes has also been reported and may provide the parasite nutrients and minerals, including lipids and iron (Rendón-Maldonado et al, 1998).

Resisting the Host Immune System

Trichomonosis is characterized by a chronic infection with no acquired immunity. Thus, the innate immune response plays a crucial role in resisting trichomonad infection. Trichomonads have developed a number of mechanisms to combat and evade the host immune response. These mechanisms include induction of apoptosis of innate immune cells, modulation of host cytokine expression, degradation of immune cell components and phenotypic variation. Following colonization, trichomonads evoke the influx of neutrophils across the vaginal epithelial barrier (Demirezen et al, 2000, Parsonson et al, 1976). The presence of lymphocytes also supports the role of macrophages in host defense. In in vitro studies, TV induces apoptotic cell death of neutrophils and macrophages, suppresses expression of the macrophage pro-inflammatory cytokines IL-12 and TNFα through inhibition of NF-κB activity, and enhances expression of anti-inflammatory cytokines IL-10 and TNFβ (Ahn et al, 2008, Kang et al, 2006, Chang et al, 2006, Chang et al, 2004). IL-12 and TNFα help direct the host immune system towards a Th1 response which is critical for combating parasite infection. Therefore, inhibition of these cytokines and a shift towards

anti-inflammatory cytokine production may be beneficial for trichomonad survival. Additional resistance of bovine TF and TV to host immune defense is provided by evasion of nonspecific and specific immune cell components through cysteine protease-mediated degradation of lactoferrin, fibronectin, fibrinogen, albumin, IgG1, IgG2 and surface bound C3 (Alderete et al, 1995, Provenzano, et al, 1995, Talbot et al, 1991). Further, TV cysteine proteases contribute to trichomonad phenotypic variation and antigenic heterogeneity, providing another layer of protection from host antibodies, by degrading prominent surface immunogens making the once "visible" trichomonad unrecognizable to the host (Alderete, et al, 1986).

Mechanisms of Disease Pathogenesis of Feline *Tritrichomonas foetus*

In addition to urogenital infections, trichomonads are also found to inhabit the gastrointestinal tract. These include *Pentatrichomonas hominis* in people and more recently *Trichomonas foetus* was identified as a pathogen in the gastrointestinal tract of domestic cats and pigs. TF is a widely prevalent cause of large bowel diarrhea in cats. Once infected, diarrhea can be long-lasting and despite periods of clinical remission, it is likely that cats remain infected for life. Only a single drug, characterized by a narrow margin of safety and emerging development of resistance, is effective for treatment. In addition to a need for more consistently effective drugs for treating feline trichomonosis, little is understood regarding how TF actually causes diarrhea. Previous studies by our lab and others suggest that the mechanisms of diarrhea in TF infection are multifactorial. However, the virulence factors responsible for these mechanisms are currently unknown. This review will explore

what is known about feline TF and how we can use knowledge of venereal trichomonad pathogenicity to further unlock mechanisms of disease in cats.

Epidemiology and transmission

In contrast to what has been described for venereal trichomonosis, very little is understood regarding the pathogenicity of trichomonads that parasitize the gastrointestinal tract. *Tritrichomonas foetus* was first recognized as a venereal pathogen of cattle in 1888 (Tachezy et al, 2002, Künstler, 1888). In 2003, a highly similar trichomonad, also called *Tritrichomonas foetus*, was recognized as an intestinal pathogen of cats (Levy et al, 2003, Gookin et al, 2001). The symptomotology and management of feline TF infection have been well described, however the mechanisms by which TF causes diarrheal disease in cats is not understood. Following experimental infection, TF infects the distal ileum and large intestine of cats and clinically causes a chronic, often waxing and waning diarrhea (Foster et al, 2004, Gookin et al, 1999). Cats can remain asymptomatic and undergo long periods of remission that permits the spread of the infection (Foster et al, 2004, Gookin et al, 2004). Transmission of feline TF occurs by the fecal-oral route. The infection has been identified in cats worldwide and is highly prevalent in cats that are obtained from high density housing environments including catteries, shelters, or breeding facilities (Gookin et al, 2004, Gookin et al, 2001). Despite the lack of environmentally stable cysts, in vitro studies have demonstrated that TF can survive in moist cat feces at ambient temperatures for at least 24 hours and in cat food for up to 5 days (Van der Saag et al, 2010, Hale et al, 2009).

Multiple findings suggest that feline TF is host adapted to the gastrointestinal tract and that direct transmission of TF from cattle to cats is unlikely to be the primary source of

transmission. Molecular and cross-transmission studies between bovine venereal TF and feline gastrointestinal TF isolates have revealed both genetic and biological differences between the organisms (Slapeta et al, 2010, Stockdale et al, 2007, Stockdale et al, 2008). Unlike the cattle genotype of TF, feline TF does not appear to naturally infect the reproductive tract of cats (Gray et al, 2010). Proximity to farms and ingestion of raw meat are not identified as risk factors for TF infection in cats. (Gookin et al, 2004). These findings suggest that feline has a unique pathogenesis and a particular tropism for the gastrointestinal tract. Thus, there is a critical need to closely evaluate mechanisms of TF disease causation within the gastrointestinal tract.

Mechanisms of disease

TF infection is sufficient to cause diarrhea in cats (Gookin et al, 2001). Infected cats show clinical signs consistent with colitis including hematochezia, relapsing malodorous diarrhea, fecal incontinence, proctitis and rectal prolapse (Foster et al, 2004, Gookin et al, 2004). The mechanisms by which TF causes diarrhea are poorly understood however in vivo and in vitro studies suggest the cause is multifactorial and involves an interplay between TF, the intestinal microbiota and the intestinal epithelium that culminates in colonic mucosal inflammation and diarrhea.

In bovine venereal TF, adherence to the uterovaginal epithelium is thought to be a critical first step in disease pathogenesis (Singh et al, 1999). Adherence is additionally recognized to be an essential prerequisite for intestinal colonization and cytopathogenicity by other enteric protozoa including *Giardia*, which uses its ventral adhesive disk to facilitate adherence to the small intestinal epithelium (Céu Sousa et al., 2001). TF is a lumen dweller

that rarely invades the subepithelial lamina propria. In naturally infected cats, TF can be found intimately associated with colonic epithelial cells along the surface epithelium and colonic crypts (Yaeger et al, 2004). In vitro and in vivo ultrastructural examination of TF infection demonstrate direct contact of trichomonads with the surface of colonic epithelial cells (Figure 1). Further, following experimental infection, TF colonizes the distal ileum, cecum and colon and diarrhea can be observed prior to the onset of the host inflammatory response (Gookin et al, 2001). These findings suggest that direct interactions of trichomonads with the intestinal epithelium play an important role in mediating diarrhea in TF infected cats and that further study into mechanisms of adherence to the intestinal epithelium is warranted.

A second possible mechanism for TF induced diarrhea is the interplay between trichomonads and the intestinal microbiota. Bacterial vaginosis is a characteristic feature of venereal trichomonosis. There are no studies investigating the relationship between feline *T. foetus* and the microbiota however colonic dysbiosis is suspected to be a characteristic feature of feline trichomonosis based on several observations. Diarrhea in TF infected cats is often waxing and waning and cats may experience extended periods of clinical remission though relapses are common and can be provoked by stressful events (Gookin et al, 2001). Antibiotic administration in TF infected cats results in improved fecal consistency and reduces the number of fecal trichomonads however these cats experience relapses of diarrhea consisting of trichomonad-containing feces following discontinuation of antibiotic treatment (Gookin et al, 2001). Further, cats switched to novel protein diets have improvement in clinical signs, which may be a result of alterations of the intestinal microbiota (Foster et al,

2004). Colonic mucosal disruption as a result of adherent and enteroinvasive TF may disturb the intestinal microbial ecosystem and promote the development of secondary bacterial infections. Additional studies will be necessary to dissect the relationship between TF and the colonic microbiota as a cause for diarrhea.

Thirdly, in vitro and ex vivo data support disruption of the colonic epithelial barrier and altered permeability as a possible mechanism for TF induced diarrhea. Following colonization of TF, colonic crypt epithelial cell hypertrophy and increased mitotic activity, microabscesses and attenuation of the superficial colonic mucosa suggesting epithelial cell cytotoxicity can be observed. Hematochezia in TF infected cats and invasion of trichomonads into the lamina propria also support the presence of epithelial cytotoxicity and disruption of mucosal barrier integrity (Foster et al, 2004, Yaeger et al, 2004, Gookin et al, 2001). In experimentally infected cats, diarrhea precedes the inflammatory response, which suggests pathogenesis in the absence of inflammation (Gookin et al, 2001). Loss of epithelial cells and disruption of epithelial tight junctions by adherent and enteroinvasive TF may increase intestinal permeability and induce diarrhea by reduced absorption of electrolytes and water (Jones et al, 2002). Unpublished studies were performed in our laboratory to evaluate the effect of feline TF on intestinal permeability. When feline colonic mucosa was infected with TF *ex vivo* in Ussing chambers, there was a significant increase in distal colonic epithelial permeability as measured by the mucosal to serosal flux of ^{3}H-mannitol (Figure 2). These findings suggest increased permeability in response to TF infection.

In the normal intestine, the villus/surface epithelium is responsible for intestinal fluid and ion absorption whereas the crypt epithelium mediates fluid secretion. Fluid absorption in

the healthy intestine exceeds intestinal secretion. In the diseased state, secretory diarrhea promotes net fluid secretion as a result of abnormal ion transport in intestinal epithelial cells (Jones et al, 2002). Mediators of secretory diarrhea stimulate the release of second messengers (ex. cyclic AMP/GMP, Ca^{++}) that act to promote anion secretion (Cl^-/HCO_3^- and/or inhibit neutral NaCl absorption. These mediators include neurohormones, phospholipids, unconjugated bile acids, enterotoxins and inflammatory cell products (prostanoids, substance P, bradykinin) (Jones et al, 2002). Intestinal inflammation is a consistent finding in TF infected cats. In a study evaluating seven cats naturally infected with TF, all cats had mild to moderate expansion of the colonic lamina propria with an infiltrate of plasma cells. Six of the cats also had moderate numbers of neutrophils within the lamina propria. (Yaeger et al, 2005). Thus, inflammatory cell mediators released from the inflamed lamina propria may contribute to secretory diarrhea in infected cats. Further evidence for the relationship between diarrhea and the presence of inflammation in TF infection is supported by the resolution of inflammation during periods of spontaneous clinical remission (Figure 3, Foster et al, 2004). The factors responsible for eliciting intestinal inflammation in TF infection are unknown. Pro-inflammatory cytokines may be released from epithelial cells and the enteric nervous system following direct contact of TF with the intestinal epithelium. In experimentally infected cats, TF antigen was observed within the superficial mucus, surface enterocytes and lamina propria of cecal and colonic mucosa (Figure 4, Gookin et al, 2001). Uptake of trichomonad antigen may directly trigger the release of pro-inflammatory cytokines from the epithelium and enteric nervous system.

Several findings also support the existence of secretory diarrhea independent of the host inflammatory response (Gookin et al, 2001). In experimentally infected cats, diarrhea precedes inflammation (Gookin et al, 2001). Further, mucoid diarrhea is a common clinical sign in TF infected cats (Figure 5). Decreased numbers of goblet cells within the large intestinal epithelium of TF infected cats suggests discharge of mucoid products in response to the presence of infection (Yaeger et al, 2004). Mucus and fluid hypersecretion may be a protective mechanism by the feline host to eliminate the trichomonad pathogen however further studies are necessary to investigate the role of secretion in response to TF infection.

Conclusion

Trichomonas vaginalis and bovine *Tritrichomonas foetus* are predacious venereal parasites that have gained mechanisms to obtain survival advantages and acquire nutrients from their mammalian hosts. Considerable research has been focused on understanding the mechanisms of venereal trichomonad pathogenicity, which is thought to be critically dependent on adherence to the uterovaginal epithelium and elaboration of virulence factors such as cysteine proteases. Despite its widespread prevalence and lack of consistently effective therapies, feline *Tritrichomonas foetus* has received considerably less attention and the virulence factors that contribute to intestinal pathogenicity are unknown. The critical role of adhesion to the host epithelium and elaboration of cysteine proteases in venereal trichomonad pathogenicity necessitates exploration of these events as pathogenic mechanisms of feline *T. foetus*, which is the overarching goal of the present thesis research.

Figures

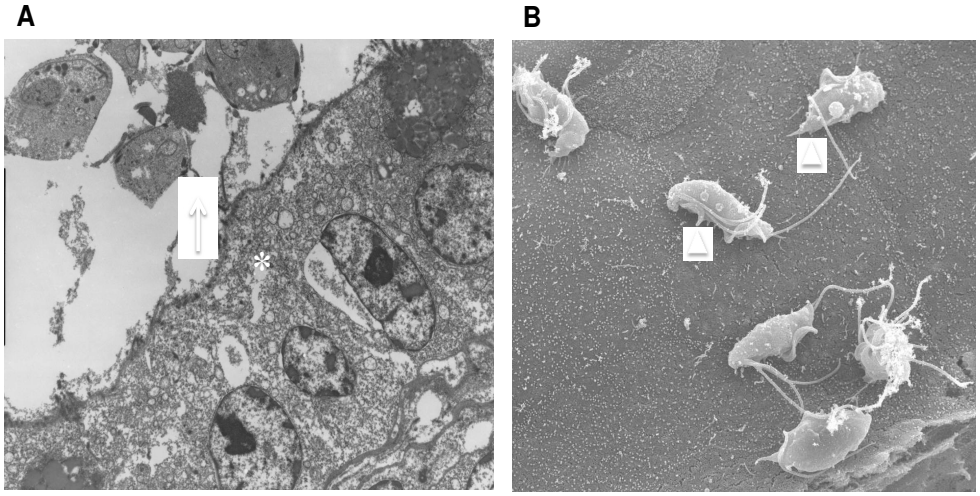

Figure 1. Ultrastructural analysis of TF infection. **(A)** Examination of colonic epithelium by transmission electron microscopy in a cat naturally infected with TF. Note the three trichomonads within the lumen of the gastrointestinal tract. A trichomonad can be observed extending its posterior flagellum (white arrow) towards the surface of the colonic epithelium (marked by white asterisk). **(B)** Porcine intestinal epithelium after infection with feline TF and examination by scanning electron microscopy. Note the cytoplasmic extensions of TF that facilitate adherence of organisms to the surface of the epithelial monolayer (white arrowheads). (2300x).

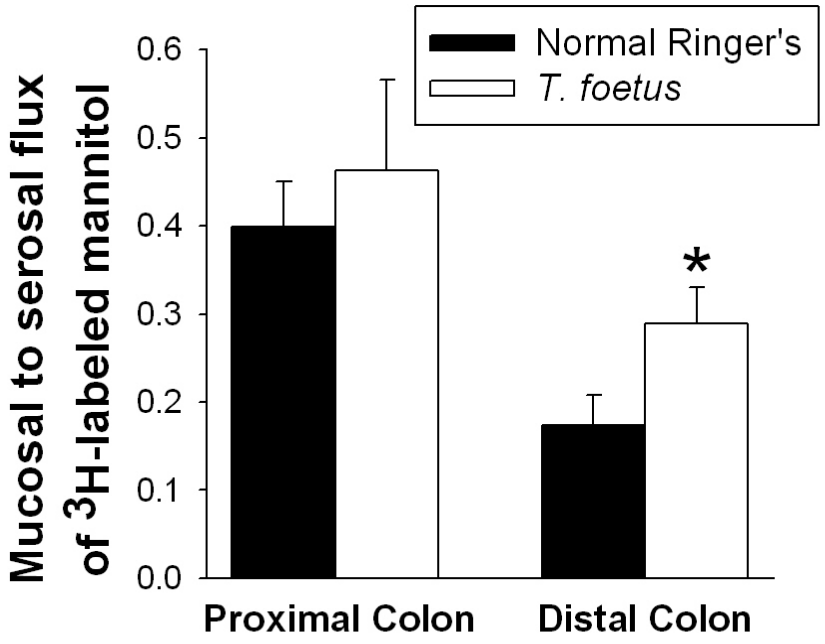

Figure 2. Effect of feline *T. foetus* on intestinal permeability.
Infection of feline distal colonic mucosa with *T.foetus* ex vivo increases permeability to ^{3}H-mannitol. Data represents 7 cats. *p<0.05

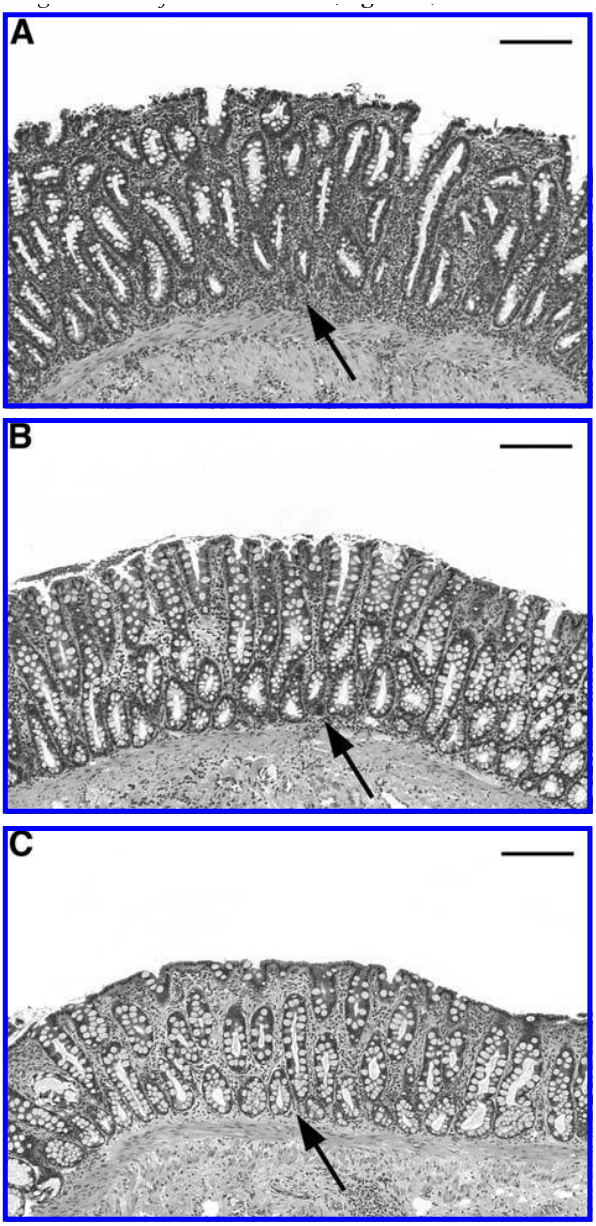

Figure 3. Presence of diarrhea parallels presence of inflammation in TF infection. Photomicrographs of colonic mucosa obtained from a TF infected cat (A) at the time of diagnosis, (B) following initial stages of resolution of diarrhea 12 months later, (C) and following complete resolution of diarrhea concomitant with clearance of TF infection 24 months after initial diagnosis. Lymphoplasmacytic inflammation in the lamina propria (black arrows) can be observed to decrease with resolution of diarrhea and infection. bar = 150 µm. Reproduced from Foster et al, 2004 with permission (JG).

