UNIVERSITY OF CALGARY

Biology and Treatment of Giardiasis in Dairy Calves

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL

FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF GASTROINTESTINAL SCIENCES

CALGARY, ALBERTA

DECEMBER, 1999

ABSTRACT

Recent research suggests *Giardia duodenalis* is associated with diarrhea in calves and may have an impact on animal performance. In addition, infected dairy calves may serve as an important reservoir for human outbreaks of giardiasis. The present studies were conducted in order to understand the biology of giardiasis in dairy calves, as well as the efficacy and potential benefits of treating infected calves with fenbendazole.

Within a commercial dairy operation, *Giardia* infections occurred naturally in 100% of calves. *Giardia* cysts first appeared in the feces of calves at approximately 30 days of age, and most calves shed *Giardia* cysts for over 100 days. The infection patterns and chronic nature of giardiasis may be related to the calves' immune response. Dam's colostrum reacted with many *Giardia* antigens and exhibited anti-*Giardia* activity *in vitro*; suggesting passive immunity may initially protect calves from *Giardia*. However, calves did not mount an increased serum immune response following *Giardia* infections, and serum antibodies failed to react with many *Giardia* antigens by the time calves were 30 days of age. *Giardia* infections alone, or in combination with other pathogens, were associated with many episodes of diarrhea in these calves. Furthermore, calves from different geographical locations were found to be infected with both a genetically distinct *Giardia* isolate, as well as an isolate known to infect humans.

Fenbendazole was highly effective in treating giardiasis in calves. Fenbendazole treatment eliminated *Giardia* cysts from the feces of infected calves at lower doses than previously reported. Also, fenbendazole treatment completely eliminated *Giardia* trophozoites from the intestines of infected calves, resulting in a physiological and clinical improvement. However, a high rate of reinfection was observed in calves following treatment. Thus, fenbendazole treatment did not result in improved animal performance.

The results of this study demonstrate that *Giardia* is an important parasite of dairy calves. *Giardia* is a chronic infection in calves, and the parasite was identified in association with diarrhea. Furthermore, calves shed *Giardia* cysts potentially infectious to humans. However, giardiasis in calves can be effectively controlled with fenbendazole, and treatment results in a clinical benefit.

ACKNOWLEDGEMENTS

First, I wish to express my sincere gratitude to my supervisor, Dr. Merle Olson, for his guidance, encouragement, and friendship during this study. I could not have had a better mentor.

I would like to thank the other members of my committee, Dr. Tim McAllister, Dr. Murray Jelinski, Dr. Andre Buret, and Dr. Ron Read, for their advice and assistance throughout the past four years. I would also like to thank Dr. Howard Ceri and Dr. Mike Belosevic for agreeing to act as members of my thesis committee.

A special thanks goes out to Carol Cockwill for all of her hard work and technical support during this project. In addition, I wish to thank all of the other employees at Agriculture and Agri-food Canada for their assistance and conscientious care of the animals.

I would like to acknowledge the technical support of Nicole Guselle, Jeanette Goh, Kathy Oke, Sandy Widder, Colleen Annett, and Carol Stremick, and would especially like to thank Liz Middlemiss for her expertise and assistance with histology and electron microscopy. In addition, I wish to thank Tiz Reiter for all of her assistance.

I am grateful to Dr. Andrew Thompson and Dr. Dave Fraser at Murdoch University in Perth, Western Australia, as well as Peter Adams and Amber Appelbee for their assistance with genetic sequencing.

I wish to express my gratitude to all of my lab mates and friends at the University of Calgary and Murdoch University. You made graduate studies very enjoyable.

Finally, I wish to acknowledge the following sources of support which made this research possible.

The Natural Sciences and Engineering Research Council of Canada

Hoechst Roussel Vet

Agriculture and Agri-food Canada

For my parents, Dan and Rosemary O'Handley

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ETEC	=	Enterotoxigenic *E. coli*
PCR	=	Polymerase chain reaction
IEL	=	Intraepithelial lymphocyte
IFN-γ	=	Interferon gamma
BZ	=	Benzimidazole
FBZ	=	Fenbendazole
FBZ-SO	=	Oxfendazole
GI	=	Gastrointestinal
Ig	=	immunoglobulin
TEM	=	Transmission electron micrograph
PBSS	=	Phosphate buffered saline solution
DNA	=	Deoxyribonucleic acid
SEM	=	Standard error of mean

1. INTRODUCTION

Diarrhea resulting from infectious disease is the most important cause of mortality and morbidity in calves [1, 2]. In many parts of the world, including North America, cattle are raised for a profit, and diseases that cause diarrhea in calves are an extremely important variable from an animal health and economic perspective. These diseases have an impact on cattle operations through animal mortality, poor herd health, and reduced performance, thus affecting the profitability of the cattle industry through decreased production [3]. Furthermore, these diseases can have an additional impact on profitability through the costs to control disease in order to increase performance [4]. Adding to the direct costs of diarrheal diseases is the recent awareness of many emerging zoonotic diseases. It is now known that some of the organisms that cause diarrhea in calves can infect humans. As a result, good management practices and control strategies in the cattle industry must be followed. However, in order to accomplish this it is important to study these diseases using a holistic approach; examining their impact on animal health, economics, and the environment.

1.1 DIARRHEAL DISEASE AND THE CALF

1.1.1 The Neonatal Immune System

Although born with an anatomically and physiologically normal immune system, calves are susceptible to many disease causing organisms because their immune system is naive and unprimed [2]. The immune system of the neonatal calf is capable of responding to antigens, however the response is slower and weaker than in the adult animal, resulting in a moderation of disease instead of prevention [2]. Factors associated with birth, such as the release of corticosteroids and stress also inhibit the immune system

of the neonatal calf. Functionally, the immune system of cattle takes time to develop, and maturity is achieved at 5 to 6 months of age [2]. During this time, calves are exposed to numerous infectious organisms, and good management practices and control strategies are necessary to ensure calves are healthy and productive.

Calves receive no passive immunity *in utero* due to an epitheliochorial type of placentation, which will not permit the transplacental transfer of antibodies. Therefore, calves must rely on colostrum for passive immunity. Colostrum is important in the newborn calf as it provides localized bactericidal and phagocytic activity in the digestive tract, transfers cell mediated immunity, and provides passive transfer of immunoglobulins [2]. Colostrum is composed of many different concentrated nutrients and immune cells such as macrophages, neutrophils, B cells, and T cells [5-8]. Colostrum also contains concentrated levels of antibodies. Bovine colostrum contains 100-700 mg of IgA per 100 ml and 3400-8000 mg of IgG per 100 ml [2]. Thus, IgG is the primary antibody in colostrum, specifically IgG_1 in bovine colostrum [1]. Immediately following birth, the digestive tract of calves allows for the absorption of colostrum through pinocytosis. However, once the digestive tract becomes stimulated the epithelial cells quickly mature preventing colostrum absorption. As a result, when calves are 6 hours old the absorptive capacity for colostrum is only 50%, and by the time calves are 24 hours old they are no longer capable of colostrum absorption [2]. Thus, calves must receive colostrum very quickly following birth to ensure successful and adequate transfer of passive immunity.

Failure to receive adequate passive immunity can be extremely detrimental to calves. Previous studies demonstrate that failure to acquire adequate transfer of passive immunity puts calves at greater risk of death and illness [1, 9, 10]. A 1994 study found that 92% of calves with diarrhea had complete or partial failure of transfer of passive immunity, and each calf that died during this study had complete failure of transfer of

passive immunity [1]. In addition, optimal transfer of colostrum has been found to maximize growth in calves and minimize the effects of disease [10].

Despite the protective effects of colostrum and the benefits of successful transfer of passive immunity, diarrhea is still very prevalent in calves, especially during the first 2 months of age [1, 10-12]. Management practices, such as nutrition and the type of housing, can have a significant influence on the incidence and severity of disease [10]. During their first month of life, calves are exposed to many infectious agents for the first time, which alone or in combination with other infectious agents can cause diarrhea. The pathogen or pathogens encountered, in addition to environmental, nutritional, and management practices, all influence the duration, severity, and outcome of the disease [12]. It is therefore important to understand the infection patterns of these pathogens, how they interact with other pathogens, and effective ways to control and prevent the spread of these pathogens in order to minimize the impact they may have on calves.

1.1.2. Bacterial Pathogens

Although exposed to a number of different bacterial pathogens, *Escherichia coli* and *Salmonella* are two of the most important causes of enteric disease in calves. Both are gram-negative, facultative anaerobes, which are classified into hundreds of serotypes [13, 14]. *E. coli* is further categorized into pathotypes based on virulence factors. In calves, the most common strain of *E. coli* that causes diarrhea is enterotoxigenic *E. coli* (ETEC) [13]. These bacteria attach to the mucosa of the small intestine using fimbral adhesive antigens and release a heat stable enterotoxin, which induces hypersecretion of electrolytes, resulting in diarrhea [15]. About 90% of ETEC in calves produce a heat stable enterotoxin and express the K99 fimbral adhesion antigen [16]. Fimbral adhesins K88, 987P, F41, F17, and F18 are not as common in ETEC isolated from calves, but are

also considered important [13, 15, 17]. The diarrhea associated with ETEC can vary in its severity, but severe watery diarrhea can result in rapid dehydration and death within 7-12 hours of onset [13]. Infected calves are usually less than 5 days of age, as binding sites for the K99 fimbrae are rapidly lost after birth [16]. Previous studies have reported ETEC in association with 41% of diarrhea cases in the United States and 31% of diarrhea cases in Canada [17]. However, reported prevalences are quite variable with ETEC as in some cattle operations it is associated with as few as 0.5% of diarrhea episodes [17].

Salmonella typhimurium is the most common species of *Salmonella* to infect calves. Generally, calves over 2 weeks of age are most likely to become infected by *Salmonella* [14]. Infection can result in an intractable diarrhea leading to metabolic acidosis and dehydration. The pathogenesis of the disease is not completely understood [16]. The importance of cytotoxins and enterotoxins, present in *Salmonella,* for the development of diarrhea is unknown [14], but *Salmonella* must invade the intestinal epithelium to cause diarrhea. It is thought that the magnitude of the invasion and associated inflammation are major factors in the development of diarrhea in calves.

1.1.3. Viral Pathogens

Rotavirus and coronavirus are two of the most commonly identified infective agents in calves with diarrhea [1, 12]. Rotavirus is often reported as the most prevalent infective agent identified in calves [18, 19], and both rotavirus and coronavirus are often identified concurrently with other pathogens [1, 12, 18, 19]. Rotavirus can infect calves as young as 12 hours of age, but is commonly identified in calves from 3 -21 days of age [1, 12]. At onset, diarrhea may be voluminous and pudding-like, becoming more watery as the disease progresses. Weight loss, acidosis, and dehydration can develop as a result

of the infection. Coronavirus also typically infects calves less than 3 weeks of age [1, 12]. Calves are severely depressed at the onset of coronavirus infection, with diarrhea lasting a period of 4 to 5 days. Dehydration can be severe during coronavirus infection, resulting in high morbidity and moderate mortality [16].

1.1.4. Parasitic Protozoa

Coccidiosis

Viral and bacterial pathogens are common causes of diarrhea in calves, but protozoan parasites are also important enteric infections. Coccidiosis is considered to be the fifth most important economic disease in cattle in the United States [20]. The infective agents responsible for coccidiosis in calves belong primarily to the genus *Eimeria*. These parasites, belonging to the Phylum Apicomplexa, commonly infect calves and are often associated with animals raised in crowded conditions [20]. Thirteen species of *Eimeria* are known to infect cattle [20], but *Eimeria bovis* and *Eimeria zuernii* are the most pathogenic. Calves are infected by ingesting fecal-associated sporulated oocysts, which often contaminate water, feed, and pastures [21]. Initially, the infective stages of *Eimeria bovis* invade the intestinal epithelium of the distal ileum, but later stages infect glandular enterocytes of the large intestine [20]. The prevalence of the parasite can be high in calves, but clinical disease does not always occur [22]. Severity of disease is dependent on the number of infective oocysts ingested [20], and clinical coccidiosis is easily recognized as it results in bloody diarrhea. During severe infections, rectal prolapse may also occur, and calves may produce an explosive stream of diarrhea that can jettison 3 to 4 feet [20].

Cryptosporidiosis

Although a coccidian parasite, *Cryptosporidium parvum* is usually discussed separately from the other coccidia that infect calves due to its prevalence and importance. The parasite has been identified in calves world wide, with infection rates of up to 100% [12, 23-26]. Infections can occur in calves as young as 4 days of age [26], however the parasite is most commonly identified in calves between 8 and 21 days of age [12, 25, 27, 28]. Clinically, *Cryptosporidium parvum* infections result in diarrhea, accompanied by lethargy, inappetence, and dehydration [26]. Mortality is not common, but severe outbreaks of *Cryptosporidium* resulting in high mortality have been reported [26]. In addition, *Cryptosporidium* is often identified concurrently with other infections, particularly rotavirus, in calves with diarrhea [1, 12].

Cryptosporidium parvum is an intracellular parasite with a complex multi-stage life cycle, characteristic of the Coccidia. Sporozoites, released following the ingestion of infective oocysts, invade the epithelium of the intestine by first attaching to the cells, then becoming enveloped by the microvilli (Figure 1B). After developing from the trophozoite to the meront stage through asexual multiplication, or schizogony, within the enterocyte, the infected cell bursts releasing numerous merozoites that invade other epithelial cells [26]. Within these new host cells, asexual reproduction is repeated. Following invasion of the intestinal epithelia, some merozoites differentiate into either microgamonts or macrogamonts. Microgamonts are multinucleate, with each nucleus incorporated into a microgamete [26], while macrogamonts are uninucleate. Following fertilization of the macrogamont by microgametes, oocysts develop. However, unlike other coccidia *Cryptosporidium* oocysts sporulate *in situ.* The oocysts contain 4 sporozoites, and once they enter the lumen of the gastrointestinal tract they are voided in the feces. At the peak of infection, calves can shed up to 10^7 oocysts per gram of feces.