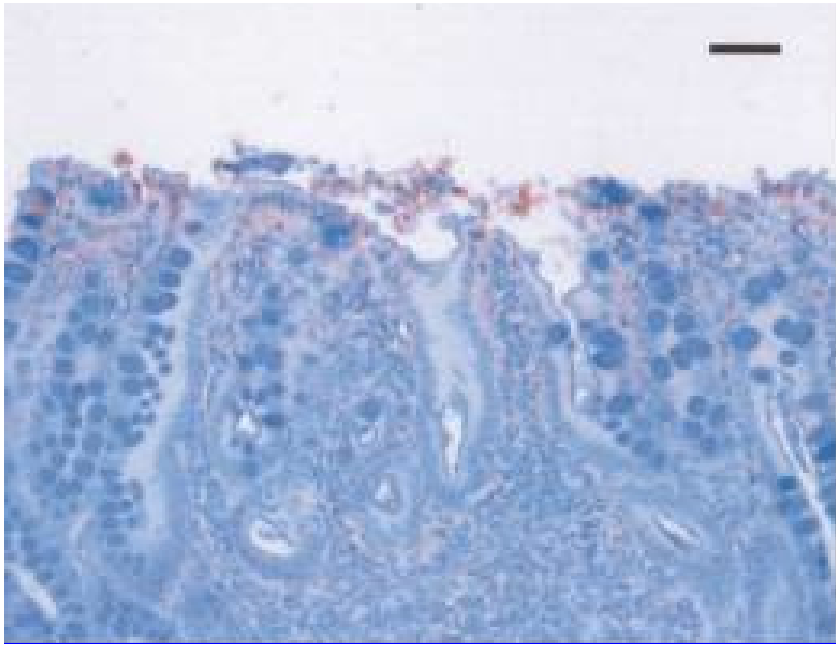

Figure 4. TF antigen is taken up by surface enterocytes and lamina propria of the colon. Photomicrograph of immunohistochemical analysis for TF antigen in colonic mucosal biopsy of cat experimentally infected with TF. Immunolabeled trichomonads (red) can be observed within the superficial mucus layer and on the surface enterocytes of the colon. TF antigen is identified within the superficial epithelium and lamina propria. Bar = 100 μm. Reproduced with permission from Gookin et al, 2001 (JG).

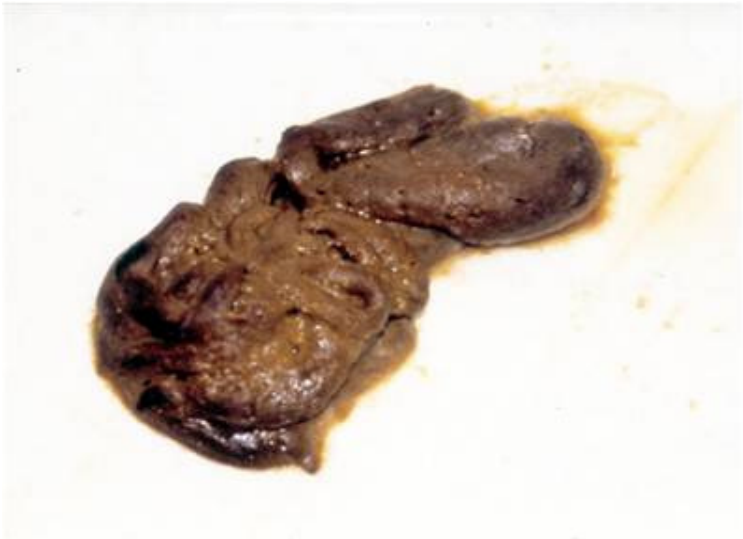

Figure 5. Hemorrhagic mucoid diarrhea in a TF infected cat.

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CHAPTER 1

Feline *Tritrichomonas foetus* adhere to intestinal epithelium by receptor-ligand-dependent mechanisms

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Abbreviations used in this paper: TF, *Tritrichomonas foetus*; PH, *Pentatrichomonas hominis*; IPEC-J2, Intestinal porcine epithelial cells; CPM, counts per minute; CFSE, Carboxy Fluorescein diacetate, Succinimidyl Ester; DMEM, Dulbecco's modified eagle medium; HBSS, Hank's balanced salt solution; EGF, Epidermal growth factor; TER, Transepithelial electrical resistance

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Abstract: *Tritrichomonas foetus* (TF) is a protozoan that infects the feline ileum and colon resulting in chronic diarrhea. Up to 30% of young purebred cats are infected with TF and the infection is recognized as pandemic. Only a single drug, characterized by a narrow margin of safety and emerging development of resistance, is effective for treatment. While the venereal pathogenicity of bovine TF is attributed to adherence to uterovaginal epithelium, the pathogenesis of diarrhea in feline TF infection is unknown. The aim of this study was to establish an *in vitro* model of feline TF adhesion to intestinal epithelium. Confluent monolayers of porcine intestinal epithelial cells (IPEC-J2) were infected with axenic cultures of feline TF that were labeled with [^{3}H] thymidine or CFSE and harvested at log-phase. The effect of multiplicity and duration of infection, viability of TF, binding competition, formalin fixation and cytoskeletal inhibitors on adherence of feline TF to IPEC-J2 monolayers was

quantified by liquid scintillation counting and immunofluorescence. [^{3}H] thymidine and CFSE-labeled TF reproducibly adhered to IPEC-J2 monolayers. Clinical isolates of feline TF adhered to the intestinal epithelium in significantly greater numbers than *Pentatrichomonas hominis*, the latter of which is a presumably nonpathogenic trichomonad. Adhesion of TF required viable trophozoites but was independent of cytoskeletal activity. Based on saturation and competition binding experiments, adherence of feline TF to the epithelium occurred via specific receptor-ligand interactions. The developed model provides a valuable resource for assessing pathogenic mechanisms of feline TF and developing novel pharmacologic therapies for blocking the adhesion of feline TF to the intestinal epithelium.

Introduction

Tritrichomonas foetus (TF) is a protozoal pathogen that is recognized internationally as a common cause of colitis in domestic cats. The reported prevalence of TF among U.S. purebred cats is as high as 30% (Gookin et al, 2004) and distribution of the infection is acknowledged to be worldwide. Intestinal trichomonosis is a chronic infection in which symptomatic cats are afflicted by waxing and waning diarrhea (Gookin et al, 2001). Despite periods of clinical remission, it is likely that many cats remain infected for life (Foster et al, 2004). The nitroimidazole antibiotic, ronidazole, is currently the only effective antimicrobial treatment for feline trichomonosis. However, ronidazole has a narrow safety margin in cats and resistant strains have been recognized both clinically and *in vitro* (Gookin et al., 2010). In addition to a need for safer and more consistently effective drugs for killing TF, little is understood regarding how TF actually causes diarrhea. In cattle where TF infection is responsible for venereal disease, adherence of TF to the uterovaginal epithelium is thought to be a critical first step in TF pathogenicity (Singh et al., 1999). Further, adherence is known to be an essential prerequisite for intestinal colonization and cytopathogenicity by many enteric protozoal organisms including *Giardia*, which uses its ventral adhesive disk to facilitate adherence to the small intestinal epithelium (Céu Sousa et al., 2001). We hypothesized that feline TF similarly adhere to the intestinal epithelium; an event that may be linked to its pathogenic effects and a potential pharmacological target for prevention or amelioration of clinical disease.

In these studies we demonstrate for the first time that feline TF adhere to intestinal epithelial monolayers. This adhesion requires TF viability but is independent of TF

cytoskeletal integrity. Adherence of TF occurs via saturable and competitive binding kinetics that suggest specific receptor-ligand interactions with the epithelium. These studies provide supportive evidence that adhesion of feline TF to the intestinal epithelium may be pharmacologically inhibited. The developed model provides a valuable resource for determining the specific mechanisms mediating adhesion of feline TF to the intestinal epithelium and whether or not blockade of adhesion ameliorates the pathogenic effects of TF.

Materials and Methods

IPEC-J2 cells

The porcine jejunal epithelial cell line (IPEC-J2) is a non-transformed primary cell line originally isolated from neonatal piglet jejunum and was obtained as a gift from Dr. Helen M. Berschneider. IPEC-J2 cells were grown in co-culture media which 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 in 5% CO_2. Prior to adhesion studies, IPEC-J2 cells were seeded onto permeable polycarbonate filters (0.4 μm pore size, 4.67cm^2; Corning Incorporated, Lowell, MA) and cultivated until confluent (Transepithelial Electrical Resistance (TER) ≥ 2,000 Ω · 4.67cm^2). For microscopic examination of adherent TF, IPEC-J2 cells were seeded onto Laboratory-Tek chamber slides (Nalg Nunc International, Rochester, NY) and grown to confluence over a period of 4 days prior to use. IPEC-J2 cells were used at passage numbers 38-50.

Trichomonads

Isolation and culture of TF and *Pentatrichomonas hominis* (PH) isolates were performed as previously described (Gookin et al., 2001). Each isolate was obtained from the feces of a naturally infected cat, cultured in modified Diamond's media supplemented with antibiotics (penicillin, amphotericin B, streptomycin), and incubated at 37°C. After ≥ 5 passes in this media, axenic cultures were established in antibiotic-free Diamond's medium. The identity of each isolate was confirmed by polymerase chain reaction (PCR) testing as previously described (Gookin et al., 2002). One *P. hominis* and six TF (F, A, C, Sti, M, Sta) isolates axenized from seven different naturally infected cats were used for comparative studies of adhesion.

Labeling of trichomonads

Trichomonads were harvested in late log-phase of growth and inoculated into modified Diamond's media containing 4 μCi/ml [^{3}H] thymidine (10 Ci/mmol) (American Radiolabeled Chemicals or GE life sciences) or 2 μCi/ml [methyl-^{3}H] thymidine (25 Ci/mmol) (GE life sciences). After 36 hours, radiolabeled trichomonads were washed three times by means of centrifugation (250×g) and reconstitution with sterile Hank's Balanced Salt Solution (HBSS) to remove unassociated radioactive tracer. Trichomonads were counted using a hemocytometer and resuspended in IPEC-J2 media at the desired concentration. For fluorescence vital staining of trichomonads, isolates were labeled with Carboxy Fluorescein diacetate, Succinimidyl Ester (CFSE, 5-20 μM) (Life Technologies, Grand Island, NY) at 37°C in the dark. Prior to use, CFSE-labeled trichomonads were washed three times with HBSS to remove unincorporated label.

Co-culture adhesion assays

For co-culture adhesion assays, [^{3}H] thymidine-labeled trichomonads were added to the apical media of IPEC-J2 cell monolayers grown to confluence on polycarbonate inserts. Co-cultures were incubated at 37°C in 5% CO_2. Following adhesion, monolayers were washed twice with sterile HBSS to remove unbound trichomonads. The inserts were then excised using a scalpel blade and placed in 20 ml scintillation vials containing Econo 2 fluid (Fisher Scientific, Pittsburgh, PA). Radioactive emissions were counted using a Wallac 1209 liquid scintillation counter and expressed in counts per minute (CPM). Radioactive emissions measured from serial dilutions of the radiolabeled trichomonads were used for each assay to generate a standard curve of CPM per trichomonad. Number of trichomonads adhered to cell monolayers were calculated by applying the CPM measured to the standard curve.

For adhesion assays using vital stained trichomonads, TF were labeled with CFSE and added to monolayers of IPEC-J2 cells grown to confluence on chamber slides. Unbound trichomonads were removed by washing chamber slides twice with sterile HBSS. The epithelial cells and trichomonads were counterstained with the nuclear stain, DAPI, and adherent trichomonads in individual chamber wells were counted in six high power fields using an epifluorescence microscope. For evaluation of CFSE as a quantitative assay, serial dilutions of CFSE-labeled trichomonads were added to 96 well microplates (Greiner Bio One, Monroe, NC) and read with a fluorescence plate reader (Fluoroskan Ascent FL, MA) using excitation and emission wavelengths of 485 and 538 nm, respectively. Baseline differences in TF adhesion to IPEC-J2 cells between experiments were controlled for by

inclusion of an untreated TF control group in each CFSE and radiolabeled TF adhesion assay.

For ultrastructural imaging of adherent TF, trichomonads were added to the apical media of IPEC-J2 cell monolayers grown to confluence on polycarbonate inserts and incubated as previously described. Following co-culture, inserts were gently washed twice with sterile HBSS to remove nonadherent trichomonads. Inserts were fixed in Trump's 4F:1G fixative at 4°C. Samples were rinsed twice for 15 min each with 1.0 M Sorenson's phosphate buffer (pH 7.2–7.4) and dehydrated in an ascending series of ethanol (50%, 75%, 95%, and 95%) for 15 min each, culminating in two washes in 100% ethanol for 30 min each. Samples were then dried in a Ladd critical-point dryer. Inserts were cut away and mounted with carbon tape on aluminum specimen stubs, and sputter coated with ~20 nm of gold-palladium using an Anatch Hummer VI sputter coater. Samples were viewed using a JEOL 6360 LV scanning electron microscope.

Adhesion characterization assays

Trichomonads were treated with the microtubule inhibitor colchicine (250 µM - 1.25 mM, Sigma-Aldrich, St. Louis, MO), the actin polymerization inhibitor cytochalasin B (10.5 µM, Sigma-Aldrich, St. Louis, MO) or their diluents, PBS and 0.5% DMSO for 1 hr at 37°C prior to co-culture. A growth curve of TF was evaluated in the presence of each drug or the appropriate diluent. To assess the cytolytic effect of treatments on TF, radiolabel liberation assays were performed. Following incubation with drug or vehicle for the designated time period, $5x10^6$ TF were placed in a microcentrifuge tube and pelleted at 6000 × g for 2 min. Radioactivity released by the trichomonads was measured in the supernatant and values were

compared to positive and negative controls (liquid nitrogen and PBS-treated TF, respectively). To evaluate the dependence of TF adhesion on trophozoite viability, trichomonads were treated with 1% formalin or diluent (deionized H_2O) for 30 seconds prior to co-culture with IPEC-J2 cells for a duration of six hours. Saturation binding of radiolabeled TF to IPEC-J2 monolayers was evaluated 6 hours post-addition of increasing numbers of trichomonads. Competition binding of TF to IPEC-J2 cells in co-culture was performed using a fixed number of radiolabeled TF in the presence of increasing numbers of non-radiolabeled trichomonads.

Statistical analysis

Data were analyzed for normality (Kolmogorov-Smirnov) and variance (Levene median) using a statistical software package and tested for significance using parametric or non-parametric tests as appropriate (SigmaStat, Jandel Scientific). Parametric data were analyzed using a Student's *t* test or one-way ANOVA. Non-parametric data were analyzed using Mann-Whitney rank sum test or Kruskal-Wallis ANOVA on Ranks. *n* = number of replicates. Results are reported as mean ± standard deviation. For all analyses, $P \leq 0.05$ was considered significant.

Results

Radiolabel and vital staining of feline T. foetus

Following inoculation into fresh medium, feline TF (isolate F) exhibited logarithmic growth between 16-36 hrs of culture culminating in a peak concentration of approximately

4.5 x 10^6 cells/ml at 48 hrs (**Fig. 1A**). Based on this finding, TF were exposed to radiolabel beginning at the time of inoculation until their time of harvest at 36 hours of growth.

Two different radioisotopes were examined for labeling of TF DNA ([methyl-^{3}H] thymidine) or DNA and RNA ([^{3}H] thymidine). Superior labeling of TF was observed with [^{3}H] thymidine compared to [methyl-^{3}H] thymidine (**Fig. 1B**). Thus, radiolabeling of TF was performed using [^{3}H] thymidine. A standard dilution curve of [^{3}H] thymidine labeled-TF demonstrated a strong positive linear relationship between the number of TF and the radioactive emissions measured (**Fig. 1C**).

CFSE was examined as a vital stain for direct imaging of TF or as a non-radioactive approach to quantifying numbers of trichomonads. Optimal labeling of TF was achieved using 20 µM CFSE in PBS for a duration of 30 min. Lower concentrations of CFSE or shorter incubation periods of labeling led to weak fluorescence of the trichomonads. Labeling of TF with CFSE demonstrated a positive linear relationship between the number of TF and fluorescence emissions as measured using a fluorometer (**Fig. 2**). Labeling of TF using either radioisotope or CFSE had no effect on TF viability.

Feline T. foetus exhibit saturable adhesion to intestinal epithelial monolayers

To determine if TF adhere to intestinal epithelium, monolayers of IPEC-J2 cells were infected with increasing numbers of [^{3}H] thymidine labeled-TF and co-cultured for durations of time ranging from 1 to 24 hours. Adhesion was observed at all chosen concentrations of TF with maximum adhesion occurring earlier as the number of infecting TF was increased (**Fig. 3A**). A 6 hour duration of co-culture was chosen for performance of adhesion assays. Additionally, TF were determined to not significantly proliferate under co-culture conditions

over this time period. In order to determine whether adhesion of TF to the intestinal epithelium was saturable, increasing numbers of [^{3}H] thymidine labeled-TF were allowed to infect IPEC-J2 monolayers in co-culture for 6 hours. Adhesion of TF to IPEC-J2 cells was saturable at numbers $\geq 40 \times 10^6$ TF (**Fig. 3B)**. An infection inoculum of 20×10^6 TF was identified as a sub-saturating number of TF that would be useful for examining the effect of pharmacological agents on TF adhesion. Infection of IPEC-J2 monolayers with 20×10^6 CFSE-labeled TF for 6 hours resulted in a multiplicity of infection of 2.26 ± 0.57 TF per IPEC-J2 cell.

To verify that the estimated change in magnitude of TF adhesion using radiolabeled TF was comparable to direct visual counts, parallel assays of [^{3}H] thymidine versus CFSE labeled-TF adhesion to IPEC-J2 cells were performed. The percent change in number of TF adhered between 1 and 6 hours of infection was comparable between visually counted CFSE-labeled TF (40% ± 12) and the radiolabel-estimated TF (39% ± 12). Examination of TF adhering to IPEC-J2 monolayers by means of scanning electron microscopy (SEM) revealed discrete multifocal regions of intensely infected IPEC-J2 cells adjoined by regions of largely uninfected epithelium (**Fig. 4**). No apparent breaches in epithelial monolayer continuity were observed by SEM following the six hour co-culture with TF.

Isolates of feline T. foetus adhere more robustly to intestinal epithelium compared to feline P. hominis

To determine the magnitude and variability of adhesion among different clinical isolates of feline TF, the adhesion of six isolates of TF were compared. Adherence of TF isolates was also compared to a single clinical isolate of feline *P. hominis* as this species

represents the only other known intestinal trichomonad of cats. All TF isolates adhered in greater numbers (mean ± SE, 389,202 ± 59,868 trophozoites) than *P. hominis* (109,784 ± 12,325 trophozoites) (**Fig. 5**). Parallel studies qualitatively examining the adhesion of CFSE-labeled TF and *P. hominis* to IPEC-J2 cells were in agreement with these findings.

Adhesion of feline T. foetus to intestinal epithelium does not require cytoskeletal activity

The dependence of TF adhesion on actin filament or microtubule activity was evaluated by exposing trophozoites to cytoskeletal inhibitors prior to infection of IPEC-J2 cells. Incubation of TF in the presence of the actin polymerization inhibitor cytochalasin B (10.5 µM) or the microtubule inhibitor colchicine (250 µM) significantly inhibited replication of the trophozoites (**Fig. 6A**). Neither inhibitor had any effect on retention of radiolabel by the trophozoites or on adhesion of TF to IPEC-J2 cells (**Fig. 6B**). Examination of co-cultures using SEM disclosed no discernable effects of the cytoskeletal inhibitors on TF morphology or adhesion.

Adhesion of feline T. foetus to intestinal epithelium requires trophozoite viability

To determine if adhesion of TF to the intestinal epithelium was an active process, trophozoites were treated with 1% formalin prior to co-culture with IPEC-J2 cells. Formalin-fixation significantly reduced adhesion of TF to IPEC-J2 cells compared to vehicle-treated TF (**Fig. 7A**). Formalin-fixation had no cytolytic effect on the trichomonads based on their retention of radiolabel. Qualitative examination of CFSE-labeled TF adhesion to IPEC-J2 cells visually supported an inhibitory effect of formalin-fixation on TF adhesion (**Fig. 7B**).

Feline T. foetus adhere to intestinal epithelium via specific receptor-ligand interactions

To determine if adhesion of TF to IPEC-J2 cells was receptor-specific and therefore could be outcompeted, we examined the effect of increasing numbers of non-radiolabeled TF on adhesion of [^{3}H] thymidine labeled-TF. The addition of 80-fold excess of unlabeled TF outcompeted the binding of radiolabeled TF by 85 ± 1.9 % consistent with a receptor-mediated mechanism of TF adhesion **(Fig. 8)**.

Discussion

To the authors' knowledge there are no published studies investigating the mechanisms of adherence of feline TF to the intestinal epithelium. Based on the use of cell culture models of bovine TF and human *Trichomonas vaginalis* infections, adherence of trichomonads to the urogenital epithelium has been identified as a critical step in venereal pathogenicity (Singh et al., 1999; Silva Filho et al., 1988; Alderete et al, 1985). Studies of feline TF are similarly suited to a cell culture model approach as the organisms are lumen-dwellers that intimately associate with the intestinal epithelium and only rarely invade into the underlying lamina propria (Yaeger et al., 2005). Further, in experimentally infected cats, diarrhea precedes the host inflammatory response (Gookin et al., 2001) suggesting that the interaction between TF and the intestinal epithelium plays an early and key role in the genesis of diarrhea. In the present study, we sought to establish an *in vitro* model of feline TF infection that could be used to define the mechanisms of adhesion of feline TF to the intestinal epithelium. Because there are currently no feline intestinal epithelial cell lines

available, we established a model system using non-transformed porcine intestinal epithelial cells (IPEC-J2). When IPEC-J2 cells are cultured to confluence on semi-permeable artificial basement membranes they form a polarized epithelial monolayer that mimics the native intestinal epithelium (Schierack et al., 2006). Both feline TF and the trichomonad of pigs, *Tritrichomonas suis*, are highly similar organisms and each demonstrates unique tropism for the gastrointestinal tract (Tachezy et al, 2002; Mostegl et al, 2011). Accordingly, porcine intestinal epithelial cells offer a particularly relevant alternative for the study of feline TF adherence to the intestinal epithelium. We demonstrated that multiple different clinical isolates of feline TF adhered robustly to IPEC-J2 monolayers in co-culture. Ultrastructural analysis demonstrated that adherence of feline TF to IPEC-J2 monolayers occurred in a patchy distribution consisting of large aggregates of trichomonads attached to the epithelium. This observation supports light microscopic descriptions of the infection *in vivo* where segmental foci of trichomonads are found to occur along the colonic epithelium (Yaeger et al., 2005).

Using the described co-culture model system, we first sought to determine whether adherence of feline TF was an active or passive event. Significant inhibition of feline TF adherence by gentle formalin fixation of the trophozoites supports the existence of an active process that requires cellular viability. Participation of cytoskeletal elements during the adhesion process has been demonstrated in other enteric protozoal infections (Katelaris et al, 1995; López-Revilla, R., 1982). Thus, we considered an active role for actin or tubulin in adherence of feline TF. Cytochalasin B and colchicine had a modest inhibitory effect on growth of TF, however adherence was not affected. These findings suggest that adherence of

TF is a metabolically active process that is not critically dependent on dynamic cytoskeletal rearrangement.

Adherence of feline TF to the intestinal epithelium may be a key step in establishing infection and inducing diarrhea. While the cytopathogenic effects of feline TF on the intestinal epithelium was not a focus of this particular study, we found it particularly interesting that feline isolates of TF adhered to the intestinal epithelium in significantly greater numbers than did feline *P. hominis*, a presumably nonpathogenic trichomonad. Future studies will be required to determine if there is any causal relationship between the magnitude of adhesion and epithelial cytopathogenicity among different TF isolates or between TF and *P. hominis*. To determine if adhesion of feline TF was receptor-specific and therefore could be outcompeted, we studied the interaction kinetics between TF and the intestinal epithelium. The addition of increasing numbers of non-radiolabeled TF displaced adherence of a fixed number of radiolabeled TF by approximately 85%. These results, along with our observation that adherence of radiolabeled TF was saturable, provide compelling evidence for the involvement of specific receptor-ligand interactions between TF and the intestinal epithelium. The identity of these ligands may represent an important step toward development of novel therapies for feline trichomonosis. Several studies of bovine venereal TF have highlighted cellular proteases and host cell surface carbohydrates as important for adherence and cytopathogenicity (Singh et al., 2005, Singh et al., 1999, Bonilha et al., 1995). Therefore, future aims will be focused on defining the role of these and other specific molecules in adhesion of TF to the intestinal epithelium.

In summary, the present study has established a unique *in vitro* model system to study the adherence of feline TF to the intestinal epithelium. Adhesion of feline TF requires that the trichomonads are viable but does not depend on a dynamic rearrangement of the actin or tubulin cytoskeleton. Further, adherence of feline TF to the intestinal epithelium is mediated by specific receptor-ligand interactions, which provide an encouraging focus for the development of pharmacologic inhibitors of TF adhesion. In future studies, the developed model will be used to evaluate the pathogenic effects of feline TF on the intestinal epithelium, to identify novel molecular inhibitors of adhesion and to determine if pharmacological inhibition of adhesion ameliorates epithelial pathogenicity.

Figures

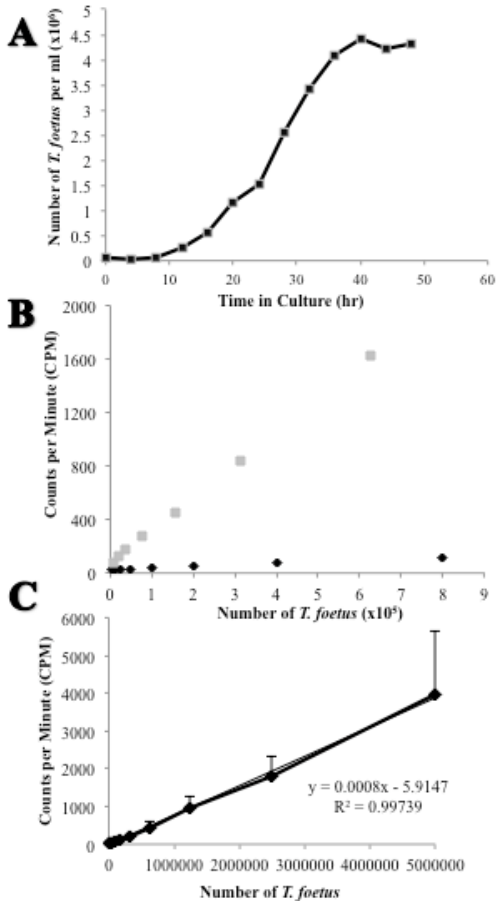

Figure 1. *In vitro* growth and comparison of radioisotopes for labeling of feline *T. foetus*. (A) A typical growth curve of *T. foetus* following inoculation of $5x10^4$ trichomonads into Advanced Diamond's media. Each data point represents 3 replicates. (B) Standard dilution curve of TF following radiolabeling with either [^{3}H] thymidine (gray square) or [methyl-^{3}H] thymidine (black diamond). (C) Standard dilution curve of TF labeled with 4 μCi/ml [^{3}H] thymidine. Each data point represents 3 replicates.

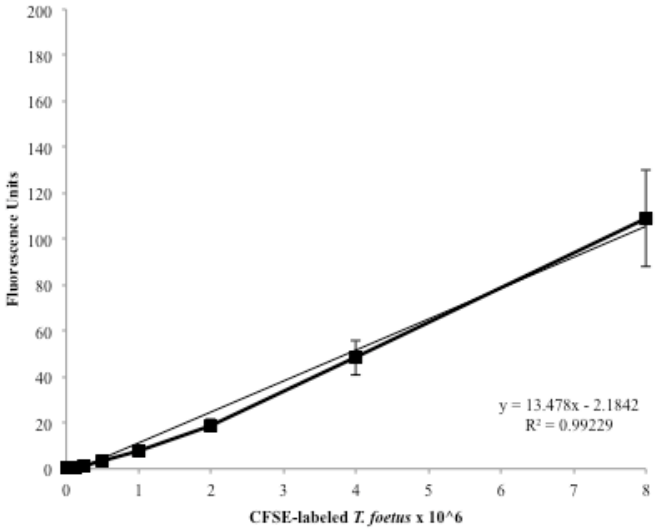

Figure 2. Fluorescence emission of CFSE-stained feline *T. foetus*. TF labeled with 20 μM CFSE were serially diluted in 96 well microplates and emissions were recorded using a fluorescence plate reader. Each data point represents 3 replicates.

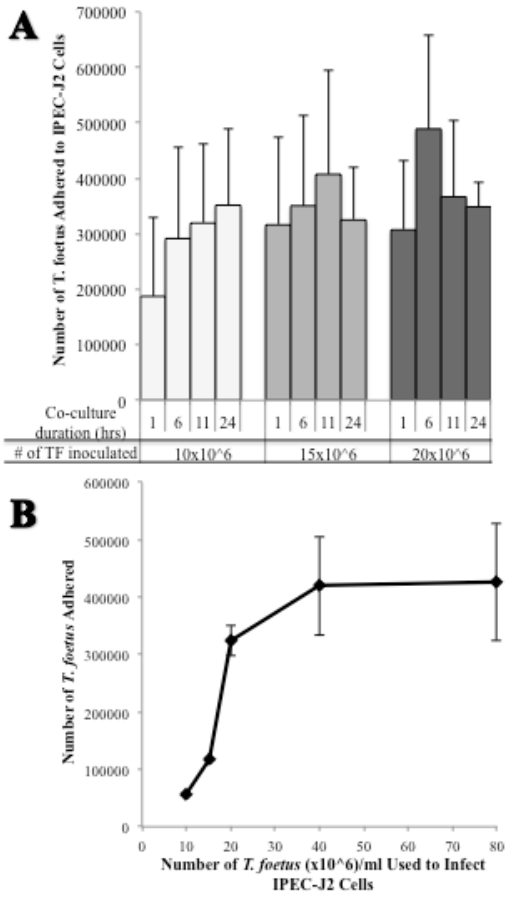

Figure 3. Effect of time and number of feline *T. foetus* on adherence to IPEC-J2 cells. (A) [^{3}H] thymidine-labeled TF were added to IPEC-J2 monolayers at infection doses of 10, 15, and 20 × 10^6 trichomonads and co-cultured for 1, 6, 11, and 24 hours. Each data point represents 3-4 replicates. (B) The adherence of [^{3}H] thymidine labeled-TF to IPEC-J2 cell monolayers (24 mm^2 polycarbonate inserts) was saturable at infecting doses ≥ 40 × 10^6 TF per ml (highest dose of TF tested was 160 × 10^6 TF per ml). Each data point represents 3-4 replicates.

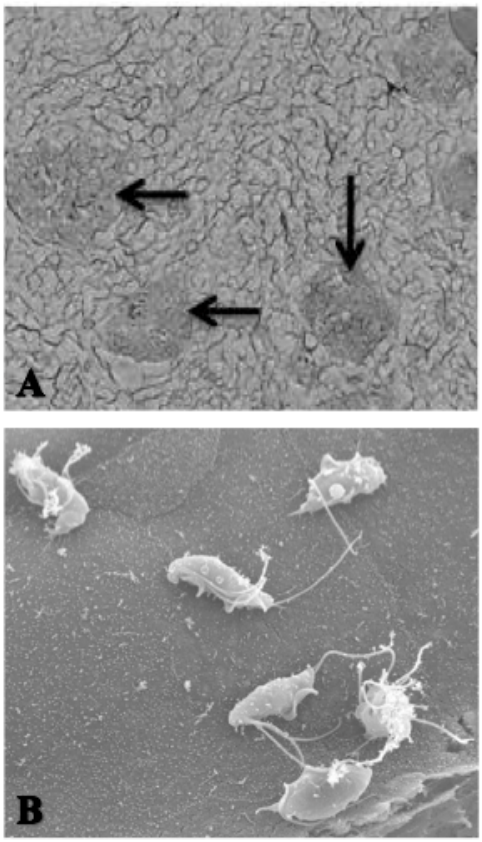

Figure 4. Scanning electron microscopy of feline *T. foetus* adhesion to IPEC-J2 monolayers. (A). Note the patchy distribution of large aggregates of trichomonads adhering to IPEC-J2 monolayers (35×). (B). Higher magnification of a cluster of six trichomonads adhering to the surface of a single IPEC-J2 cell (2300×).

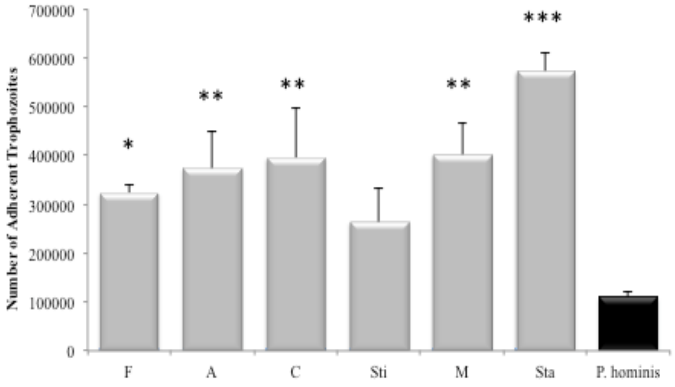

Figure 5. Adhesion characteristics of multiple feline *T. foetus* and a single feline *P. hominis* isolate. All TF isolates adhered in greater numbers to IPEC-J2 cell monolayers than did *P. hominis*. Each column represents 3-8 replicates per clinical isolate. *p<0.05 **p<0.01***p<0.001 compared to *P. hominis* (One Way ANOVA and post-hoc Holm-Sidak test).

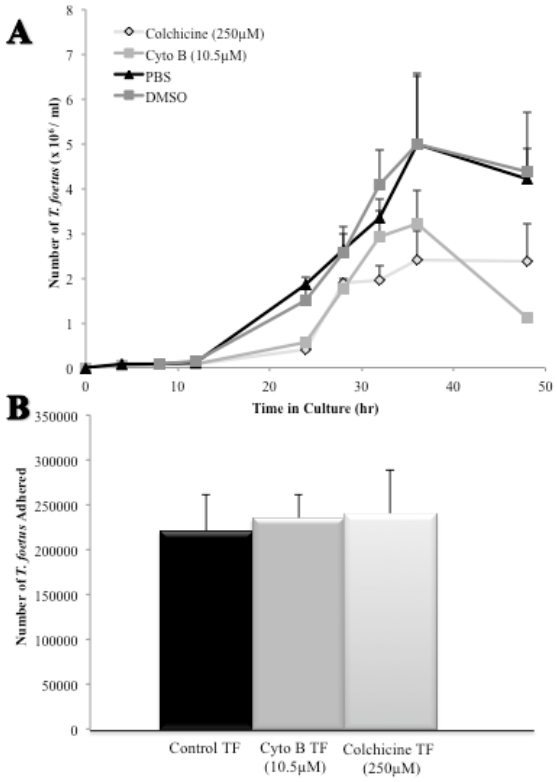

Figure 6. Proliferation of feline *T. foetus* but not adhesion is repressed by cytoskeletal inhibitors. (A) Growth of TF in the presence of cytoskeletal inhibitors is reduced over time compared to vehicle-treated controls. n=3 replicates at each time point. (B) Adhesion of [^{3}H] thymidine labeled-TF to IPEC-J2 cells was similar to control following exposure of TF to cytoskeletal inhibitors. Each column represents 8-10 replicates.

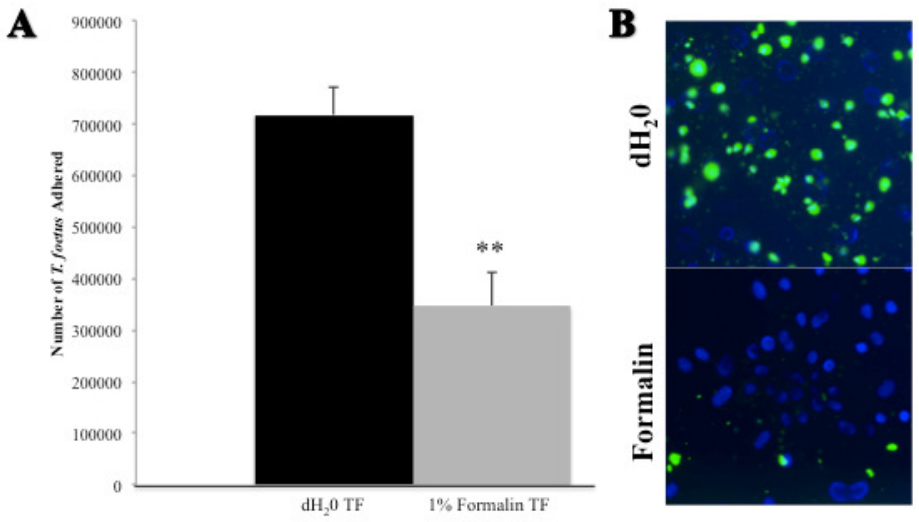

Figure 7. Trophozoite viability is required for *T. foetus* adhesion. (A) Adhesion of formalin-treated [^{3}H] thymidine labeled-TF to IPEC-J2 cells is significantly reduced compared to vehicle treated (deionized water) TF. Each column represents 6 replicates. **p<0.01 compared to vehicle treated control (Student's *t* test). (B) A representative, qualitative fluorescence microscopy image demonstrating CFSE-labeled TF (green) adhering to monolayers of porcine intestinal epithelial cells (IPEC-J2). IPEC-J2 cells can be identified by the nuclear counterstain, DAPI (blue). Adhesion is reduced following treatment of TF with 1% formalin (10× magnification).

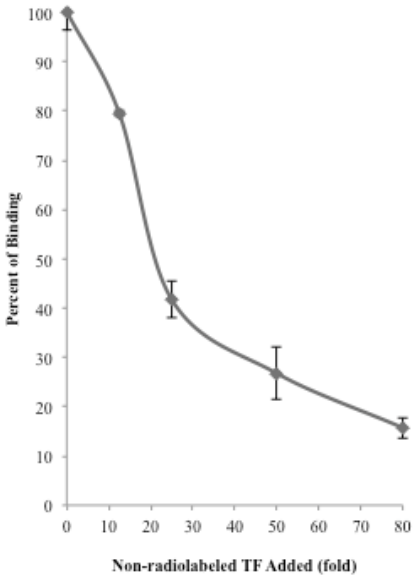

Figure 8. Non-radiolabeled *T. foetus* outcompete [^{3}H] thymidine labeled- *T. foetus* for binding to IPEC-J2 cells. Adhesion of [^{3}H] thymidine labeled-TF to IPEC-J2 cells is inhibited by the addition of increasing numbers of non-radiolabeled TF. Each data point represents 3-4 replicates.