The diarrhea associated with *Cryptosporidium* infections results from a number of different factors. Villous atrophy, villous fusion, shortened microvilli, and sloughing of enterocytes have been observed in calves with cryptosporidiosis [29, 30]. *Cryptosporidium* infections in calves are also associated with intestinal inflammation, and an increase in intraepithelial lymphocytes (IELs) has been observed during infection [30-32]. The intestinal lesions associated with the infection reduce absorption and impair uptake of Vitamin A and carbohydrates [26]. In addition, *Cryptosporidium* infections can result in impaired glucose stimulated Na^+ and H_2O absorption, and a net secretion of Cl^- has been observed during the height of infection [31].

Although *Cryptosporidium* is highly prevalent and a major cause of diarrhea in calves, infections are self-limiting, and calves are resistant to reinfection after recovery [33]. In immunocompetent hosts, infections usually persist for less than 10 days [27] as both humoral and cellular components of the immune system are involved in elimination of the parasite [33, 34]. Both CD4+ and CD8+ T cell subsets are upregulated in response to the parasite [32], and mucosal infiltration by neutrophils has also been observed [26, 32]. In addition to these cellular responses, serum and mucosal levels of IgG, IgM, and IgA increase following infection, and these antibody responses coincide with the decline in oocyst shedding and resolution of diarrhea [33].

Unlike the Coccidia of the genus *Eimeria*, which are largely host specific, *Cryptosporidium parvum* infects a wide range of host species, including humans [26]. As a result, infections in calves may pose a serious public health risk. Infected calves commonly shed from 10^5 to 10^7 oocysts per gram of feces at the height of infection [26]. These oocysts are extremely resistant to natural stresses and chemical disinfectants, including standard levels of chlorine used to disinfect drinking water [35]. Studies have

demonstrated that humans can become infected with *Cryptosporidium* through direct contact with infected calves [36]. Also, studies have identified *Cryptosporidium* oocysts to be common in surface water [37, 38], and many water-borne outbreaks of cryptosporidiosis have occurred [39-41]. In humans, *Cryptosporidium* infection results in self-limiting diarrhea in immunocompetent hosts. However the immunocompromised or immunosuppressed may develop chronic life-threatening disease [35]. In individuals suffering from AIDS, or another immunosuppresive condition, infection with *Cryptosporidium* can result in mortality, due in part to a lack of effective treatments for cryptosporidiosis [26].

Giardiasis

Another protozoan parasite, *Giardia duodenalis,* has recently emerged as a potentially important parasite of cattle. Many studies have identified *Giardia* in domestic livestock [23-25, 42-46] with a prevalence of up to 89% reported in calves [43]. However, the pathogenicity, infection patterns, and zoonotic potential of giardiasis in cattle are not completely understood. Calves with giardiasis may develop diarrhea or general signs of ill health, but many calves infected with *Giardia* develop no signs of intestinal disease [24, 25, 42, 43]. Therefore, it is not known if *Giardia*, on its own, is an important pathogen of cattle. Many infective agents cause diarrhea in calves, and because concurrent infections are common, it is often difficult to determine the cause of diarrhea in calves [12, 18, 19, 44]. Calves as young as 4 days of age have become infected with *Giardia,* but unlike many other common infections, giardiasis is also prevalent in older calves [23, 25, 43]. Due to its high prevalence, *Giardia* may be an important zoonotic disease. In fact, isolates from domestic livestock are morphologically, phenotypically, and genotypically identical to human isolates [46, 47]. However, recent work also suggests *Giardia* from livestock may be host specific [48]. Thus, much is to be learned

about the infection patterns, pathology, and zoonotic potential of giardiasis in cattle before its importance to the cattle industry can be determined.

1.2. *GIARDIA DUODENALIS*

The flagellate protozoan parasite *Giardia duodenalis* (= *Giardia lamblia* = *Giardia intestinalis*) is one of the most commonly identified gastrointestinal pathogens in North America [49, 50]. Discovery of this parasite is credited to Lambl, who in 1851 provided the first confirmed report of the parasite's existence [51]. However, it is believed that Leeuwenhoek may have been the first to observe the parasite in 1681, after examining his own diarrheic stool [50, 52]. It was once believed that *Giardia* was not pathogenic, as many people infected with the parasite are asymptomatic [50]. It was not until the 1970s, when people returning from the former Soviet Union had clinical giardiasis, that the parasite became recognized as a pathogen [50]. Today, this ubiquitous parasite is well known as a common disease of travelers, children in day-care centers, and as a frequently identified pathogen in water-borne outbreaks of gastrointestinal illness [50, 51].

1.2.1. Life Cycle

Giardia has a simple, direct life cycle in which the parasite alternates between the motile feeding trophozoite stage and the infective, environmentally resistant, cyst stage. Infection occurs when cysts are ingested by the host. Ingestion of as few as ten cysts can lead to infections in humans and animals [53]. Following ingestion, the cysts are activated for excystation by the acidity of the stomach [54]. The emergence of the trophozoites from the cysts is then triggered by the more alkaline pH and proteolytic

activity of the duodenum [54]. The trophozoites multiply by binary fission and colonize the entire small intestine. Trophozoites adhere to the intestinal epithelium using a specialized adhesive disk located on their ventral surface (Figure 1A). It is proposed that a surface membrane associated lectin, activated by trypsin, may mediate attachment of trophozoites to the enterocyte through phosphate residues [55]. As trophozoites are motile they may detach from the epithelial surface and move through the intestinal fluid [50, 56]. Within the intestine, trophozoites feed through pinocytosis [57], utilizing numerous digestive vacuoles located on their dorsal surface (Figure 1A). Trophozoites also take up bile salts through active and passive mechanisms, which may be important for completion of the parasite's life cycle [58, 59]. The life cycle of *Giardia* is concluded within the lumen of the ileum and large intestine where trophozoites encyst and the cysts are voided in the feces.

1.2.2. Morphology and Taxonomy

Giardia duodenalis trophozoites are binucleate, pear shaped organisms that possess four pairs of flagella. They range in size from 9-21 μm in length by 5-15 μm in width. Two prominent nuclei occupy most of the anterior portion of the trophozoite, and can be visualized under light microscopy. The nuclei contain equivalent amounts of DNA and both are transcriptionally active [54]. Two organelles, called median bodies, are found near the center of the trophozoite. Although their function is unknown, median bodies are unique to *Giardia* and are comprised mainly of microtubules [54]. The most prominent feature of the trophozoite is its ventral adhesive disk (Figure 1A). In cross-section, the ventral adhesive disk is a concave structure, tapering at the edges, and is composed of a single layer of evenly spaced microtubules [54]. The adhesive disk is composed largely of tubulin as well as proteins called giardins, which are also unique to the *Giardia* species [54]. The cysts are ovoid in shape and measure approximately 8-12

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μm in length and 6-8 μm in width. They are quadri-nucleate, as the trophozoite undergoes a single division upon encystation, and various internal structures such as the flagella and median bodies can be visualized using light microscopy [50].