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CHAPTER 2

Cysteine Proteases Mediate Feline *Tritrichomonas foetus* Adhesion-Dependent Cytopathogenicity to Intestinal Epithelial Cells

MARY KATHERINE TOLBERT, STEPHEN STAUFFER, and JODY GOOKIN

Abbreviations used in this paper: CPM, counts per minute; IPEC-J2, Intestinal porcine epithelial cells; DMEM, Dulbecco's modified eagle medium; HBSS, Hank's balanced salt solution; EDTA, ethylenediaminetetraacetic acid; EGF, Epidermal growth factor; PMSF, phenylmethylsulfonylfluoride; RIPA, radioimmunoprecipitation assay

Abstract: *Tritrichomonas foetus* is a mucosal protozoan that parasitizes the feline ileum and colon resulting in chronic diarrhea. Unfortunately, despite the growing prevalence of *T. foetus* and the emergence of drug-resistant strains in cats, little is known regarding the pathogenic mechanisms of *T. foetus* towards the intestinal epithelium. Understanding the mechanisms that contribute to the cytopathic effects of *T. foetus* towards the intestinal epithelium holds promise for development of novel treatment strategies to prevent or ameliorate intestinal trichomonosis. The aims of this study were to investigate the ability of *T. foetus* to damage intestinal epithelial cells and to determine the mechanisms that contribute to cytotoxicity in an effort to identify pharmacologic targets for the treatment of intestinal trichomonosis.

The cytopathogenic effect of feline *T. foetus* on confluent monolayers of intestinal porcine epithelial cells (IPEC-J2) was analyzed by light microscopy and immunoblotting *T. foetus* -infected IPEC-J2 cells for the M30 antigen of cleaved epithelial cytokeratin 18. Substrate-polyacrylamide gel electrophoresis was performed using trichomonad protein

extracts in the presence or absence of protease inhibitors (E64, 0.3-0.6mM; ethylenediaminetetraacetic acid, 0.5mM; phenylmethylsulfonylfluoride, 0.5-1.5mM; diisopropyl fluorophosphate, 0.01-10mM; pepstatin A, 0.01-0.1mM) to identify class-specific protease activities of feline *T. foetus*. Confluent monolayers of porcine intestinal epithelial cells were infected with axenic cultures of feline [^{3}H] thymidine labeled- *T. foetus* pretreated with the protease inhibitors. Adhesion was quantified by scintillation counting of adherent *T. foetus*. A minimum of three replicates was performed for each experiment. Data were analyzed using commercially available statistical software.

The results demonstrate that feline *T. foetus* adhered to and severely damaged IPEC-J2 monolayers through induction of apoptosis. Protein patterns from *T. foetus* isolates (n=4) were similar revealing multiple proteases of apparent high and low molecular weights that were identified as serine and cysteine proteases (CP), respectively. Treatment of *T. foetus* with CP inhibition had no effect on *T. foetus* growth but blocked adhesion of *T. foetus* to intestinal epithelial cells and significantly inhibited enterocyte apoptosis. A presumably nonpathogenic feline trichomonad isolate, *Pentatrichomonas hominis*, with previously reported poor adherence capabilities, produced little protease activity compared to *T. foetus* and had no detectable cysteine protease activity.

We demonstrate for the first time the mechanisms by which *T. foetus* adheres and induces cytopathogenicity of the intestinal epithelium. Our results support a central role for cysteine protease activity in *T. foetus* adhesion-dependent cytotoxicity of the intestinal epithelium and may suggest a novel molecular target for therapeutic intervention of intestinal trichomonosis.

Introduction

Trichomonads are ancient eukaryotic protists that inhabit the warm, moist and anaerobic regions of their hosts including the urogenital and gastrointestinal tracts. Several species of Trichomonadida exist including both pathogenic and commensal organisms. Parasitic trichomonads of mammals and birds include *Trichomonas vaginalis*, *Tritrichomonas mobilensis*, *Trichomonas gallinae*, *Tetratrichomonas gallinarum* and *Tritrichomonas foetus* (Schwebke et al, 2004). Among these, the venereal pathogens *T. vaginalis* and *T. foetus* are the most widely studied of the pathogenic trichomonads. *T. vaginalis,* the causative agent of human trichomonsis, is the most frequent non-viral sexually transmitted disease which affects over 248 million people worldwide (World Health Organization, 2005). *Tritrichomonas foetus*, the venereal pathogen of cattle, causes similar pathology as that observed in TV-infected humans and can result in considerable economic losses in infected herds. Cytoadherence to the urogenital epithelium and elaboration of cellular proteases are thought to be critical events in the pathogenicity of venereal trichomonads (Singh et al, 2005, Arroyo et al, 1995, 1989). Gastrointestinal trichomonads receive considerably less attention and the virulence factors that contribute to their pathogenicity are unknown. *Pentatrichomonas hominis* is an intestinal trichomonad considered to be a commensal of both humans and domestic mammals, however the reasons for the lack of virulence in *P. hominis* compared to pathogenic trichomonads are currently unclear. *Tritrichomonas foetus* is a minimally invasive mucosal protozoan parasite that colonizes the feline ileum and large intestine and causes chronic large bowel diarrhea (Gookin et al, 2001; Foster et al, 2004). *T. foetus* has a worldwide distribution yet no

commercially available drugs consistently treat the infection. *T. foetus* was also recently identified as an intestinal pathogen of pigs with a tendency towards tissue invasiveness when present as a co-infection (Mostegl M, 2011). Thus, understanding the mechanisms that contribute to the cytopathic effects of *T. foetus* towards the intestinal epithelium holds promise for development of novel treatment strategies to prevent or ameliorate intestinal trichomonosis in a number of hosts. Unfortunately, despite the growing prevalence of *T. foetus* and the emergence of drug-resistant strains in cats, little is known regarding the pathogenic mechanisms of *T. foetus* towards the intestinal epithelium. Thus, the aims of this study were to investigate the ability of *T. foetus* to damage intestinal epithelial cells and to determine the mechanisms that contribute to cytotoxicity in an effort to identify pharmacologic targets for the treatment of intestinal trichomonosis.

Using an in vitro co-culture model system, the present work has identified the mechanisms by which *T. foetus* induces cytopathogenicity of the intestinal epithelium. *T. foetus* stimulated IPEC-J2 cells to undergo apoptosis, an effect which was found to be both dependent on adhesion of the parasite to the intestinal epithelium and release of trichomonad cysteine proteases. Inhibition of cysteine protease activity significantly reduced adhesion and ameliorated cytopathogenicity. Our results support a central role for cysteine protease activity in *T. foetus* adhesion-dependent cytotoxicity of the intestinal epithelium and may suggest a novel molecular target for therapeutic intervention of intestinal trichomonosis.

Materials and Methods

IPEC-J2 cells

The porcine jejunal epithelial cell line (IPEC-J2) is a non-transformed primary cell line originally isolated from neonatal piglet jejunum and was obtained as a gift from Dr. Helen M. Berschneider. IPEC-J2 cells were grown in co-culture media which 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 (100 IU/ml), streptomycin (100 mg/ml) and 5% fetal bovine serum and incubated at 37°C in 5% CO_2. Prior to adhesion studies, IPEC-J2 cells were seeded onto permeable polycarbonate filters (0.4 μm pore size, 4.67cm^2; Corning Incorporated, Lowell, MA) and cultivated until confluent. For microscopic examination of trichomonad-induced cytotoxicity, IPEC-J2 cells were seeded onto Laboratory-Tek chamber slides (Nalg Nunc International, Rochester, NY) or permeable polyester filters (0.4 μm pore size, 1.12cm^2; Corning Incorporated, Lowell, MA) and grown to confluence. IPEC-J2 cells were used at passage numbers 38-60.

Trichomonads

Isolation and culture of *T. foetus* and *Pentatrichomonas hominis* isolates were performed as previously described (Tolbert et al., 2012). Trichomonads were harvested in mid to late-logarithmic phase by centrifugation at 250 x g and washed twice in Hanks' Balanced Salt Solution (HBSS). The trichomonads were suspended in HBSS at desired concentrations for experimental purposes. One *P. hominis* and four TF (F, Sti, D, A) isolates

axenized from five different naturally infected cats were used for comparative studies of protease activity.

Labeling of trichomonads

Labeling of trichomonads was performed as previously described (Tolbert et al, 2012). Briefly, trichomonads were harvested in late logarithmic-phase of growth and inoculated into modified Diamond's media containing 4 μCi/ml [^{3}H] thymidine (17 Ci/mmol) (American Radiolabeled Chemicals). After 36 hours, radiolabeled trichomonads were washed three times by means of centrifugation (250 × g) and reconstitution in HBSS to remove unassociated radioactive tracer. Trichomonads were counted using a hemocytometer and resuspended in IPEC-J2 media at the desired concentration.

Light microscopic evaluation

For examination of *T. foetus*-induced epithelial cytoxicity, IPEC-J2 cells were grown to confluence on chamber slides and inoculated with $20x10^6$ TF that had been pretreated with E64 (300μM) or vehicle (dH_20). Uninfected cells and cells treated with *P. hominis* for up to 36hr at 37°C were used in control experiments. At the end of the incubation period, the wells were gently washed twice with warm HBSS to removed non-adherent trichomonads and cellular debris and the remaining cells were fixed with 10% neutral buffered formalin for 10 min at room temperature prior to examination of the monolayer using a Nikon inverted light phase-contrast microscope.

Apoptosis assays

Trichomonads ($20x10^6$) were inoculated into the apical media of IPEC-J2 cell monolayers grown to confluence on polycarbonate inserts and co-cultured for durations of 0,

2, 6, 12, 24, and 36 hours. *T. foetus* were pretreated or not with E64 (300μM) or vehicle (sterile deionized H_20) for 2 hr at 37°C prior to inoculation onto IPEC-J2 cell monolayers. Following co-culture, supernatants were collected and monolayers were disrupted with radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors (Halt Protease Inhibitor, Thermo Fisher Scientific, Rockford, IL). Protein lysates were extracted and quantified were performed as previously described for trichomonads. The lysates were treated with a reducing agent (Nupage reducing agent, Life Technologies, Carlsbad, CA) and LDS buffer (Life Technologies, Carlsbad, CA) and heated at 70°C for 10 minutes prior to gel electrophoresis using 4-12% Bis-Tris polyacrylamide gels (Life Technologies, Carlsbad, CA) at 200V for 1 hr. Proteins were transferred to nitrocellulose membranes at 30V for 1 hr. Following transfer, nitrocellulose membranes were blocked overnight in blocking buffer (StartingBlock T20, Thermo Fisher Scientific, Rockford, IL) at 4°C. Immunoblotting was performed using M30 cytodeath primary antibody (1:500, Roche Diagnostics, Indianapolis, IN) in TBST for 4hr at 25°C followed by goat anti-mouse horseradish peroxidase conjugated antibody (1:10,000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 30 minutes at 25°C. Immunoblots were developed using an enhanced chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL) and exposed to radiographic film. Quantitative densitometric analysis of M30 protein bands was performed using SigmaScan software (SyStat, Inc., Chicago, IL) and expressed in arbitrary units. The isolated effect of *T. foetus* secretory proteins were determined by co-culture of IPEC-J2 cells with 20 x 10^6 *T. foetus* separated by a 0.4 μm culture plate insert (Corning Incorporated, Lowell, MA) or by inoculation with secreted proteins collected from 20x10^6 *T. foetus* over a period of 24 and 36 hours. In all

assays, *Cryptosporidium parvum*-infected IPEC-J2 cells and untreated cells were used as positive and negative controls for M30 expression, respectively.

Co-culture adhesion assay

Adhesion assays were performed as described previously (Tolbert et al, 2013). For co-culture adhesion assays, $20x10^6$ [^{3}H] thymidine-labeled *T. foetus* were inoculated into the apical media of confluent (TER≥2,000Ω·4.67cm^2) IPEC-J2 cell monolayers seeded onto polycarbonate inserts. Co-cultures were incubated at 37°C in 5% CO_2. Following adhesion, monolayers were washed twice with sterile HBSS to remove unbound trichomonads. The inserts were then excised using a scalpel blade and placed in 20 ml scintillation vials containing Econo 2 fluid (Fisher Scientific, Pittsburgh, PA). Radioactive emissions were counted using a Wallac 1209 liquid scintillation counter and expressed in counts per minute (CPM). Radioactive emissions measured from serial dilutions of the radiolabeled trichomonads were used for each assay to generate a standard curve of CPM per trichomonad. Number of trichomonads adhered to cell monolayers were calculated by applying the CPM measured to the standard curve.

Protein extract preparation

Trichomonads ($20x10^6$) in mid-logarithmic-phase trichomonads were washed twice in HBSS, lysed in radioimmunoprecipitation assay RIPA buffer (1x PBS, 1% Ipegal, 0.5% sodium deoxycholate, 0.1% SDS), sonicated twice, and incubated for 30min at 4°C. Supernatants were collected following pelleting of insoluble material at 15,800 x g for 10min at 4°C. Protein lysate concentrations were determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Rockford, IL) using bovine serum albumin as a standard. Lysates

were diluted in LDS buffer in the absence of a reducing agent and immediately used for substrate-gel electrophoresis or were stored as single-use samples of approximately 400 μg at -80°C. Secreted components were prepared from trichomonads as previously described with some modifications (Yu Y., 1997; Talbot J.A., 1991). *T. foetus* (20 x 10^6) were washed once in HBSS and then incubated in DMEM/F12 supplemented with 10 mM L-cysteine hydrochloride and 10 mM ascorbic acid (pH 7.2) at 37°C for 2 hours. After incubation, trichomonads were centrifuged at 1000 x g for 10 minutes, the supernatant was aspirated and filtered through a 0.22 μM pore size filter and filtered supernatants were centrifuged at 15,000 x g for 10 min @ 4°C prior to use in substrate-gel electrophoresis and apoptosis studies. Supernatants were used only when obtained from trichomonads that retained a minimum of 95% motility as assessed by light microscopy.

Substrate-gel electrophoresis

Trichomonad proteases were separated and analyzed under non-denaturing and non-reducing conditions in 10% Tris-glycine gels containing 0.1% gelatin (Life Technologies, Carlsbad, CA) as the protein substrate. Protein samples (40 μg) were electrophoretically separated at a voltage of 125V for 90 minutes. Following electrophoresis, gels were immersed in renaturing buffer (Life Technologies, Carlsbad, CA) for 30 min at 25°C to allow proteases to become activated. Following renaturing, gels were equilibrated in developing buffer (Life Technologies, Carlsbad, CA) for 30 min at 25°C followed by an overnight incubation at 37°C in fresh developing buffer. After overnight incubation, gels were washed 3 times in deionized water (dH_20) for 5 minutes each and then stained for a minimum of seven hours in Coomassie blue. Gels were then incubated in dH20 overnight. Proteolysis

was visualized as clear bands against a stained background. Identification of protein classes was accomplished by pretreatment of trichomonads or protein lysates with class specific protease inhibitors immediately prior to electrophoresis. Each of cysteine (E64 0.015-1.0 mM), metallo- (EDTA 0.015-5.0 mM), serine (PMSF 0.1-10mM; DFP 0.01-10mM) or aspartic (pepstatin A 0.01-1.5 mM) protease inhibitors were applied for 15 min at 37°C. As negative controls, protein extracts were treated identically with the respective protease inhibitor diluents.

Statistical analysis

All experimental data were analyzed for normality (Kolmogorov-Smirnov) and variance (Levene median) using a statistical software package and tested for significance using parametric or non-parametric tests as appropriate (Systat, Inc, Chicago, IL). Parametric data were analyzed using a Student's *t* test or one-way ANOVA with a post-hoc Holm-Sidak test. Non-parametric data were analyzed using Mann-Whitney rank sum test or Kruskal-Wallis ANOVA on Ranks. n = number of replicates. Results are reported as mean ± standard deviation. For all analyses, $P \leq 0.05$ was considered significant.

Results

T. foetus induces cytopathic effects on intestinal epithelial cells in association with activation of apoptosis

To determine if *T. foetus* induce cytopathic effects on intestinal epithelial cells, confluent monolayers of IPEC-J2 cells were grown in chamber slides, infected with 20 x 10^6 log-phase *T. foetus* and co-cultured for durations ranging from 0 to 36 hours, and then

examined by means of light microscopy. Light microscopic evaluation of *T. foetus* following incubation with IPEC-J2 cells revealed extensive destruction of the cell monolayer and numerous adherent trichomonads. In contrast to *T. foetus*-infected IPEC-J2 cells, feline *Pentatrichomonas hominis* adhered poorly to IPEC-J2 cells and cell monolayers remained intact (Figure 1).

To determine the general mechanism of *T. foetus*-induced epithelial cytopathogenicity, infected IPEC-J2 cells were further examined for evidence of cellular activation of apoptosis. Immunoblot analysis of infected IPEC-J2 cells demonstrated generation of a caspase-specific cleavage product of cytokeratin 18 (M30). M30 antigen was observed beginning at 12 hours with maximal cleavage occurring at 36 hours (Figure 2A). M30 antigen was not detected in uninfected IPEC-J2 cells or in isolated trichomonads at any phase of growth (Figures 2B and 5A).

T. foetus cytopathic effects require trophozoite-epithelial cell interaction

We next sought to determine if the cytopathic effects of *T. foetus* were mediated by direct interaction of trophozoites with intestinal epithelial cells versus a soluble mediator released by *T. foetus*. In contrast to the pro-apoptotic effect of *T. foetus* on IPEC-J2 cells in direct co-culture, cytokeratin cleavage was largely prevented when trichomonads were separated from direct contact with IPEC-J2 cells by a filter or when only secretory products of *T. foetus* were used to treat IPEC-J2 monolayers (Figure 2C).

Feline T. foetus express multiple protease activities

Cellular proteases are commonly implicated in the pathogenicity of trichomonads that cause venereal disease, however, their role in mediating cytopathic effects of gastrointestinal

trichomonads has not been explored. To characterize the protease activity of feline gastrointestinal trichomonads, whole cell protein lysates from 4 different feline *T. foetus* isolates and 1 feline *Pentatrichomonas hominis* isolate were separated by gelatin-SDS-PAGE using 10% tris-glycine gels copolymerized with 0.1% gelatin. The proteolytic activity of each of the feline *T. foetus* isolates were similar and characterized by at least five different bands of proteolysis with non-denatured molecular weights of approximately 52, 65, 85, 110, 120 kDa and a broader coalescing band with molecular weights of ≤ 20 kDa (Figure 3A). In contrast, whole cell lysates of *P. hominis* revealed weak proteolysis at bands corresponding to non-denatured with molecular weights of approximately 54, 64, and 85 kDa (Figure 3A). The lower molecular weight proteolytic bands observed in feline *T. foetus* isolates were absent in *P. hominis*.

T. foetus protease activities are attributed to serine and cysteine proteases

To determine the identity of the cellular protease activities produced by feline *T. foetus* and *P. hominis*, the protein lysates were treated with class-specific protease inhibitors or their diluents prior to substrate-gel electrophoresis. Treatment of both *T. foetus* and *P. hominis* protein lysates with PMSF (5mM) and DFP (1mM) inhibited proteolytic bands of 50kDa and greater, thereby identifying these activities as attributed to serine proteases. The molecular weight bands of ≤ 20kDa observed in *T. foetus* protein lysates were inhibited by pretreatment with E64, identifying these activities as attributed to cysteine proteases (Figure 3B). Failure of pepstatin A or EDTA to inhibit the proteolytic activity of either *T. foetus* or *P. hominis* suggested the absence of active aspartic or metalloproteases in whole cell proteases of these trichomonads. Treatment of live trichomonads with each protease inhibitor had

identical inhibitory effects on gel protease activities compared to treatment of protein lysates. These results suggest that both feline *T. foetus* and *P. hominis* trichomonads produce serine proteases but only *T. foetus* produces cysteine proteases.

T. foetus cysteine protease activity is cell-associated

Because *T. foetus* cytopathic effects required direct interactions between trophozoites and the intestinal epithelium, we sought to determine if either cysteine or serine protease activities of *T. foetus* were strictly cell-associated rather than secreted. When whole cell lysates of *T. foetus* were compared with cell-free *T. foetus* conditioned media, only cysteine protease activity was strictly cell associated while serine protease activity was associated with both cellular and secretory protein fractions (Figure 3C).

T. foetus cysteine proteases mediate intestinal epithelial cytotoxicity

To determine if TF cysteine protease activity mediates intestinal epithelial cytotoxicity, live *T. foetus* were pretreated with E64 (300μM) or diluent (dH_20) prior to co-culture in direct contact with IPEC-J2 cells. Treatment of trichomonads alone with E64 had no effect on motility or growth of *T. foetus* compared to treatment with vehicle alone for periods of up to 48 hours (Figure 4). Inhibition of *T. foetus* cysteine protease activity significantly reduced epithelial cell apoptosis as determined by the presence of cleared cytokeratin 18 at 36 hours of co-culture compared to *T. foetus* treated with vehicle alone (Figure 5A and B). Light microscopy evaluation of IPEC-J2 cells that were infected with *T. foetus* pretreated with E64 also revealed amelioration of monolayer destruction (Figure 5C). Pre-incubation of *T. foetus* with serine protease inhibitors (PMSF, 5mM; DFP, 1mM) at concentrations required for neutralization of protease activity was lethal which obviated our

ability to examine any additional contribution of *T. foetus* cell-associated serine protease to epithelial toxicity.

T. foetus cysteine protease activity mediates cytopathic effects by promoting adhesion of T. foetus to intestinal epithelial cells

Because the cytotoxicity of *T. foetus* requires direct interaction with the epithelium, is dependent on cysteine protease activity, and cysteine protease activity is primarily cell-associated, we sought to determine if cysteine proteases mediate their cytopathic effects by promoting adhesion of *T. foetus* to the intestinal epithelium. To test this hypothesis, adhesion of [^{3}H]-thymidine labeled TF to IPEC-J2 cells was assayed following pretreatment of TF with E64 (300μM) or diluent. Inhibition of *T. foetus* cysteine protease activity significantly blocked adhesion of trichomonads to the intestinal epithelial cells (Figure 6). To determine the specificity of this effect, [^{3}H]-thymidine labeled TF were additionally pretreated with serine (PMSF, 1.5mM), metallo- (EDTA, 500μM), and aspartic (pepstatin A, 100μM) protease inhibitors at standard inhibitory concentrations prior to co-culture with IPEC-J2 cells (Coradi ST, 2006; Jiménez, J.C., 2000). Pretreatment of trichomonads with these inhibitors had no effect on adhesion compared to vehicle-treated control. Trichomonad motility was not affected by EDTA, Pepstatin A, and PMSF at standard inhibitory concentrations.

Discussion

T. foetus is a prevalent cause of chronic large bowel diarrhea in cats with no consistently effective drugs available to treat infection. Knowledge of the cytopathogenic mechanisms employed by *T. foetus* is crucial for developing novel therapies to treat the infection. Although there is a growing body of evidence to support the role of cytoadherence and parasite released cellular proteases in the cytopathogenicity of the venereal trichomonads, *Trichomonas vaginalis* and bovine *Tritrichomonas foetus*, the mechanisms of cytopathogenicity of intestinal trichomonads are unknown (Singh et al, 2005, Arroyo et al, 1995, Talbot et al, 1991, Arroyo et al, 1989). Using an *in vitro* model system of porcine intestinal epithelial cells, we examined both the cytopathic effects of feline trichomonads towards the intestinal epithelium and the role of cellular proteases in parasitic virulence. Light microscopic examination and crystal violet quantification of *T. foetus* following co-culture with IPEC-J2 cells revealed progressive destruction of the monolayer over time. Numerous adherent trichomonads were observed within and surrounding the areas of destruction. In contrast, *Pentatrichomonas hominis*, a presumably nonpathogenic trichomonad of humans and domestic mammals, adhered poorly to IPEC-J2 cells and left the monolayers intact. Having demonstrated that *T. foetus* damages intestinal epithelial monolayers, we next sought to determine the general mechanism underlying *T. foetus*-induced epithelial cytopathogenicity. Cell death can be attributed to two distinct mechanisms: apoptosis and necrosis. Apoptosis is a more controlled form of cellular death that is initiated by the host following various physiological and pathological conditions. Apoptotic cells can be cleared by phagocytic cells prior to cell lysis and thus limits the host

inflammatory response, which may support the survival of parasites in chronic infection (Hotchkiss et al, 2009). For this reason, microbial pathogens have developed mechanisms to induce apoptosis of host target cells. A key feature of cellular apoptosis is cleavage of critical cytoskeletal proteins that leads to subcellular compartment collapse whereas cellular necrosis is characterized by cellular lysis and loss of plasma membrane integrity that promotes leakage of cytoplasmic components (Hotchkiss et al, 2009, Adams et al, 2007). To determine whether *T. foetus*-induced epithelial cell death is caused by apoptosis or necrosis, we assayed IPEC-J2 cells following co-culture with *T. foetus* for cytoplasmic lactate dehydrogenase (LDH) release as a marker of cellular lysis and cleaved epithelial cell cytokeratin 18 as a marker of apoptosis. *T. foetus* had no effect on LDH release as compared to control, however cleavage of cytokeratin 18 was significantly increased following incubation with *T. foetus*. These data suggest that *T. foetus*-induced epithelial cytopathogenicity is caused by stimulation of host cell apoptotic pathways.

Next, the question of whether contact between *T. foetus* and intestinal epithelial cells is necessary for cytopathogenicity was examined. Secretory products isolated from *T. foetus* trophozoites were added to IPEC-J2 monolayers. In addition, because secreted proteins are likely to be labile substances, live trichomonads separated from intestinal epithelial cells by a Transwell insert system were also analyzed. In these conditions, the activation of apoptosis was significantly ameliorated compared to trichomonads that were allowed to come into contact with epithelial cells. These results indicate that the majority of *T. foetus* cytopathogenicity is mediated by contact-dependent mechanisms.

We previously demonstrated the involvement of specific receptor-ligand interactions in the adhesion of feline *T. foetus* to the intestinal epithelium (Tolbert et al, 2013). In the present study, we sought to identify ligands that might mediate this adhesion event. Parasitic proteases are known to play numerous indispensable roles in the cytopathogenicity of venereal trichomonads including serving a critical role as adhesion proteins and mediating target cell cytotoxicity (Singh et al, 2005, Arroyo et al, 1995, Arroyo et al, 1989). Therefore, we characterized the identity of the cellular protease activities of feline *T. foetus* and *P. hominis*. Zymograms of feline intestinal trichomonads revealed that all isolates of *T. foetus* were qualitatively similar and produced multiple proteases that were identified as serine and cysteine proteases. In contrast, the nonpathogenic trichomonad, *P. hominis*, had weak proteolytic activity compared to *T. foetus* and no cysteine protease activity. These findings furthered our interest in evaluating the role of protease activity in *T. foetus* cytopathogenicity.

Because we determined that the majority of *T. foetus* cytopathic effects required direct contact with intestinal epithelial cells, we sought to determine if either cysteine or serine protease activities of *T. foetus* were strictly cell-associated rather than secreted. We observed that serine proteases were both cell-associated and secreted while cysteine proteases were strictly cell-associated. When evaluating the role of proteases in cytoadherence, we determined that only cysteine protease inhibition with E64 significantly reduced adherence to intestinal epithelial cells. Since we demonstrated both a role for direct contact in *T. foetus* cytopathogenicity and the importance of cysteine protease activity in cytoadherence, we evaluated the effect of cysteine protease activity on *T. foetus*-induced epithelial cytotoxicity. Pre-treatment of *T. foetus* with E64 significantly ameliorated

activation of apoptosis as compared to control-treated cells. These findings may suggest that *T. foetus* cysteine protease activity mediates cytopathic effects by promoting adhesion of *T. foetus* to intestinal epithelial cells. We cannot discount the possibility that serine proteases may also contribute to adhesion-dependent cytopathogenicity because cysteine protease inhibition did not completely ameliorate cytopathogenicity. However, since serine proteases induced rapid cell death of trichomonads, we were unable to evaluate its contribution to adhesion-dependent cytotoxicity using this model system.

In this study, we have revealed novel mechanisms by which *T. foetus* induces cytopathogenicity towards the intestinal epithelium. We demonstrate that *T. foetus* adheres to and damages intestinal epithelial cells via activation of apoptosis. Further, we have identified an important role for cysteine proteases as virulence factors in *T. foetus* induced adhesion-dependent cytotoxicity. These results suggest a putative molecular target for therapeutic intervention of feline trichomonosis. Further studies will be necessary to determine the identity and function of individual cysteine proteases in feline *T. foetus* cytopathogenicity and the efficacy of cysteine protease inhibition on clinical models of infection.

Figures

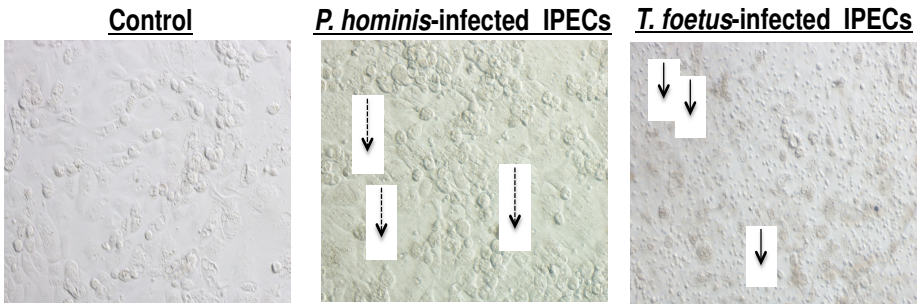

Figure 1. *T. foetus* induces cytopathic effects on intestinal epithelial cells. Light microscopy examination of the interaction of feline trichomonads with the intestinal epithelium. Note the numerous adherent trichomonads and extensive destruction of IPEC-J2 monolayers infected with *T. foetus*. In contrast, IPEC-J2 monolayers infected with the poorly adherent trichomonad, *P. hominis,* are confluent without any signs of damage. Dashed lines: Pentatrichomonas hominis; Solid lines: Tritrichomonas foetus (20x).

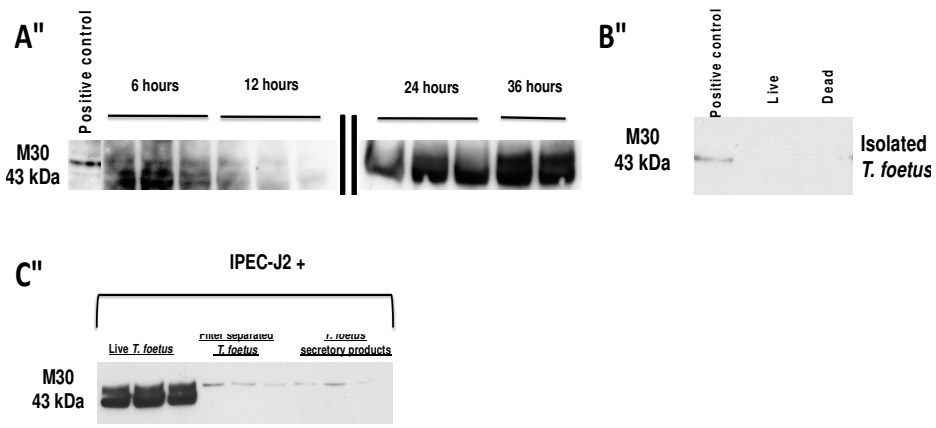

Figure 2. *T. foetus* induces contact-dependent activation of apoptosis in intestinal epithelial cells. (*A*) Immunoblot of IPEC-J2 cells for the M30 antigen of cleaved cytokeratin 18 after incubation with *T. foetus* for 6-36 hours. Maximal cleavage was observed at 36 hours. Uninfected IPEC-J2 cells showed no evidence of M30 at any time point (as shown in Figure 6A). (B) M30 antigen was not detected in isolated trichomonads at any phase of growth. (C). Immunoblot for the M30 antigen of cleaved cytokeratin 18 following 36 hr co-culture of IPEC-J2 with *T. foetus*, filter separated *T. foetus* and secretory *T. foetus* products.

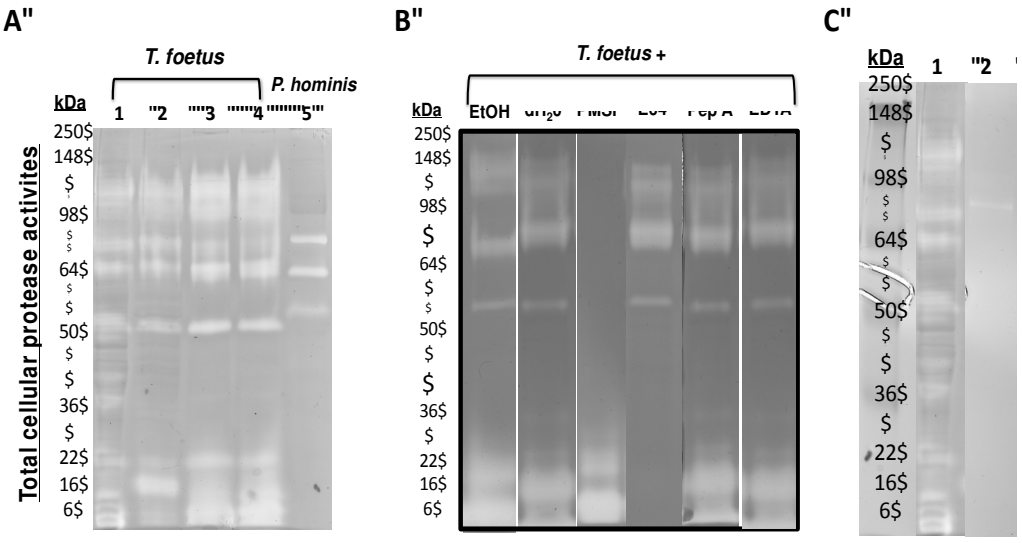

Figure 3. Cellular protease activity of feline intestinal trichomonds. (A) Representative gelatin zymography of cellular protein lysates from trichomonad isolates. Lanes 1-4, *T. foetus* isolates from four domestic cats; Lane 5, feline *Pentatrichomonas hominis*; (lane represent loadings as shown for lanes 1-4). (B) The effect of inhibitors on protease activity of cellular protein lysates from *T. foetus*. Lane 1-2, no inhibitors (vehicle-treated controls; ethanol: diluent for pepstatin A and PMSF; dH_20: diluent for E64 and EDTA); Lane 3, 5mM PMSF; Lane 4, 300µM E64; Lane 5, 10µM pepstatin A; Lane 6, 5mM EDTA. (C) Lane 1, Cellular protein lysate from *T. foetus*; Lane 2, Secretory product of feline *T. foetus* from corresponding isolate.

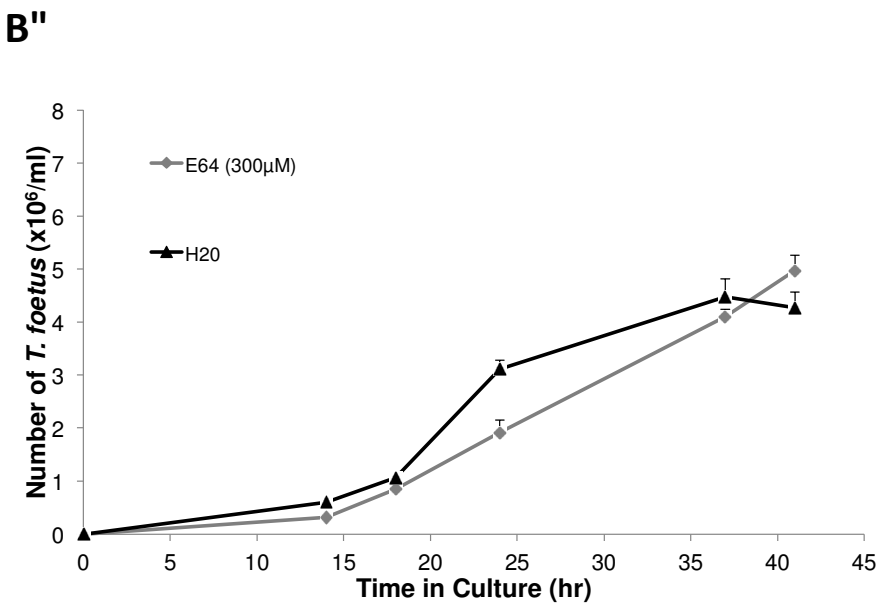

Figure 4. Growth of *T. foetus* in the presence of cysteine protease inhibition. A growth curve of *T. foetus* following inoculation of $5x10^4$ trichomonads into Advanced Diamond's media in the presence of 300µM E64 or its diluent (H_2O). Each data point represents 3 replicates.

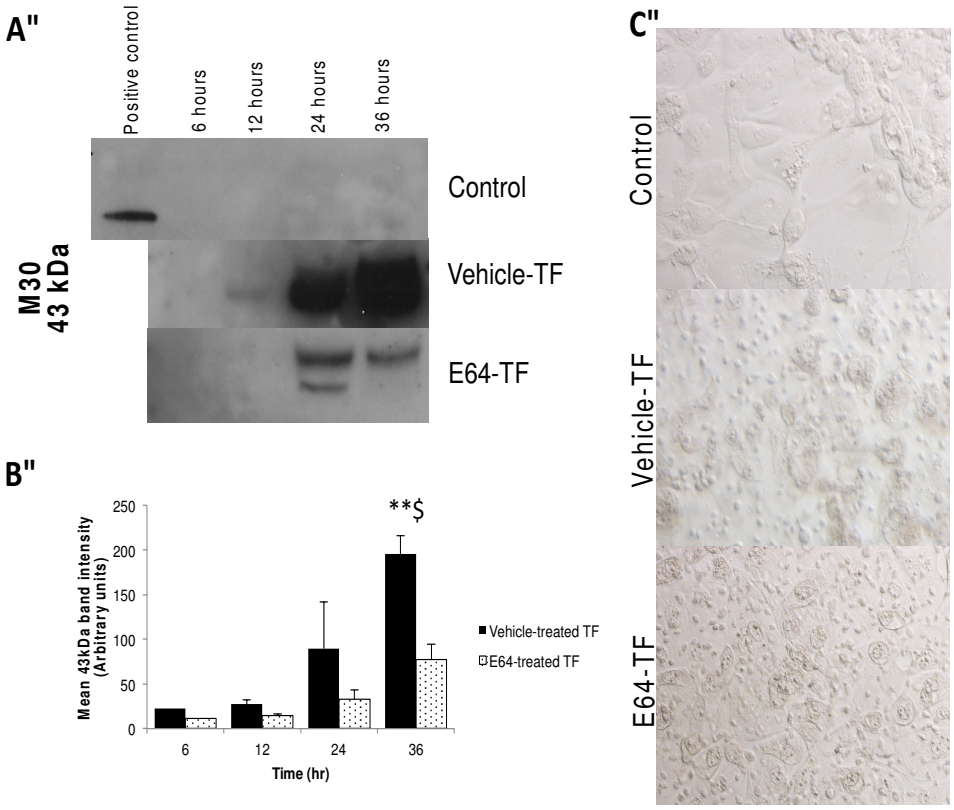

Figure 5. Cysteine protease activity mediates *T. foetus* cytoadherence. *T. foetus* pretreated with E64 or vehicle were added to IPEC-J2 monolayers at an infection dose of $20x10^6$ and and co-cultured for 6, 12, 24, and 36 hours. (A) Immunoblot of control, vehicle-treated *T. foetus* and E64-treated *T. foetus* infected IPEC-J2 cells for the M30 antigen of cleaved cytokeratin 18. n=3 each. (B) Densitometric analysis of immunoblot from figure 6A. **$p<0.01$ compared to E64-treated *T. foetus* (One Way ANOVA and post-hoc Holm-Sidak test). (B) Data are reported as mean ± SD. (C) Representative light microscopy images of control, vehicle-treated *T. foetus* and E64-treated *T. foetus* infected IPEC-J2 cells (40x magnification).