The taxonomy of *Giardia* was confusing during the first half of this century as *Giardia* species names were based on host occurrence [57]. However, work by Filice [60] proposed the existence of 3 distinct species based on morphological characteristics. *Giardia agilis*, described only from amphibians, is composed of a long trophozoite body and long teardrop-shaped median bodies [57]. *Giardia muris*, infecting rodents, birds, and reptiles, contains two small rounded median bodies located near the center of the trophozoite. The third species described by Filice is *Giardia duodenalis*. This species infects a wide variety of mammalian hosts, including humans, and may also infect birds [61]. *Giardia duodenalis* trophozoites are characterized by "claw hammer" shaped median bodies lying transversely across the trophozoite [57].

During the past decade, molecular studies have demonstrated that *Giardia duodenalis* is a genetically diverse species. Using a range of genetic criteria, *Giardia duodenalis* isolates from humans and animals have been found to fall within one of two major genetic assemblages. These two assemblages have previously been described as the "Polish" and "Belgian" [62] groups, as well as Assemblages A and B [63]. This genetic evidence suggests that *Giardia duodenalis* may actually be comprised of two separate species, however more biological and epidemiological studies are required in order to demonstrate that these genetic groups are, in fact, distinct.

1.2.3. Transmission

Successful transmission of *Giardia* requires that the infective cysts be ingested. To help ensure their transmission, *Giardia* cysts are resistant to the external environment

and remain infective for months in cold water or in cool, damp surroundings [50, 51]. Cysts are commonly ingested through a direct fecal-oral route, however contaminated water and contaminated food are also possible vehicles for parasite transmission. High population densities, poor hygiene, and feeding behaviors such as coprophagia can also promote transmission of *Giardia*.

Fecal-oral transmission is the most common mode of *Giardia* infection in both humans and animals [50, 52, 64]. In humans, children are most often infected via this route of transmission, and a high prevalence of giardiasis in day-care centers has been reported [52]. Animals kept in close confinement are also exposed to large numbers of infective cysts, thus increasing the chances of disease transmission.

Epidemic outbreaks of giardiasis in human populations are often associated with waterborne transmission [65-67]. Processed drinking water, raw municipal water, and back country streams and lakes can all contain infective *Giardia* cysts [38, 68, 69]. In one study, over 80% of surface water locations examined in Canada and the United States contained *Giardia* cysts [37]. The number of *Giardia* cysts found in drinking water samples can exceed 1000 cysts per liter [68], while sewage samples have contained more than 88,000 cysts per liter [68]. Waterborne transmission is also aided by the fact *Giardia* cysts can survive for months in waters which contain low concentrations of bacteria and organic contaminants, and cysts are resistant to standard chlorine concentrations used to purify drinking water [50]. Water mammals, such as beavers, have been implicated in many waterborne *Giardia* infections [37, 67]. However, waterborne transmission of *Giardia* is more likely the result of infected human and/or animal effluent [37, 38, 70].

Inappropriate handling of food and washing food with contaminated water has been implicated in food-borne outbreaks of giardiasis [50, 52]. In addition, carnivorous animals may be susceptible to food-borne infection when they ingest infected prey. It may also be possible that feces from animals, such as small rodents, may contaminate cereal grains, leading to potential human and animal infections.

1.2.4. Clinical signs

In the past, the pathogenicity of *Giardia* was called into question because, following infection with *Giardia*, many hosts experience only mild symptoms, and many hosts experience no symptoms at all. It is likely that these asymptomatic infections comprise the majority of *Giardia* cases [64]. In symptomatic cases, the clinical signs of giardiasis can include severe diarrhea, steatorrhea, abdominal cramps, nausea, and weight loss [49, 50, 52, 71]. These symptoms may persist for a few weeks, in the case of acute giardiasis, or evolve into chronic reoccurring disease [64]. Studies also demonstrate an association between allergic disease and urticaria in horses, birds, and humans [52, 72], and fatal *Giardia* infections were previously reported in chinchillas and birds [61, 73]. The variation in clinical signs experienced by the host is likely the result of both host and parasite factors.

1.2.5. Pathophysiology and Pathogenesis

The pathophysiology of giardiasis has been described in a variety of animal host models. The infection results in a number of morphological and physiological changes to the small intestine which culminate in a malabsorptive diarrhea. Trophozoites do not invade the epithelium of the small intestine, but attach themselves to both the microvillous and basolateral membranes of the enterocyte [74, 75]. Trophozoites may

use a surface lectin to mediate attachment [55, 76] to the mucosa of the intestinal tract. Despite the non-invasive nature of the parasite, once *Giardia* trophozoites have colonized the small intestine of the host a wide range of morphological abnormalities can result. Under light microscopy, changes to the intestinal architecture differ extensively. In some studies, severe villous atrophy has been observed in association with giardiasis, but in other studies villous architecture appears normal [75]. For instance, in the gerbil, villous shortening was observed in the duodenum, along with crypt hyperplasia in the duodenum, jejunum, and ileum [74], but in the same study, the jejunum remained unchanged and there was an increase in villous height in the ileum [74]. Studies in the mouse have also demonstrated a decrease in villous height in the duodenum as well as the jejunum associated with giardiasis [77]. However, in other studies employing the mouse and gerbil model, and in most human cases of giardiasis, an increased crypt depth is observed with normal villous architecture or relatively mild villous shortening [75, 78].

Despite a large variation in intestinal architecture in response to *Giardia* at the light microscopic level, more consistent observations have been made using the electron microscope. A diffuse shortening of epithelial microvilli appears to be characteristic of giardiasis, even when villous architecture appears normal [75]. An early study demonstrated that the surface area measurements of the epithelial brush border from human biopsies were significantly smaller in patients infected with *Giardia* [79]. This reduction in the microvillous brush border was also observed in a number of animal models of giardiasis [74, 80-82]. Although trophozoites attaching to the epithelium can distort and disrupt the microvilli with their adhesive disk [80], changes to the microvilli are diffuse and also occur at sites where trophozoites are not attached [74, 82].

Corresponding with the diffuse shortening of the microvillous border is a reduction in disaccharidase activity in the microvillous membrane [75]. In the gerbil and

the mouse, reduced lactase, maltase, and sucrase activities are observed during *Giardia* infection [77, 82, 83]. In addition, glucose, water, sodium, and chloride absorption was found to decrease in association with reduced brush border surface area [74]. In the neonatal rat, giardiasis resulted in a decreased basal transport of electrolytes and water, however mucosal disaccharidase activity varied, and maltase activity increased with infection [84].

Aside from the morphological alterations of the intestine described above, giardiasis is associated with other factors which contribute to pathology. Accelerated gastric emptying and increased intestinal transit have been observed in the gerbil model of giardiasis [85]. Increased contractility of smooth muscle was also observed in this study, which may contribute to abdominal cramps associated with the disease [85]. Furthermore, there is evidence suggesting that giardiasis is associated with bacterial overgrowth, which could lead to alterations of the intestinal architecture, and bile salt deconjugation could reduce lipolysis [58, 75]. Collectively, these morphological and physiological alterations associated with giardiasis result in malabsorption, maldigestion, and hypermotility, the consequence being diarrhea observed in symptomatic cases of this disease.