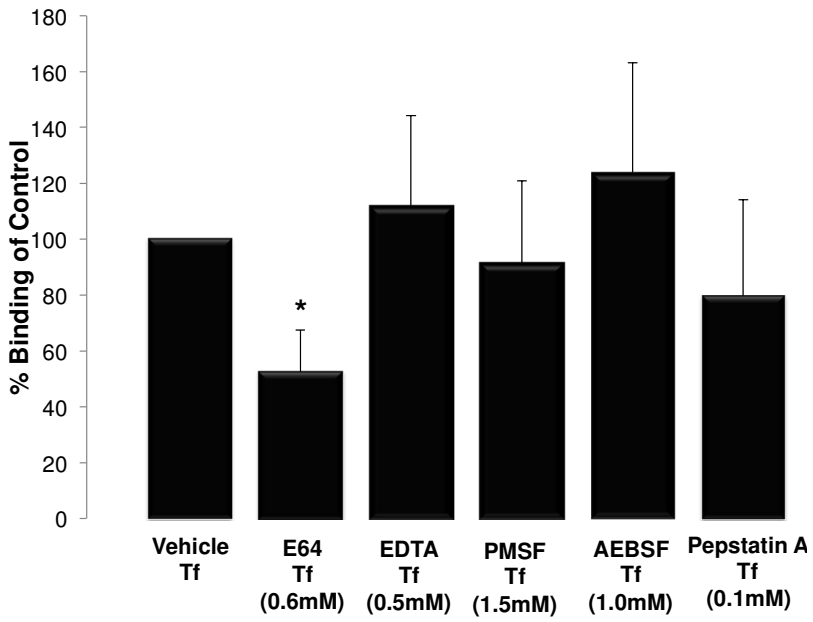

Figure 6. *T. foetus* adhesion is mediated by cysteine protease activity. Adhesion of [^{3}H] thymidine labeled-*T.foetus* to IPEC-J2 cells is significantly reduced following pretreatment with E64 (cysteine protease inhibitor) compared to vehicle-treated control. No effect is observed on *T.foetus* adhesion with pretreatment using protease inhibitors of other classes. *$p<0.05$ compared to vehicle-treated *T.foetus* (Mann Whitney Rank Sum). Each data point represents 4-11 replicates.

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CHAPTER 3

Feline *Tritrichomonas foetus* Adhere to Intestinal Epithelium by Sialic Acid-Dependent Mechanisms

MARY KATHERINE TOLBERT, STEPHEN STAUFFER, and JODY GOOKIN

Abbreviations used in this paper: CPM, counts per minute; IPEC-J2, Intestinal porcine epithelial cells; DMEM, Dulbecco's modified eagle medium; HBSS, Hank's balanced salt solution; Epidermal growth factor; PBS, phosphate buffered solution; MAH, Maackia amurensis lectin II; NeuAc, N-Acetylneuraminic acid; SNA, Sambucus nigra; TER, Transepithelial electrical resistance

Abstract: *Tritrichomonas foetus* is a flagellated protozoan that parasitizes the feline colon resulting in chronic foul-smelling diarrhea. Once infected, diarrhea can be long-lasting and despite periods of clinical remission, it is likely that cats remain infected for life. While adhesion to mucosal epithelium is believed to be a key event in the venereal pathogenicity of bovine *T. foetus*, the pathogenesis of diarrhea in feline *T. foetus* infection is unknown. The reproductive and gastrointestinal tract are rich in glyconjugates that contain sialic acid as their terminal residue. Bovine *T. foetus* express lectins that selectively recognize and bind to sialic acid and adhesion can be inhibited by antibodies directed against these sialic-acid specific lectins. Aims of this study were to investigate the role of sialic acid in the adhesion of feline *T. foetus* to the intestinal epithelium.

Assays on the expression of sialic acid on porcine intestinal epithelial cell (IPEC-J2) monolayers were performed using biotinylated lectin cytochemistry. Confluent IPEC-J2 cells

(TER≥2,000Ω·4.67cm^2) were infected with axenic cultures of feline *T. foetus* that were labeled with [^{3}H]thymidine (4μCi/ml for 36hrs) and harvested at log-phase. The effect on *T. foetus* cytoadhesion of sialic acid (*N*-acetylneuraminic acid, 2-50mM), D+ galactose (5-200 mM), 2,3,-dehydro-2-ceoxy-NeuAc (1-5mM) or desialyation of IPEC-J2 monolayers were quantified by liquid scintillation counting of adherent *T. foetus*. A minimum of four replicates was performed for each experiment. Data were analyzed using commercially available statistical software.

IPEC-J2 monolayers demonstrated the presence of surface sialic acid with good reactivity using sialic acid specific lectins, SNA and MAH. Sialic acid and 2,3,-dehydro-2-ceoxy-NeuAc inhibited adhesion of ^{3}[H] thymidine-labeled *T. foetus* to IPEC-J2 monolayers by approximately 50% and 65%, respectively. Predigestion of IPEC-J2 surface sialic acid with *V. cholarae* and *C. perfringens* sialidases significantly inhibited adhesion of *T. foetus* to intestinal epithelial cells. These findings suggest that *T. foetus* adhesion is mediated in part by sialic acid and that further study of sialic acid as a putative pharmacological target for treatment of feline trichomonosis is warranted.

Introduction

Tritrichomonas foetus is an etiologic agent of colitis and chronic diarrhea in domestic cats. Feline *T. foetus* is prevalent worldwide yet no consistently effective drugs are available to treat the infection (Gookin, J.G., 2010). Following ingestion, trichomonads colonize the distal ileum, cecum and colon (Gookin, J.G., 2001) where they are observed to intimately associate with the mucosal surface and lumen of colonic crypts (Yaeger, M., 2004). Previous work in our laboratory has demonstrated that feline *T. foetus* adhere to intestinal epithelial cells by a specific receptor-ligand mediated interaction (Tolbert, M.K., 2013). Epithelial cells display a variety of glyconjugates on their cellular surfaces with which *T. foetus* could potentially adhere. Sialic acid is the most abundant monosaccharide expressed as the terminal residue on glycoproteins and glycolipids that decorate the intestinal epithelial glycocalyx. Sialic acid is a generic term encompassing a large family of nine carbon ketosugars that are distinguished based on modifications of the amino or hydroxyl group (Schauer, R., 2000). Sialic acids are richly expressed in the gastrointestinal tract where they provide pivotal functions related to immune recognition, cell-to-cell communication, and contribute to epithelial cell barrier integrity (Lewis, A.L., 2012; Varki, A., 2009). Thus, it is not surprising that microbial pathogens have developed mechanisms to both disrupt and exploit host sialic acids. Adhesion to mucosal epithelium is believed to be a key mechanism of pathogenesis of trichomonad infections. The bovine venereal pathogen *Tritrichomonas foetus* and the intestinal pathogen of squirrel monkeys, *Tritrichomonas mobilensis*, express lectins that selectively recognize and bind to sialic acid. Adhesion of bovine *T. foetus* and *T. mobilensis* is inhibited by antibodies directed against these sialic-acid specific lectins (Babál, P., 1999).

The role of sialic acid in feline *T. foetus* infection is unknown. We hypothesized that intestinal epithelial cell surface sialic acid plays a similar role in feline *T. foetus* adhesion and may represent a novel molecular target for the treatment of feline trichomonosis.

In these studies, we demonstrate for the first time that adhesion of feline *T. foetus* to the intestinal epithelium can be abrogated by the use of exogenous sialic acid to inhibit *T. foetus* sialidase activity. Further, de-sialyation of the intestinal epithelial glycocalyx significantly reduces *T. foetus* adhesion. These findings suggest that *T. foetus* adhesion is mediated in part by sialic acid and that further study of sialic acid as a putative pharmacological target for treatment of feline trichomonosis is warranted.

Materials and Methods

IPEC-J2 cells

The porcine jejunal epithelial cell line (IPEC-J2) is a non-transformed primary cell line originally isolated from neonatal piglet jejunum and was obtained as a gift from Dr. Helen M. Berschneider. IPEC-J2 cells were grown in co-culture media which 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 in 5% CO_2. Prior to adhesion studies, IPEC-J2 cells were seeded onto permeable polycarbonate filters (0.4 μm pore size, 4.67cm^2; Corning Incorporated, Lowell, MA) and cultivated until confluent. For microscopic examination of trichomonad-induced cytotoxicity, IPEC-J2 cells were seeded onto Laboratory-Tek chamber slides (Nalg Nunc

International, Rochester, NY) and grown to confluence. For transepithelial electrical resistance (TEER) studies, IPEC-J2 cells were seeded onto permeable polyester filters (0.4 μm pore size, 1.12cm^2; Corning Incorporated, Lowell, MA) and grown to confluence (TER≥2,000Ω·1.12cm^2). IPEC-J2 cells were used at passage numbers 38-60.

Trichomonads

Isolation and culture of TF isolates were performed as previously described (Tolbert et al., 2013). Trichomonads were harvested in mid to late log phase by centrifugation and washed twice in Hanks' Balanced Salt Solution (HBSS). The trichomonads were suspended in HBSS at desired concentrations for experimental purposes.

Labeling of trichomonads

Labeling of trichomonads was performed as previously described (Tolbert et al, 2013). Briefly, trichomonads were harvested in late log-phase of growth and inoculated into modified Diamond's media containing 4 μCi/ml [^{3}H] thymidine (17 Ci/mmol) (American Radiolabeled Chemicals). After 36 hours, radiolabeled trichomonads were washed three times by means of centrifugation (250×g) and reconstitution in HBSS to remove unassociated radioactive tracer. Trichomonads were counted using a hemocytometer and resuspended in IPEC-J2 media at the desired concentration.

Lectin cytochemistry

To demonstrate cell-associated sialic acid on the surface of IPEC-J2 monolayers, the biotinylated lectins Sambucus nigra (SNA), that preferentially binds sialic acid α (2-6) linked to galactose or *N*-Acetylgalactosamine, and Maackia amurensis lectin II (MAH), that preferentially binds sialic acid α (2-3) linked to galactose, were used. IPEC-J2 cells seeded

on chamber slides were cultured to confluence, washed gently with HBSS, and fixed in 10% buffered formalin at 25C for 10 minutes. Following fixation, slides were treated with 70% ethanol, washed and incubated in phosphate-buffered solution (PBS, pH 7.4) for 10 minutes, incubated in streptavidin and biotin block for 15 minutes each and blocked with a carbohydrate-free blocking for 60 minutes at RT. Slides were incubated with the biotinylated lectins for 60 minutes at 37°C. Slides were then washed in PBS + 0.05% Tween (TPBS) twice for 5 minutes each. Slides were incubated in strepavidin peroxidase for 30 minutes at 25°C and washed with TPBS twice for 5 minutes each. A peroxidase precipitating substrate was applied for approximately three minutes until the desired color reaction developed. Slides were washed in dH_20 once for 5 minutes, counterstained with alcian green, dehydrated, and mounted. Slides treated identically with diluent from the biotinylated lectin preparations were used as negative controls. Biotinylated lectins and immunocytochemistry reagents were obtained from Vector Laboratories (Burlingame, CA).

Co-culture adhesion assay

Adhesion assays were performed as described previously (Tolbert et al, 2013). For co-culture adhesion assays, an infection dose of $20x10^6$ TF [^{3}H] thymidine-labeled trichomonads were added to the apical media of IPEC-J2 cell monolayers grown to confluence (TER≥2,000Ω·4.67cm^2) on polycarbonate inserts. Co-cultures were incubated at 37°C in 5% CO_2 for six hours. Following adhesion, monolayers were washed twice with sterile HBSS to remove unbound trichomonads. The inserts were then excised using a scalpel blade and placed in 20 ml scintillation vials containing Econo 2 fluid (Fisher Scientific, Pittsburgh, PA). Radioactive emissions were counted using a Wallac 1209 liquid

scintillation counter and expressed in counts per minute (CPM). Radioactive emissions measured from serial dilutions of the radiolabeled trichomonads were used for each assay to generate a standard curve of CPM per trichomonad. Number of trichomonads adhered to cell monolayers were calculated by applying the CPM measured to the standard curve. Baseline differences in TF adhesion to IPEC-J2 cells between experiments were controlled for by inclusion of an untreated TF control group in each radiolabeled TF adhesion assay.

Sialic acid adhesion characterization assays

[^{3}H] thymidine-labeled TF were treated with exogenous sialic acid (*N*-Acetylneuraminic acid, 2-50mM, Sigma-Aldrich, St. Louis, MO), D+ galactose (5-200mM, Sigma Aldrich, St. Louis, MO) or their diluents (pH matched H_20) for 2hr at 37° C prior to co-culture. Following incubation, trichomonads were washed once by means of centrifugation (250 × g) and reconstitution in HBSS and allowed to adhere to IPEC-J2 monolayers.

To further evaluate the effect of sialic acid binding on TF cytoadhesion, [^{3}H] thymidine-labeled TF were pretreated with the sialidase inhibitor, 2,3,-dehydro-2-ceoxy-NeuAc (1-5mM, Sigma Aldrich, St. Louis, MO) or its diluent, pH matched dH20 for 1 hr at 37°C. Following incubation, trichomonads were washed as described for sialic acid and allowed to adhere to IPEC-J2 monolayers. A growth curve of TF was evaluated in the presence of each adhesion-inhibiting drug or the appropriate diluent.

Desialyation of IPEC-J2 monolayers

For adhesion assays, IPEC-J2 monolayers were washed with HBSS and incubated for 2 at 37° C with sialidase preparations from *Clostridium perfringens* (Sigma) and *Vibrio*

cholarae (Sigma) at a final concentration of 1U/ml or their diluents, PBS with 0.2% BSA and PBS with 4mM $CaCl_2$, respectively. These enzymes exert broad substrate specificity, cleaving terminal sialic acid residues which are α-2,3- α-2,6- or α-2,8- linked to oligosaccharides, glycolipids or glycoproteins. Following incubation, monolayers were gently washed twice with HBSS and were used in co-culture assays as described previously. To determine the effect of sialidase treatment on IPEC-J2 permeability, IPEC-J2 monolayers were treated as described with *V. cholarae* sialidase or its diluent and TEER was measured hourly for an 8 hr time period using an electrovoltometer (EVOM; WPI instruments, Sarasota, FL).

Statistical analyses

All experimental data were analyzed for normality (Kolmogorov-Smirnov) and variance (Levene median) using a statistical software package and tested for significance using parametric or non-parametric tests as appropriate (Systat, Inc, Chicago, IL). All data were found to be parametric and were analyzed using a Student's *t* test. *n* = number of replicates. Results are reported as mean ± standard deviation. For all analyses, $P \leq 0.05$ was considered significant.

Results

IPEC-J2 cells express surface sialic acid

To determine if IPEC-J2 cells could be used in our co-culture model system to evaluate the role of sialic acid as a receptor for *T. foetus* adhesion, we assayed for the expression of sialic acid on IPEC-J2 monolayers (Figure 1). The monolayers were probed

with sialic-acid specific biotinylated lectins SNA and MAH II. MAH II exhibited strong reactivity with IPEC-J2 monolayers. Examination by light microscopy (Figure 1A) revealed discrete multifocal regions of stained epithelial cells adjoined by smaller regions of unstained epithelial cells. Less reactivity was observed in cells probed with SNA (Figure 1B) however the heterogenous distribution visualized with MAL II was apparent which may reflect the heterogenous population present within IPEC-J2 cultures. Minimal to no reactivity was observed in control treated cells (Figure 1C).

T. foetus adhesion is dependent on sialic acid

To determine the dependence of *T. foetus* adhesion on recognition of epithelial cell surface sialic acid, *T. foetus* were pretreated with sialic acid (NeuAc; 2-50mM) or vehicle prior to co-culture with intestinal epithelial cells. Pretreatment of *T. foetus* with NeuAc at concentrations as low as 2 mM significantly reduced the number of trichomonads adhered to IPEC-J2 monolayers compared to vehicle-treated *T. foetus* (Figure 2). Maximum inhibition of *T. foetus* was achieved at 50 mM NeuAc and resulted in a 50% reduction in adhesion to IPEC-J2 monolayers. In contrast, D+ galactose, a glyconjugate commonly found as the subterminal glycoconjugate adjacent to terminal sialic acid residues, at concentrations as high as 200mM had no effect on *T. foetus* adhesion (Figure 3).

To determine the potential efficacy of sialidase inhibition as a novel therapy for inhibiting adhesion of feline *T. foetus* to the intestinal epithelium, trichomonads were pretreated with the sialidase inhibitor, 2,3,-dehydro-2-ceoxy-NeuAc (Figure 3). 2,3,-dehydro-2-ceoxy-NeuAc is a sialidase inhibitor that serves as a parent compound for commercially available sialidase inhibitor drugs. Trichomonads were pretreated with 2,3,-dehydro-2-ceoxy-

NeuAc, washed and then allowed to adhere to IPEC-J2 monolayers. The number of trichomonads adhering to IPEC-J2 monolayers was significantly inhibited by approximately 65% by pretreatment with the sialidase inhibitor compared to vehicle-treated *T. foetus*. Incubation of *T. foetus* in the presence of either NeuAc or 2,3,-dehydro-2-ceoxy-NeuAc at concentrations observed to inhibit adhesion had no effect on replication or viability of the trichomonads (Figure 5).

Epithelial sialic acid mediates T. foetus adhesion

We next asked the question of whether sialic acid was utilized as a receptor or was immediately cleaved by *T. foetus* to reveal subterminal glycoconjugates that may be more preferable as binding sites. Desialyation of IPEC-J2 monolayers was performed using sialidase from *V. cholarae* or *C. perfringens* prior to co-culture with *T. foetus*. Removal of sialic acid residues from the surface of IPEC-J2 monolayers resulted in significantly reduced numbers of adhered trichomonads compared to vehicle-treated monolayers (Figure 6A and B). There was no evidence that sialidase treatment resulted in any toxic effects to the IPEC-J2 cells as there was no significant difference in transepithelial electrical resistance (TEER) over the 8 hr incubation period in sialidase-treated IPEC-J2 monolayers compared to control. These results suggest that inhibition of *T. foetus* adhesion to de-sialyated monolayers is a result of removal of the sialic acid-binding site rather than loss of IPEC-J2 cells.