The mechanisms responsible for the pathophysiological changes in hosts infected with *Giardia* are not fully understood. It is likely that both parasite and host components contribute to the pathophysiology of the disease and thus, the onset of clinical signs. There is some evidence to suggest that *Giardia* may produce toxins. The lysosomal vacuoles located along the dorsal surface of the trophozoite contain hydrolytic enzymes [86, 87]. Thiol proteinases, when secreted into the intestine by these vacuoles, may exhibit toxic effects to surface glycoproteins and damage the intestinal microvilli [75, 87]. The diffuse shortening of the microvilli in response to *Giardia* infection would

suggest secretion of such a toxin. A gene, encoding a sarafotoxin-like protein, has also been characterized [88], and sonicated trophozoites and trophozoite extracts have been shown to cause increased smooth muscle contractility and disaccharidase deficiency [85, 89]. Sonicated trophozoites are also cytotoxic to a number of different cell types [85]. The mannose-binding lectin used to mediate the attachment of *Giardia* trophozoites to enterocytes may also cause damage, as similar intestinal abnormalities have been observed in response to plant lectins [75].

1.2.6. Host response and Immunology

Although a strong immune response is necessary in eliminating *Giardia* infections [34], it may also play a role in the production of disease. Humoral immunity plays an important role in the elimination of *Giardia* trophozoites from the intestine of the host [34, 90]. Serum and mucosal anti-*Giardia* IgM, IgA, and IgG antibodies are elevated in experimentally and naturally infected hosts following infection [34, 90-92], and mucosal IgA antibodies appear to play a major role in control and elimination of *Giardia* from the intestine [93-96]. Antibody mediated killing of *Giardia* can occur in the presence of complement [97], however independent antibody cytotoxicity has also been demonstrated [98]. Serum antibodies may also play a role in elimination of *Giardia* infection as immune serum and an IgG_1 monoclonal antibody were found to reduce the number of trophozoites in the small intestine in *Giardia* infected mice [99]. Both IgG and IgA have been found to coat the surface of trophozoites within the intestine [34], suggesting that IgG must gain entry to the intestine during infection. It has been proposed that IgG may leak through the damaged epithelia during *Giardia* infection [100], but intestinal secretion of IgG has been demonstrated in both the diseased and normal gastrointestinal tract [101].

Although elimination of *Giardia* from the host is not mediated by components of the cellular immune system, it is dependent upon them in as much as depletion of CD4+ cells reduces IgA synthesis, thus prolonging the infection [94, 96]. During the acute phase of the infection, CD8+ T cells within the epithelium increase, but during the elimination phase CD4+ T cells become more numerous [90]. Mice with an impaired CD4+ response develop chronic giardiasis, while normal mice clear the infection within 4 to 5 weeks [34]. The secretion of the cytokine interferon-gamma (IFN-γ) by CD4+ cells appears to play an important role in elimination of *Giardia*. Blockade of IFN-γ using a monoclonal antibody was found to enhance the intensity of *Giardia* infection in B10 mice [96]. Furthermore, macrophages have been shown to phagocytose *Giardia*, and IFN-γ can activate macrophages for giardicidal activity [102, 103]. Thus, the cellular immune response may play an important role in eliminating *Giardia*.

The host response also appears to play a role in the pathogenesis of disease during giardiasis. The increase in the number of intraepithelial lymphocytes (IELs) observed during infection has been implicated in the pathophysiology of the disease [75]. There are however conflicting reports. In many studies IEL proliferation in response to *Giardia* infection does not occur [78, 84, 96], and when an increase in IELs was observed, they were found to be in a resting state, unlike activated IELs associated with celiac disease [104]. IFN-γ is associated with villous atrophy and crypt hyperplasia [32, 105]. Therefore, IFN-γ released by CD4+ cells may contribute to the pathology of the disease. Also, proteases and free oxygen radicals released by macrophages have been demonstrated to cause enterocyte damage in mice with symptomatic giardiasis [106]

The host response to giardiasis may also contribute to allergic disease. Increased levels of circulating IgE antibodies to common allergens have been observed in atopic children with giardiasis [72]. An increase in mucosal mast cells, mediators of IgE

hypersensitivity, have also been observed in gerbils experimentally infected with *Giardia*. Increased macromolecular uptake in association with giardiasis was also observed using the gerbil model [78], and this greater exposure to intestinally absorbed antigens may play a role in hypersensitivity associated with *Giardia* [72, 78]. In addition, increased mast cell proliferation is associated with villous atrophy, crypt hyperplasia, malabsorption, and diarrhea in gluten enteropathy and colitis [107, 108] and may be associated with these conditions in giardiasis.

1.2.7. Zoonosis

Not only is *Giardia duodenalis* a common infection of humans throughout the world, but the parasite infects a wide range of mammalian species. In the wild, *Giardia duodenalis* infections have been reported in a number of different animals including beavers, muskrats, bears, wolves, elk, and seals [38, 109, 110]. In domestic animals, cats, dogs, sheep, and horses are all hosts for *Giardia* [43, 45, 111, 112]. This vast assortment of host species has raised great concerns regarding the zoonotic potential of this parasite. However, the zoonotic potential of *Giardia* has been vigorously debated in the past [113, 114], and recent studies, many employing molecular techniques, provide evidence to support and refute the zoonotic potential of giardiasis.

Many researchers consider *Giardia duodenalis* to be zoonotic because isolates obtained from a variety of hosts species are morphologically identical to human isolates [42, 46, 57, 61]. In fact, a *Giardia* isolate obtained from a sulfur-crested cockatoo in Australia was morphologically characteristic of *Giardia duodenalis* implicating the zoonotic transmission between birds and humans [61]. Additional evidence indicating *Giardia* to be zoonotic is the fact the parasite causes disease in animals similar to the disease in humans. Diarrhea is the major clinical sign of giardiasis in dogs, cats, and

sheep [46, 111], and ovine giardiasis so closely resembles the disease in humans it was proposed to be used as a model of human infections [46]. Antigenically, sera from infected lambs were shown to recognize human *Giardia* isolates and human sera recognized *Giardia* isolated from sheep [46]. Furthermore, in a cross transmission study a human volunteer was experimentally infected with *Giardia* from an animal source [115].

Molecular techniques have also been used to provide evidence that *Giardia* is zoonotic. Genetic techniques such as restriction enzyme analysis and polymerase chain reaction (PCR) have demonstrated genetically identical *Giardia* isolates from both humans and animals [116-119]. During a waterborne outbreak of giardiasis in British Columbia, molecular tools were used to demonstrate *Giardia* isolated from a beaver, located upstream from the outbreak, was identical to isolates obtained from the water and infected humans [67]. Thus, molecular tools along with morphological evidence and cross transmission studies provide a great deal of evidence that giardiasis is a zoonosis.

Despite the above evidence, many researchers still refute the claim that *Giardia* is zoonotic [120], and other evidence has been brought forward suggesting *Giardia* may be species specific. Studies from Sweden, Japan, and the Czech Republic suggest transmission of *Giardia* between infected dogs and their owners does not occur [121-124]. This suggestion is supported by an Australian study in which a genetically distinct *Giardia* isolate was found to infect dogs, but not humans, in the same community [119]. Genetic techniques have also been used to demonstrate that livestock may be infected with a unique *Giardia* genotype that has yet to be identified in humans [48].

It is likely that the debate over the zoonotic potential of *Giardia* will continue, as genetic techniques such as PCR and restriction enzyme analysis are currently unable to

provide definite evidence that giardiasis is a zoonoses. What is evident, is that *Giardia* is genetically heterogeneous, and this variability occurs independently of host species or geographical location [47, 68, 116, 125, 126]. In fact, different *Giardia* isolates have been shown to occur in the same patient [127, 128]. Whether or not *Giardia* is a zoonotic parasite, species specific, or composed of isolates which are both will remain unresolved until more definite evidence can be provided.

1.2.8. *Giardia* and Cattle

Little is known regarding the importance of *Giardia duodenalis* to the cattle industry. As mentioned previously, studies indicate that this parasite is highly prevalent [23, 25, 43] and may be a cause of diarrhea in calves [44]. Researchers have found that *Giardia* infections in cattle are associated with some of the same morphological abnormalities observed in humans and laboratory animals, such as villous atrophy and increased IELs [30] . In a ruminant model of *Giardia*sis, infected lambs experienced decreased weight gain, impaired feed efficiency, and a reduction in carcass weight, suggesting *Giardia*sis may have an economic impact [129] . The high prevalence of the parasite in calves may pose a public health risk, but it is not yet known if bovine *Giardia* is zoonotic. Nevertheless, *Giardia*sis may be an important cause of diarrhea in calves, resulting in potential health and economic impacts. Treatment of the infection may be necessary in calves to prevent economic losses, improve animal health, and minimize the parasites zoonotic potential.

1.2.9. Treatment

Currently, there are a number of chemotherapeutic agents used to treat giardiasis in both humans and animals (Table 1). These agents work through a variety of

mechanisms against *Giardia* and are usually effective. Metronidazole is perhaps the most widely used anti-*Giardia* therapy in humans and animals. Metronidazole acts against *Giardia* by interfering with electron transport in the parasite [130]. This drug appears to arrest *Giardia* during mitotic activity at the G2+M phase of its life cycle [130, 131], and cytotoxic products resulting from the reduction of metronidazole may result in the loss of helical structure, strand breakage, and DNA impairment in the trophozoite [130]. Although widely used and effective, metronidazole has some side effects. Metronidazole has an unpleasant metallic taste, and is therefore difficult to give to animals [132]. The drug is also a mutagen and a carcinogen [132], and metronidazole therapy in dogs has resulted in moderate to severe intestinal and neurologic damage [133, 134]. Neurotoxicity has also been demonstrated with chronic exposure to metronidazole [133].

Quinacrine, an anti-malarial drug, has also been used to treat giardiasis in humans, dogs and cats. Quinacrine acts against the parasite by intercalating into its DNA. It forms a hydrogen bonded complex with *Giardia* DNA preventing DNA replication, transcription, and protein synthesis [130].

Nitrofurans, such as furazolidone have also been used to treat giardiasis. Furazolidone may act against *Giardia* trophozoites through lipid oxidation [130]. Enzyme activation, membrane damage, and DNA damage to *Giardia* trophozoites are associated with furazolidone treatment. The advantage to furazolidone is that it is available in suspension [130], however it is not as effective against *Giardia* as metronidazole or quinacrine. Furthermore, furazolidone is a carcinogen and can longer be used in domestic animals.

Several studies demonstrate that the benzimidazoles are effective in treating *Giardia* infections [135-138]. It is believed that the benzimidazoles are effective against *Giardia* because they inhibit tubulin [139]. Tubulin is major component of the *Giardia* cytoskeleton, and many of its organelles, including its adhesive disc, contain tubulin [54]. As a result, *Giardia* trophozoites exposed to benzimidazoles experience alterations in shape and the disappearance of their ventral adhesive disc [136], thus trophozoites are unable to adhere to cell surfaces. Benzimidazoles may also alter the bioenergetics of *Giardia* by acting as lipid-soluble protein ionophores [140]. Studies have demonstrated that the benzimidazoles are more effective as anti-*Giardia* agents than any previous treatments. Benzimidazoles have been found to be 30 to 50-fold more potent than metronidazole and 30 to 40-fold more effective than quinacrine *in vitro* [138]. Nocodazole, for example, is the most potent of the benzimidazoles; the concentration of nocodazole required to kill 50% of trophozoites *in vitro* (IC_{50}) being 0.07 μM. However nocodazole does not maintain its anti-*Giardia* activity beyond 48 h and is toxic to mammals [141]. Mebendazole, albendazole and fenbendazole are also very effective against *Giardia in vitro* and are currently used as anthelmintics. The IC_{50} for *Giardia* of these three compounds ranges from 0.19 μM for mebendazole, 0.25 μM for albendazole, and 0.30 μM for fenbendazole [141]. All three compounds maintain their anti-*Giardia* activities past 48 h and failure to induce resistance for both albendazole and fenbendazole has been demonstrated [141]. Oxfendazole and thiabendazole are only moderately effective against *Giardia in vitro*, with the IC_{50} for oxfendazole reported to be 4.20 μM [141].