Discussion

Epithelial mucosal surfaces and their mucus secretions contain a variety of glycosylated molecules that have sialic acid as the outermost monosaccharide (Varki et al,

2012). The terminal position of sialic acid on mucosal surfaces has made them an inevitable target for microbial pathogens including the venereal pathogen of cattle, *Tritrichomonas foetus*, which expresses lectins that selectively recognize and bind to sialic acid (Babál P et al, 1999). Host sialic acid can be used as a binding site and/or can be cleaved by pathogens to reveal subterminal glycoconjugates for adhesion. Further, cleavage and release of free sialic acid can be utilized by pathogens as a nutrient source (Almagro-Moreno et al, 2009, Chava et al, 2004). The gastrointestinal tract and its mucous lining are particularly rich in sialic acid (Lewis et al, 2012). Having previously demonstrated the role of specific receptor-ligand interactions on the adhesion of feline *T. foetus* to the intestinal epithelium (Tolbert et al, 2013) and acknowledging the abundance of sialic acid on gastrointestinal mucosal surfaces, we chose to examine the role of epithelial sialic acid as a receptor for feline *T. foetus* adhesion.

To determine if IPEC-J2 cells could be used in our co-culture model system to evaluate the role of sialic acid as a receptor for *T. foetus* adhesion, we assayed for the expression of sialic acid on IPEC-J2 monolayers. We demonstrated that IPEC-J2 cells express sialic acid and were an appropriate cell line for our co-culture model system. IPEC-J2 cells displayed predominantly sialic acid α (2-3) linked to galactose. Exploration with other cell culture lines that express sialic acid with varying glycosidic linkages and subterminal glycoconjugates may give more insight into the sialic acid- dependent mechanisms of adhesion of *T. foetus* to the intestinal epithelium.

N-acetylneuraminic acid (NeuAc) is the most common sialic acid identified on mammalian cell surfaces hence this molecule was used as the representative sialic acid in our

studies. Trichomonads were pretreated with NeuAc in order to saturate sialic acid-binding lectins if present. Significant inhibition of feline *T. foetus* adherence was demonstrated by pretreatment of trophozoites with NeuAc. This finding suggests a role for sialic acid in the interaction between *T. foetus* and the intestinal epithelium. To further evaluate this effect, trichomonads were pretreated with the sialidase inhibitor, 2,3,-dehydro-2-ceoxy-NeuAc . Sialidases are enzymes that cleave sialic acid residues from cell surface glycoconjugates. Sialidase inhibitors are sialic acid analogues that can block the active site of the sialidase on the pathogen surface. 2,3,-dehydro-2-ceoxy-NeuAc is a sialidase inhibitor that serves as a parent compound for commercially available sialidase inhibitor drugs including TamiFlu® and Relenza (von Itzstein M, 2007). Thus, we sought to determine the potential efficacy of sialidase inhibition as a novel therapy for the treatment of feline *T. foetus* infection. Sialidase inhibition of *T. foetus* resulted in significantly reduced numbers of adhered trichomonads compared to control. Pretreatment of *T. foetus* with sialic acid analogs and desialyation of epithelial monolayers did not completely inhibit *T. foetus* adhesion, which suggests that this event is multifactorial.

It is unclear whether sialic acid is utilized as a receptor or is immediately cleaved by feline *T. foetus* to reveal subterminal glyconjugates that may be more preferable as binding sites. *T. foetus* co-cultured with de-sialyated IPEC-J2 monolayers resulted in significantly decreased numbers of adhered trichomonads compared to vehicle-treated IPEC-J2 monolayers. Further, pretreatment with galactose, a commonly identified subterminal monosaccharide expressed beneath sialic acid residues, had no inhibitory effect on *T. foetus* adhesion. These findings may further support the role for sialic acid as a receptor for *T.*

foetus adhesion to the intestinal epithelium. Future work assaying for the presence of cleaved sialic acid on epithelial monolayers following exposure to *T. foetus* or for the detection of neuraminidase activity in feline *T. foetus* as observed in venereal *T. foetus* may provide more information regarding the cleavage of sialic acid following the initial adhesion event (Dias Filho et al, 1999).

In conclusion, our preliminary results suggest a role for epithelial surface sialic acid in the adhesion of *T. foetus* to the intestinal epithelium. As in venereal trichomonad pathogenicity, adhesion of feline *T. foetus* to intestinal epithelial cells appears to be a multifactorial event. Thus, future studies are necessary to determine the precise course of events in sialic acid-dependent adhesion and to explore other mechanisms of adhesion in *T. foetus* pathogenicity.

Figures

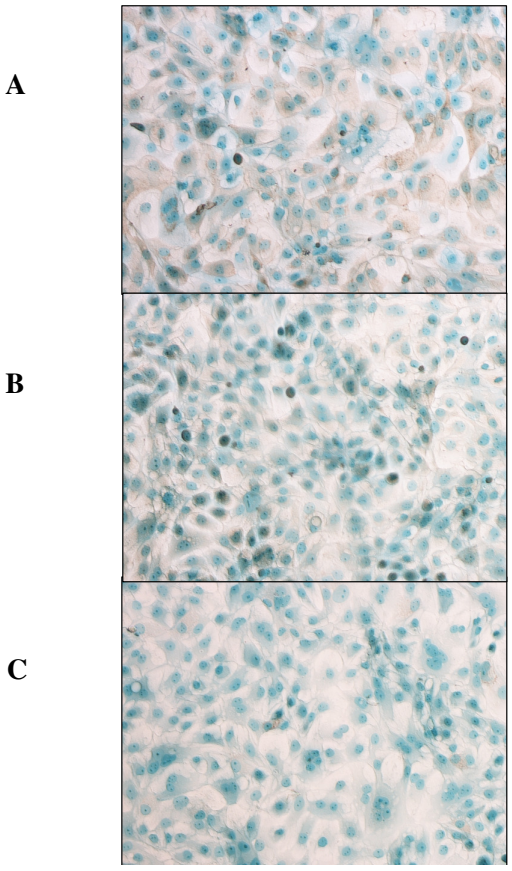

Figure 1. Cytochemical detection of sialic acid-rich glycoconjugates on the surface of IPEC-J2 cells. Biotinylated lectins: (A) Maackia amurensis lectin II (MAH) and (B) Sambucus nigra (SNA) or (C) control.

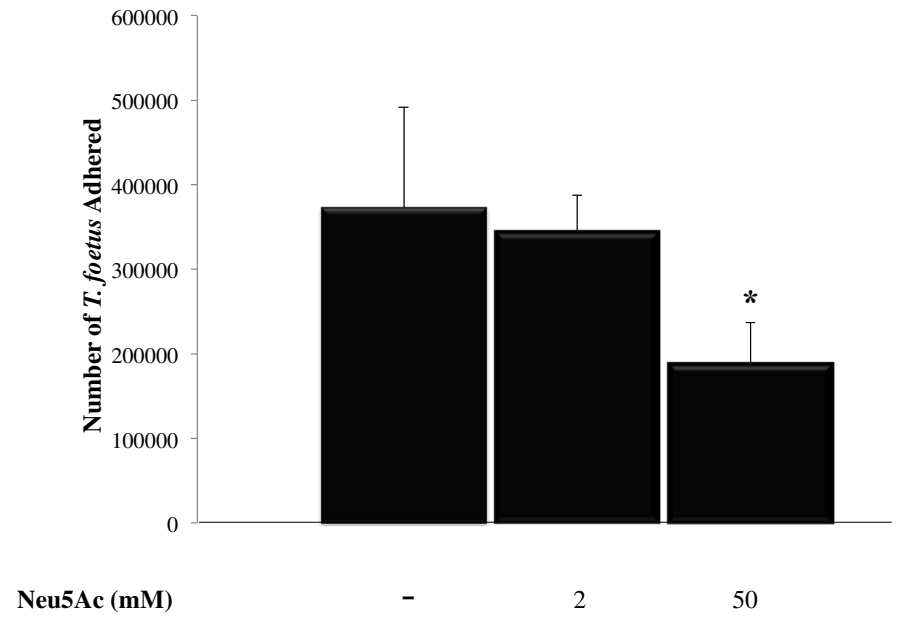

Figure 2. *T. foetus* adheres to the intestinal epithelium via sialic acid. Adhesion of [3H] thymidine labeled-TF to IPEC-J2 cells following pretreatment with exogenous sialic acid is significantly reduced compared to vehicle treated (deionized water) TF. Each column represents 4 replicates. *$p<0.05$ compared to vehicle treated control (Student's t test).

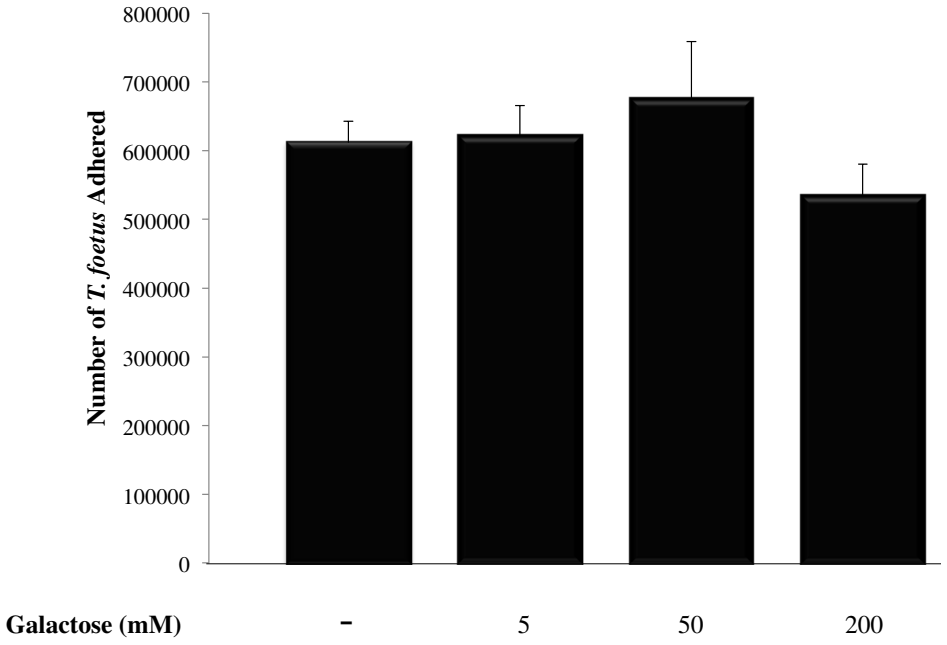

Figure 3. Galactose has no effect on *T. foetus* adhesion. Adhesion of [^{3}H] thymidine labeled-TF to IPEC-J2 cells was similar to control following pretreatment of TF with 5-200mM D+ galactose. Each column represents 5-9 replicates.

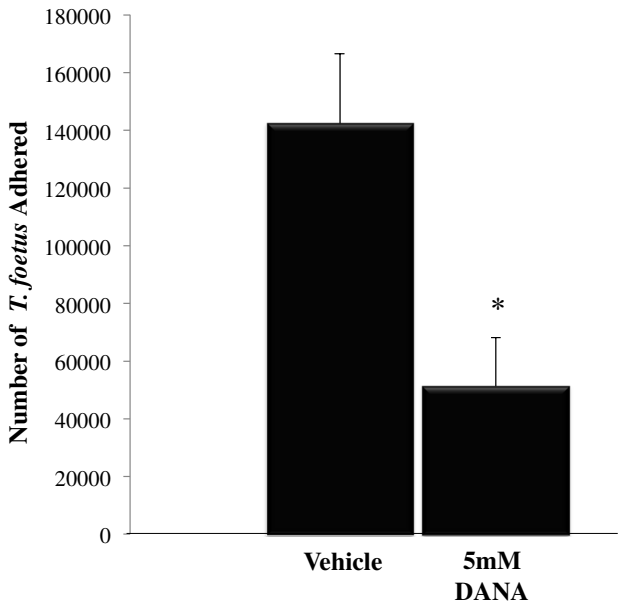

Figure 4. ***T. foetus*** **adheres to intestinal epithelium via sialic acid-dependent mechanisms.** Adhesion of sialidase inhibitor (DANA)-treated [3H] thymidine labeled-TF to IPEC-J2 cells is significantly reduced compared to vehicle treated (deionized water) TF. Each column represents 6 replicates. *$p<0.05$ compared to vehicle treated control (Student's t test). N=6 replicates.

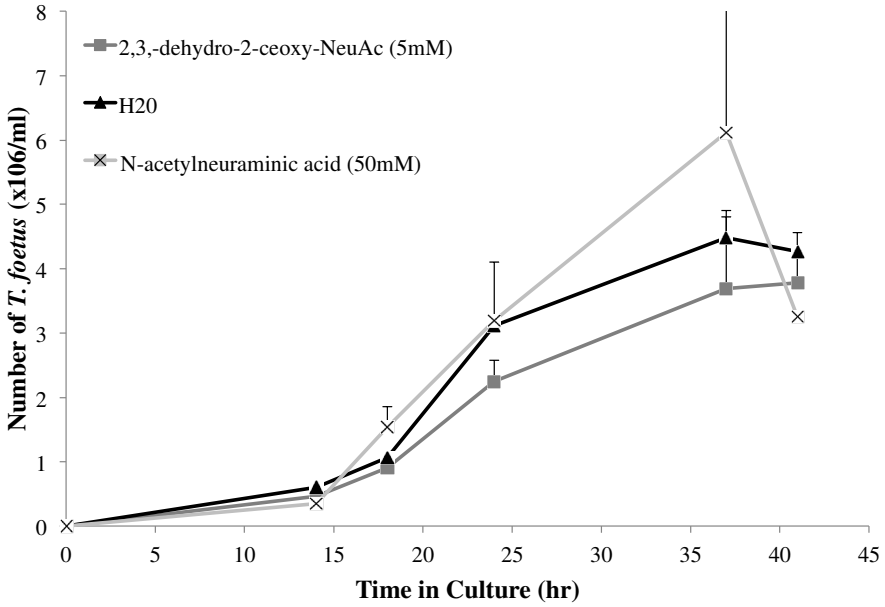

Figure 5. Growth of *T. foetus* in the presence of sialic acid analogues. Growth of TF in the presence of sialic acid (N-acetylneuraminic acid) or the sialidase inhibitor (2,3,-dehydro-2-ceoxy-NeuAc) is unaffected compared to vehicle-treated controls. N=3 replicates at each time point.

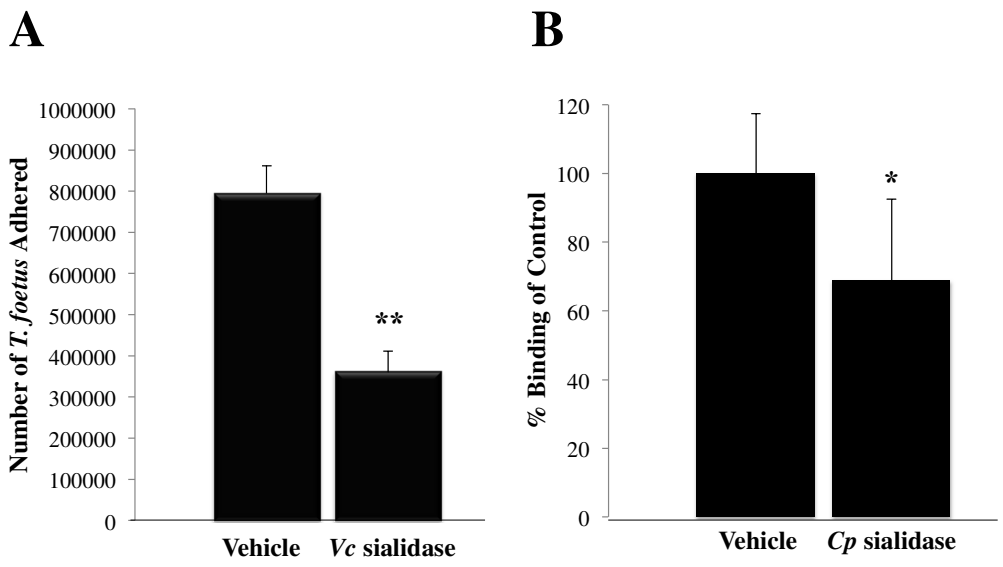

Figure 6. *T. foetus* adheres to sialic acid expressed on IPEC-J2 cells. Adhesion of [3H] thymidine labeled-TF to desialyated IPEC-J2 cells is significantly reduced compared to vehicle treated (deionized water) control IPEC-J2 cells. (A) Adhesion of TF to V. cholarae desialyated and control-treated IPEC-J2 cells. (B) Adhesion of TF to C. perfringens desialyated and control-treated IPEC-J2 cells. Each column represents 5-6 replicates. *p<0.05 **p<0.01 compared to vehicle treated control (Student's t test).

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CHAPTER 4

Preliminary Investigations Pertaining to the Culture of Primary Feline Intestinal Epithelial Cells

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Abbreviations used in this paper: BMP, Bone Morphogenic Pathway; DAPI, 4',6-diamidino-2-phenylindole, DMEM, Dulbecco's modified eagle medium; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGTA, ethyleneglycotetraacetic acid; FIEC, feline intestinal epithelial cell; GMA, glycolmethacrylate; HBSS, Hank's balanced salt solution; OCT, optimal cutting temperature; PBS, phosphate buffered solution; Wnt, Wingless and Int

Abstract: The successful culture of a primary feline intestinal epithelium cell line (FIEC) would be a key asset to feline and human translational research. Cats serve as hosts and/or models for many important intestinal pathogens that are infectious to humans including *Toxoplasma gondii*, *Cryptosporidium* spp., *Giardia duodenalis*, Enteropathogenic *E. coli*, and feline immunodeficiency virus. Primary epithelial cells have been historically difficult to culture, thus most studies use malignant cell lines. Although these models have been exceedingly informative, this environment leads to altered gene expression resulting in the inability of cells to obtain a differentiated and polarized phenotype. Novel developments in our understanding of the of the intestinal stem cell niche and cell signaling networks have led to the cultivation of tissue aggregates (organoids) that more accurately model in vivo

biology. Preliminary results produced by ourselves and our collaborators have incorporated these approaches. We have successfully isolated and maintained feline intestinal crypts, which contain the stem cells of the intestine, and have fostered the in vitro growth of multicellular structures (enteroids) as well as epithelial cell sheets that architecturally recapitulate the native feline large intestinal epithelium. The long-term goals of these studies are to generate a long-lived primary epithelial cell culture model of the feline intestinal epithelium that can be used by the research community for the study of feline gastrointestinal disease pathogenesis and treatment.

Introduction

The mammalian intestinal tract is lined by a single layer of epithelial cells that are responsible for the life-sustaining absorption of nutrients and water while simultaneously preventing the translocation of bacteria, endotoxins and antigens into the body. Failure of these absorptive and barrier functions is a primary cause or consequence of nearly all gastrointestinal diseases including acute gastroenteritis, inflammatory bowel disease, food allergy/intolerance, intestinal neoplasia, and gastrointestinal infection. These as well as other causes of gastrointestinal disease are predicted to be amongst the most prevalent reasons for presentation of companion animals to a veterinarian. Unfortunately, growth of our knowledge of companion animal gastrointestinal pathophysiology has been slow, largely descriptive, and limited for the most part to the study of patients with naturally occurring diseases of differing severity and uncertain etiology. Consequently, there is a compelling need for new approaches to gastrointestinal research that enable veterinary clinician-scientists to define the cellular mechanisms of disease pathogenesis without requiring the use of live animals.