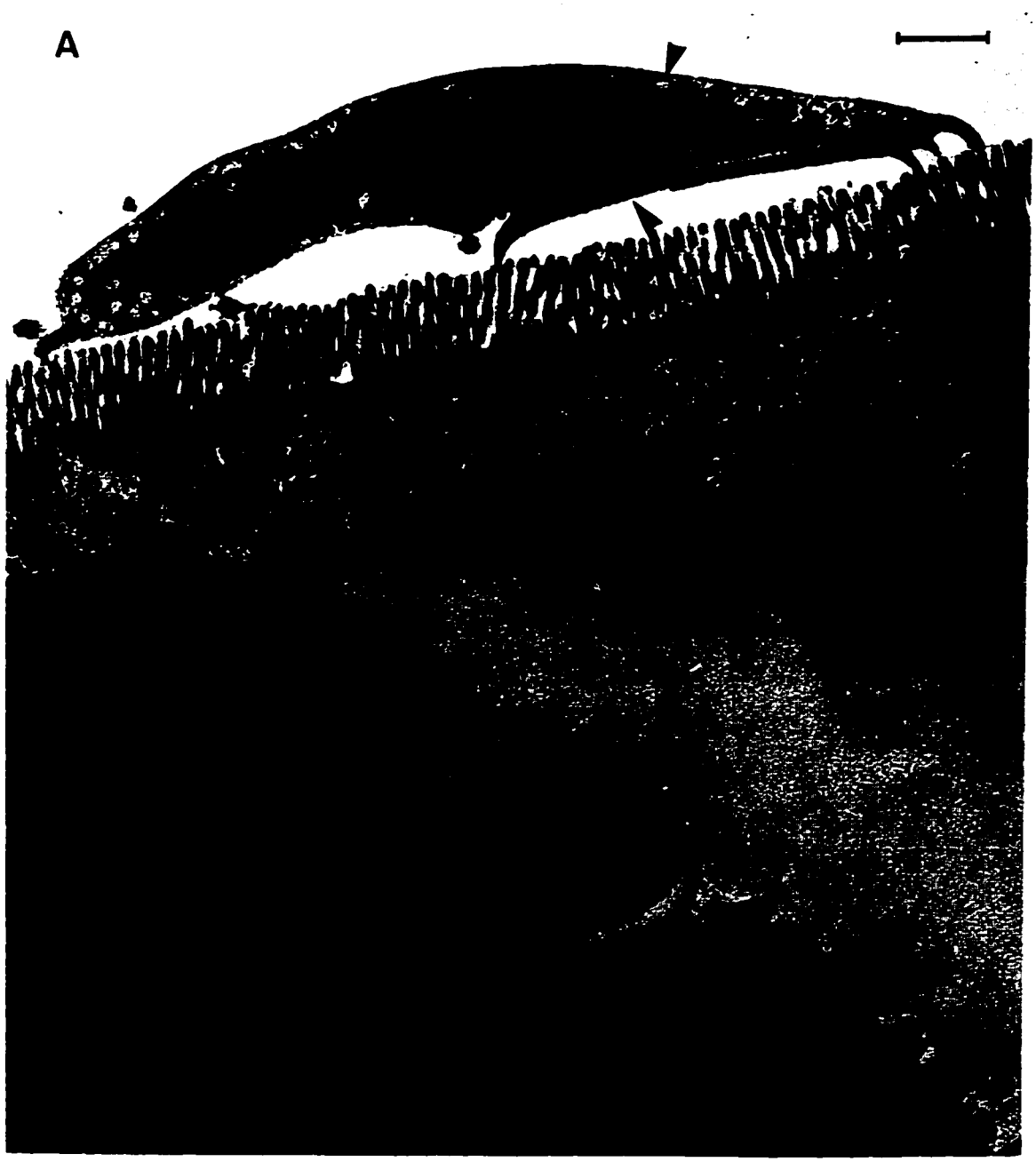
The benzimidazoles have also been studied for anti-*Giardia* activity *in vivo.* Albendazole is effective against giardiasis in humans when 200 mg is administered orally once daily for 5-10 days [52]. Albendazole is also effective against *Giardia* in dogs when administered orally at 25 mg/kg twice daily for 3-5 days, and fenbendazole administered

orally at 50 mg/kg once daily for 3-5 days was also found to clear *Giardia* infections from dogs [135]. Calves can also be treated for giardiasis using benzimidazoles. Both albendazole and fenbendazole were found to be effective at eliminating *Giardia* cysts from the feces of infected calves when administered at 20 mg/kg once daily for 5 days [137].

Although widely used as anthelmintic agents in humans and animals, some benzimidazoles exhibit side effects. Nocodazole is a potent inhibitor of mammalian tubulin, thus it is potentially toxic [142]. The acute toxicity of albendazole, mebendazole, and oxfendazole is low, however they can produce embryo toxicity and teratogenesis in laboratory animals, dogs, and cattle, and should not be used in pregnant animals or humans [135, 141, 143]. These side effects have yet to be demonstrated with fenbendazole, and it may be the drug of choice for treating giardiasis in calves.

Figure 1.

Transmission electron micrographs of 2 common intestinal protozoan parasites in calves. (A) *Giardia duodenalis* trophozoite in the jejunum of an infected calf. Note the prominent ventral adhesive disk (bottom arrow), as well as the numerous digestive vacuoles located on its dorsal surface (top arrow) Bar = 1 μm. (B) A coccidian parasite (*Cryptosporidium parvum* or *Eimeria* spp) within the jejunal brush border of an infected calf. Bar = 1 μm.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1.3. FENBENDAZOLE

1.3.1. History and Chemistry

First described in 1974 [144], fenbendazole has been widely used to treat cattle for helminth infections, especially gastrointestinal nematodes. Fenbendazole is a broad spectrum anthelmintic, effective against a wide range of helminth species and is extremely safe for use in cattle. Fenbendazole (FBZ, methyl 5-[phenylthio]-benzimidazole-2-carbamate) is a colorless powder, insoluble in water, with a melting point at 233^0 C. It belongs to a class of drugs known as the benzimidazole carbamates. All benzimidazoles (BZs) are composed of the same bicyclic ring system in which imidazole is fused to benzene at its 4- and 5- position [145]. Modification of this basic structure at the 2- and 5- positions has resulted in the discovery of many broad spectrum anthelmintics. The first of the BZ anthelmintics discovered was thiabendazole (TBZ) (2-(4'-thiazolyl)benzimidazole), in 1961 [145]. The researchers discovered that condensing thiazole-4-carboximide in the presence of *0*-phenylenediamine (initial component of benzimidazole construction), created a very active drug [146]. However, it was later discovered that hydroxylation of thiabendazole at the 5- position rendered the drug inactive [145]. The resulting research to overcome this problem led to further modifications of the BZ molecule and the discovery of many other BZ anthelmintics. Among these were the benzimidazole carbamates which, in addition to alterations at the 5-position, were modified by the addition of $NHCO_2CH_3$ to the 2-position [145]. Benzimidazole carbamates were found to be very active anthelmintics, but with further modification at the 5-position by the addition of a phenoxy group, the efficacy of the compound was improved even more [144]. The result of this modification was fenbendazole.

1.3.2. Pharmacokinetics

Fenbendazole is one of the most efficacious of the BZs. A 5 mg/kg dose of FBZ is equivalent to a thiabendazole dose of 100 mg/kg [147]. It can also be administered in a number of different ways, including a 10% suspension, 0.5% pellets, and a 20% premix without reducing its efficacy [148]. A single oral dose of 5 mg/ kg is between 99 and 100% effective against most gastrointestinal nematodes [148-153]. Low continuous doses administered in water, medicated feed blocks, and slow release boluses are also effective against parasites [154-156].

Compared to other BZ anthelmintics such as thiabendazole, fenbendazole is more slowly absorbed following oral administration [147] and this may contribute to the drug's efficacy. High concentrations of the drug remain in the gastrointestinal tract for a longer period of time as compared to other benzimidazoles. Half the maximal concentration of fenbendazole can be detected in the rumen of cattle 4 hours following administration, while half maximal concentrations for thiabendazole are reached 1 hour after administration [147]. In the GI tract as a whole, fenbendazole concentrations reach half maximal levels over 30 hours following administration [147]. Therefore, parasites in the GI tract are exposed to high concentrations of the drug for long periods of time. Consequently, less fenbendazole needs to be administered to cattle for the drug to be effective.