In human medicine, cell culture models of the intestinal epithelium have been used for decades to investigate mechanisms of disease pathogenesis induced by infectious agents, chemotherapeutics, inflammatory cytokines and toxins; to develop therapy for the treatment of inflammatory bowel disease; and identify putative gene mutations that lead to the development of intestinal cancer (Chopra et al, 2010). Because primary (non-malignant) intestinal epithelial cells are notoriously difficult to establish in culture, most of these studies use transformed (malignant or immortalized) cell lines that are grown on plastic or synthetic

basement membranes. While highly informative, genetic mutations and artificial conditions limit the ability of these models to recapitulate the architecture, differentiated phenotype and function of the native intestinal epithelium (Nickerson et al, 2001, Hammond et al, 2001, Sato et al, 2009); a fact that always calls into question translation of research findings from bench to bedside.

In the past few years, basic scientists have made major breakthroughs in our understanding of the structural and growth requirements of the intestinal epithelium. Although all of the factors controlling the intestinal stem cell niche have not been fully elucidated, the expression of several signaling molecules along the crypt-villus axis appears to be critical. These signaling pathways, which include the Wnt (Wingless and INT-1), Notch, and Bone Morphogenic Pathway (BMP), are each involved in unique aspects of stem cell maintenance, proliferation, post-mitotic differentiation, and migration (Kim et al, 2007, Van der Flier et al, 2009). Through efforts to innovatively recapitulate these signaling events in vitro using exogenous peptides, growth factors and novel extracellular matrices, stem cell biologists have successfully established renewable cultures of primary epithelial stem cells that proliferate, differentiate and function just like the intestinal epithelium in vivo (Sato et al, 2009, Gracz et al, 2010, Formeister et al, 2009, Ishizuya-Oka et al, 2008). Our study aim was to apply this innovative cultivation strategy to promote the in vitro culture of primary feline intestinal epithelial cells (FIEC) into a model intestinal epithelium.

Here we report on our preliminary successes, which include the isolation of feline intestinal crypts, which contain the stem cells of the intestine, and the in vitro growth of multicellular structures, as well as epithelial cell sheets, that architecturally recapitulate the

native feline large intestinal epithelium. The long-term goals of these studies are to generate a long-lived primary epithelial cell culture model of feline intestinal epithelium that can be applied by the research community to the study of infectious and inflammatory bowel diseases.

Materials and Methods

Crypt isolation and feline intestinal epithelial cell culture

Preparation and culture of individual cells from whole mucosa was performed as previously described with some modifications (Gracz et al, 2010, Sato et al, 2009). Large intestinal mucosa was obtained from cats within 10 minutes of euthanasia at a local animal shelter. The mucosa was rinsed thoroughly in oxygenated Hank's Balanced Salt Solution (HBSS) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml) and gentamycin (5 μg/ml). 3 cm tissue sections were incubated in HBSS containing 1mM each of ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich) and ethyleneglycotetraacetic acid (EGTA, Sigma Aldrich) for 60-75 minutes at room temperature to facilitate exfoliation of crypts. Crypts were pelleted at 9000 x g for 5 min at 4°C. The pellet was resuspended in Matrigel at a ratio of 250 crypts/ 75 μL Matrigel (BD Biosciences, San Jose, CA). The cell suspension was vortexed briefly and 75 μL droplets were added per well to 24-well polystyrene plates. The Matrigel was allowed to polymerize at 37°C for 10 minutes. Following polymerization, a base medium containing advanced Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12) supplemented with 5 μg/ml each of insulin, transferrin, and selenium, EGF (5 ng/ml), penicillin (125 U/ml), streptomycin (125 μg/ml), 5% fetal bovine serum, N2 and B27 minus vitamin A supplements (Life

Technologies, Carlsbad, CA), and growth factors including R-spondin-1 (1 μg/ml, R&D, Minneapolis, MN), Wnt3a (100 ng/ml, R&D), EGF (50 ng/ml, Life Technologies) Y27632 (10 μM, Sigma-Aldrich, St. Louis, MO), and Noggin (100 ng/ml, Peprotech, Rocky Hill, NJ) was overlaid. Cultures were incubated at 37°C in 5% CO2. Media was changed approximately every four days. Growth factors were added to the media every 48 hours at the same concentrations with the exception of R-spondin, which was added at a concentration of 500 ng/ml following initial crypt seeding.

Microscopy and immunofluorescence

At varying time points after cultivation of FIEC, the epithelial cells and multicellular structures were examined by light microscopy using a Nikon inverted light phase contrast microscope to assess for cellular viability and morphology. At day 14 of culture, Matrigel containing multicellular structures and epithelial-like sheets was carefully removed by a tissue scraper and processed for histology through incubation in 2% paraformaldehyde at 4°C for 16-18hrs. Samples were washed in phosphate buffered solution (PBS), embedded in glycolmethacrylate (GMA), and sectioned. 2 μM sections were placed on microscope slides and stained with H&E and Alcian Blue. To determine how closely feline intestinal epithelial cell (FIEC) cultures recapitulated the phenotype of the native feline intestinal epithelium, specimens of intact intestinal mucosa obtained from the same cat from which FIEC cultures were derived were processed for histology and immunofluorescence. Intestinal tissue (proximal duodenum, ileum, cecum, and proximal and distal colon) was dissected, opened longitudinally, and flushed with HBSS. The tissue was then incubated in 4% paraformaldehyde for 14-18°C at 4°C followed by a 24 hr incubation in a 30% sucrose

solution at 4°C. Tissues were embedded in optimal cutting temperature (OCT) medium, frozen in liquid nitrogen, and cryosectioned. 5 μM sections were placed on positively charged microscope slides and stored at -80°C for subsequent histology and immunofluorescence. Immunofluorescence labeling was performed using standard approaches. Briefly, frozen sections were rinsed with PBS, permeabilized with 0.3% Triton X-100 in PBS for 20 minutes at RT, rinsed with PBS, and incubated in blocking buffer consisting of 2-5% non-immune serum from the same species of origin as the secondary antibody for 1 hr at RT. Primary antibodies (goat anti-muc2, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, sc-15334; rabbit anti-sox9, EMD Millipore, Billerica, MA, AB5535; goat anti-carbonic anhydrase II (M-14), Santa Cruz Biotechnology, sc-17244; rabbit anti-lysozyme, Diagnostic Biosystems, Pleasanton, CA, RP 028; rabbit anti-chromagranin A, ImmunoStar, Inc., Hudson, WI, 20086; mouse anti-β-catenin, Cell Signaling Technology, Danvers, MA, L54E2; goat anti-villin, Santa Cruz Biotechnology, Inc., sc-7672; and isotype control antibodies, Life Technologies) were used at 1:100 (villin, muc2, lysozyme), 1:200 (carbonic anhydrase II, β-catenin), 1:500 (sox9) and 1:1000 (chromagranin A) dilutions for 2 hr at RT. All secondary antibodies (donkey anti-goat Cy3, 705-166-147; goat anti-mouse Cy2, 115-225-003; goat anti-mouse Cy3, 115-166-003; goat anti-rabbit Cy3, 711-165-152; all from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used at a 1:750 dilution for 30 min at RT. Following exposure to primary and secondary antibodies, slides were washed four times in PBS for 5 minutes each. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratores, Inc., Burlingame, CA). All slides were examined with a fluorescence microscope. Frozen sections of mouse intestine prepared

identically to feline tissue were run in parallel as positive controls to determine the ability of antibody to recognize feline tissue.

Results

Single isolated feline intestinal crypts form enteroids and epithelial-like cells

When feline colonic crypts were seeded at 250 crypts/well, they began to form multicellular structures that resembled previously described enteroids by day 3-7 of culture (Figures 1A, 2A) (Sato et al, 2009, Gracz et al, 2010). Light microscopy of many of these structures revealed a wide multicellular crypt-like base, lining a central lumen (Figure 1B), that grew to the surface of the Matrigel. These structures were observed to grow in size with increasing duration of culture and were dependent on the continued presence of serum. Sheets of cells with a cobblestone, epithelial-like morphology could be also observed within the Matrigel matrix (Figures 1C).

Enteroids resemble the native feline intestine

H&E staining of primary FIEC cultures demonstrated multicellular organization of enteroids and sheets of epithelial-like cells (Figures 2-4). Alcian blue staining revealed the presence of goblet cells within FIEC cultures similar to that observed in vivo (Figure 3). These findings suggest that primary FIEC phenotypically resemble the native feline colonic epithelium. Further studies are warranted to determine how closely primary FIEC cultures architecturally and phenotypically recapitulate feline intestine in vivo.

Tissue samples from murine intestine were used as positive controls when testing the ability of antibodies to recognize feline intestinal tissue (Figure 5). We found that all immunofluorescence antibodies recognized and labeled murine intestine at expected

locations along the intestinal epithelium. In contrast, only muc2, chromagranin A and β-catenin recognized feline tissue (Figure 6). Muc2 immunolabeling demonstrated good expression within epithelial cells in all segments of the feline intestine while Chromagranin A labeling could only be observed in the duodenum and colon. β-catenin labeling was detected in all segments of the feline intestine with highest expression at presumed intercellular adherens junctions. Further manipulations of our methods or adoption of other labeling techniques will be necessary to characterize the feline intestine such that they can be used for comparative analysis of FIECs.

Discussion

There is a critical and compelling need for veterinary scientists to define cellular mechanisms of disease pathogenesis using approaches that do not require the use of live animals. Creation of a feline intestinal epithelial cell culture model would inarguably be a major advance for feline gastroenterology research and would provide an outstanding alternative to live animal use. This model could be applied to studies of feline gastrointestinal pathobiology, pharmacology, toxicology, nutrition, cancer, immunology, inflammation, and infectious disease. We have successfully isolated and maintained feline intestinal crypts, the stem cells of the intestine, and have fostered the in vitro growth of multicellular structures (enteroids) as well as epithelial cell sheets that resemble the native feline large intestinal epithelium. Our short-term goals are to continue the characterization of in vitro FIEC cultures to see how closely their architecture and function recapitulates the feline intestinal epithelium in vivo. Further, we will continue to refine methods by which primary FIEC cultures can be established by individual investigators or alternatively passed, cryo-preserved, and shared

among the research community. Finally, we will optimize our methods to better characterize the feline intestine in vivo. The long-term goals of these studies are to generate a long-lived primary epithelial cell culture model of feline small and large intestinal epithelium that can be applied by the research community to the study of feline gastrointestinal disease pathogenesis and treatment. An immediate vision by our laboratory is to study the susceptibility and response of FIEC cultures to pathogens of human and feline importance.

Figures

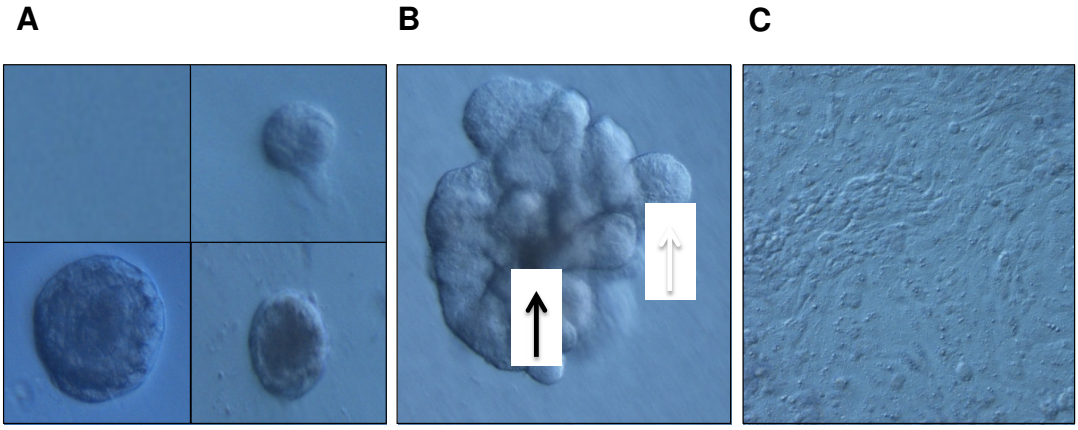

Figure 1. Feline crypts form enteroids and epithelial sheets. (A) Single crypts form enteroids by day 3-7 of culture. (B) Enteroids consist of crypt domains (white arrow) surrounding a central lumen (black arrow) lined by surface-like epithelium. (C) Single crypts grow into sheets of intimately associated cells with polygonal, cobble stone morphology consistent with intestinal epithelial cells.

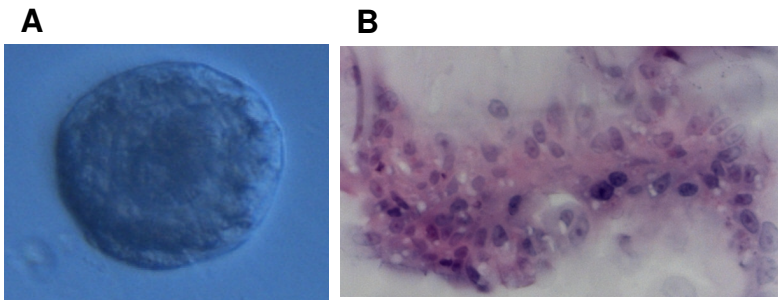

Figure 2. In vitro growth of feline enteroids. (A) Feline colonic enteroid (40x magnification). (B) H&E staining demonstrates multicellular organization of enteroids (40x magnification).

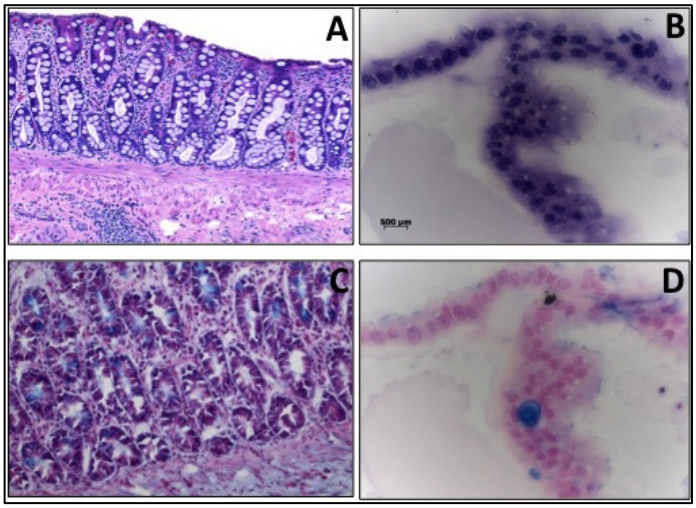

Figure 3. Light microscopy evaluation of epithelial-like cells. H&E staining demonstrates feline colonic epithelium (A) and organization of primary FIEC into crypt and surface domains (B) in vitro that resembles native tissue. (C) Alcian Blue staining of feline colonic epithelium and primary FIEC (D) (goblet cells observed in blue). 20x magnification.

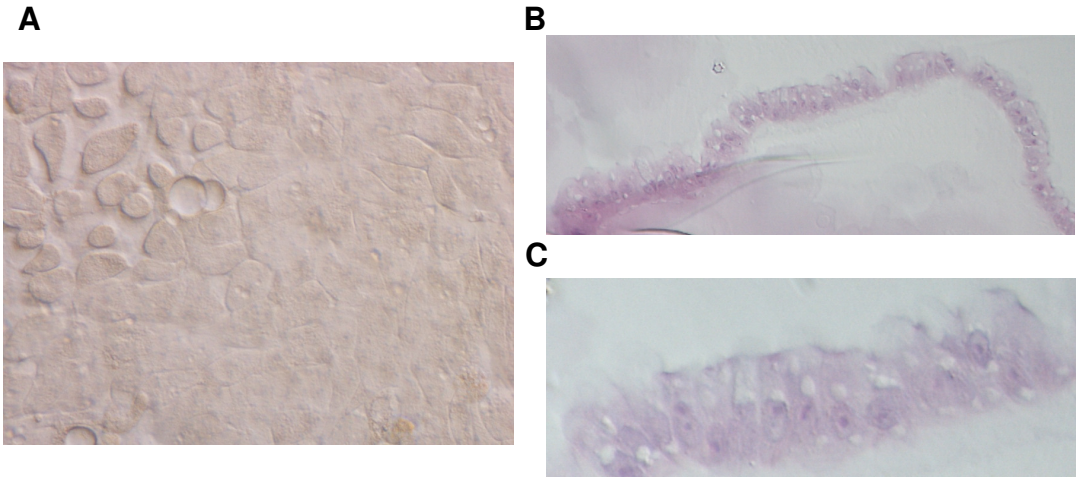

Figure 4. Epithelial-like cells resemble in vivo architecture. (A-B) Light microscopy and H&E staining (B-C) of primary FIEC demonstrates epithelial-like morphology. (20x magnification, A-B; 40x magnification, C).

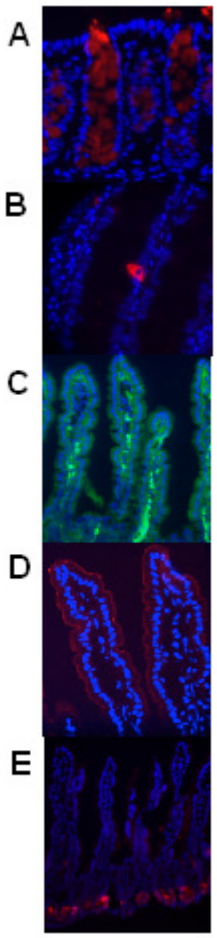

Figure 5. Immunofluorescence of murine intestine.

Expression of (A) Muc2, cecum, 20x magnification, (B) Chromagranin A, duodenum, 20x magnification, (C) β-catenin, jejunum, 20x magnification, (D) Villin, jejunum, 20x magnification, (E) Lysozyme, duodenum, 10x magnification.

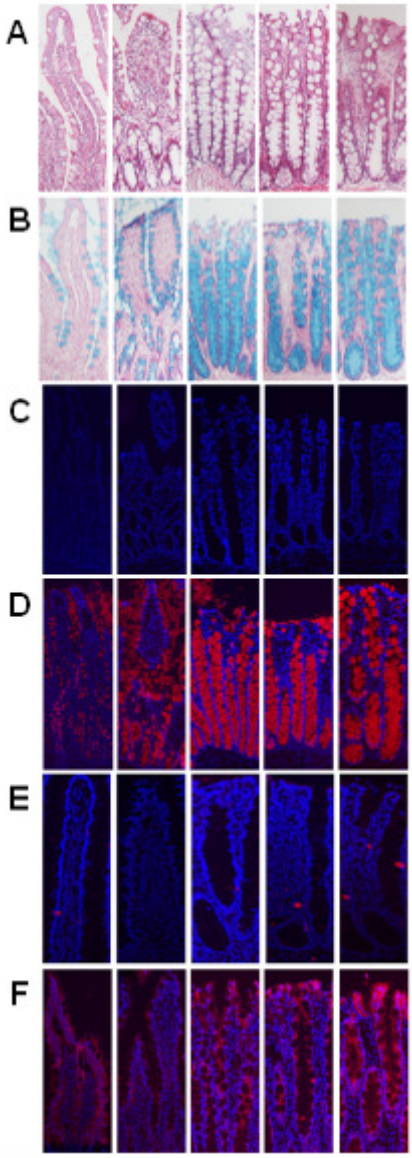

Figure 6. Immunofluoresence of feline intestine. Histology (A-B) and immunofluorescence (C-F) characterization of feline intestine (arranged from left to right: duodenum, ileum, cecum, proximal colon, distal colon). (A) H&E staining (B) Alcian blue staining (C) Expression of isotype control, (D) Muc2, (E) Chromagranin A, (F) β-catenin. 10x magnification.

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