Although parasites are exposed to high concentrations of luminal FBZ, absorbed FBZ is also important in maintaining the efficacy of the drug. Maximal plasma concentrations of FBZ are observed from 24-29 hours after administration [147, 157, 158]. Plasma concentrations remain at this level for a long period of time, reaching half

maximal concentrations 30 hours after administration. This absorbed FBZ is metabolized by the liver through sulfur oxidation or p-hydroxylation [159], and the major metabolite is oxfendazole (FBZ-SO). FBZ-SO also possesses potent anthelmintic activity [157, 158, 160, 161] and is secreted back into the lumen of the GI tract via the circulation or the bile ducts [161]. The sulfur oxidation of FBZ is reversible, thus when FBZ-SO returns to the GI tract it can be reduced back to FBZ [157]. The constant recycling of FBZ and FBZ-SO between the circulation and the lumen of the gut increases the exposure time of both anthelmintics to parasites. The secretion of FBZ-SO into the lumen via the circulation and bile ducts also increases the efficacy of FBZ against parasites that invade the mucosa or the liver.

1.3.3. Mode of Action

Despite their existence for over 30 years, the mechanisms by which benzimidazoles, including FBZ, work to eliminate parasites was not completely understood until recently. In helminths many biochemical pathways are disrupted by FBZ which affects the metabolism of the worms. For example, treatment with FBZ can reduce the capacity for helminths to actively uptake glucose and other low molecular weight nutrients [140, 162]. Other studies indicate that FBZ inhibits the enzyme fumarase reductase, which is a metabolically essential final electron acceptor in many parasites [163]. It was also demonstrated that FBZ can act as a lipid soluble proton conductor, disrupting the cellular electrochemical gradient and inhibiting synthesis of ATP [140]. In addition to these, there are likely a variety of other biochemical pathways disrupted by FBZ. However, central to the disruption of biochemical pathways, the mode of action, and the efficacy of FBZ is the drug's ability to bind tubulin.

Tubulin is a protein dimer and is the basic subunit of microtubules. Microtubules are essential for almost all eukaryotic cellular processes [164]. They are involved in formation of the mitotic spindles and necessary for cell division. Microtubules are major components of the cytoskeleton and locomotory organelles, maintaining cell shape and motility. Many cellular functions such as secretion, nutrient absorption and intracellular transport are also dependent on microtubules. Thus, disruption of the tubulin-microtubule equilibrium can lead to a variety of changes in the biochemistry and physiology of the cell. The resultant loss of homeostasis, if maintained, can result in cell death [139].

Microtubules undergo continuous addition and subtraction of tubulin units at opposite ends of the developing tubule [139]. The tubulin-microtubule equilibrium is controlled by endogenous regulatory proteins and cofactors such as GTP, Mg^{2+}, Ca^{2+}, calmodulin, and microtubule-associated proteins (MAPs) [139]. To disrupt the tubulin-microtubule process, drugs need only to bind to tubulin dimers at the associating end of the microtubule, thus capping the microtubule as it dissociates from the opposite end [139, 165]. Fenbendazole competitively binds to the colchicine binding sites on the tubulin dimer [139, 165, 166]. The location of the colchicine binding site is still unknown, but it is proposed that colchicine and benzimidazoles block access to sulphydryl groups on tubulin, which are necessary for polymerization [166]. Fenbendazole is among the most potent of the benzimidazoles in terms of tubulin binding [167, 168], and oxfendazole also exhibits a high affinity for binding tubulin [167]. As a result, high concentrations of FBZ are not required for maximal occupation of tubulin binding sites. In contrast, less efficacious BZs such as thiabendazole have a lower affinity for tubulin and larger concentrations are required to inhibit polymerization [167]. This affinity for benzimidazoles to bind tubulin directly correlates with their dose [167]. Thus, FBZ administered at 5 mg/kg is as efficacious as TBZ administered at a far greater

dose. The affinity of FBZ for tubulin also explains why FBZ is effective against parenteral nematodes such as *Dictyocaulus viviparus* or blood feeding nematodes such as *Haemonchus contortus,* as large concentrations of FBZ or FBZ-SO in the plasma are not required in order to effectively eliminate these helminth species.

Most benzimidazoles have high affinities for tubulin. However, tubulin is a ubiquitous protein and early studies demonstrated that many of these drugs could inhibit polymerization of mammalian tubulin as well. In fact, a study demonstrated a concentration of 5mM FBZ to cause 60% inhibition of mammalian tubulin *in vitro* compared to 50% inhibition of *Ascaris gali* tubulin [168]. As a result, many questions have been raised as to why FBZ is not toxic to cattle. It has since been discovered that the kinetics of benzimidazole binding to mammalian tubulin differs from parasite tubulin [165, 169]. Fenbendazole binds to parasite tubulin through a pseudo-irreversible interaction that remains stable after charcoal extraction [165]. The FBZ-tubulin complex dissociates slowly, independent of host pharmacodynamics, and the parasite is eliminated [139, 169]. The binding of FBZ to mammalian tubulin differs in that a charcoal non-stable complex is formed [139, 165]. As levels of FBZ decrease through excretion or metabolism by the host, the complex rapidly dissociates preventing toxicity [165].

1.3.4. Safety

As a result of its low affinity binding to mammalian tubulin, FBZ is a very safe drug. In toxicity studies, the LD_{50} for FBZ administered to mice and rats was found to be greater than 10,000 mg/kg [144]. Oral doses of FBZ failed to produce any teratogenic effects in rats given 2500 mg/kg, and oral doses in cattle of 2000 mg/kg did not produce any signs of toxicity [170]. Long-term administration of the drug has also failed to

produce any signs of toxicity or teratogenicity in cattle, and treatments of 20 mg/kg had no effect on cattle fertility [170].

1.3.5. Resistance

Fenbendazole resistance amongst helminths is not widely reported in cattle, but many cases of benzimidazole resistance have been demonstrated in sheep [171-175]. The mechanisms that confer resistance are not completely understood, but it is thought a reduction in charcoal stable binding of BZs to parasite tubulin is responsible for the onset of resistance [165, 167, 176, 177]. In resistant parasites, the number of high affinity receptors on tubulin is reduced, resulting in decreased high affinity, pseudo-irreversible binding of the drug [167, 172, 174]. Polymorphic nematode populations, containing worms with stable and non-stable charcoal binding sites, have been observed [178]. As the proportion of worms with non-stable binding sites are selected by drug use, resistance develops within the population [171]. In these worms, dissociation of the BZ-tubulin complex occurs rapidly, as observed in mammalian tubulin, and the drug is no longer toxic.

Genetic analyses confirm the existence of a polymorphic nematode population. Also, studies have identified the specific loci on the beta-tubulin gene responsible for resistance. In benzimidazole resistant nematodes, the beta-tubulin gene gru-1 was found to contain a gene sequence for three amino acids different from susceptible worms [179, 180]. How this mutation alters the kinetics of fenbendazole binding is not completely understood, but it may cause a structural alteration of the BZ binding ligand on tubulin [181].

Table 1: Chemotherapeutic agents used to treat giardiasis in humans and animals.

Species	Agent	Dosage	Duration
Human	Quinacrine	6 mg/kg, PO	3X daily for 5 days
	Metronidazole	15 mg/kg, PO	3X daily for 5 days
	Tinidazole	50 mg/kg, PO	single dose
	Furazolidone	8 mg/kg, PO	4X daily for 10 days
	Albendazole	200 mg/kg, PO	1X daily for 5-10 days
Dogs / Cats	Quinacrine	6.6 mg/kg, PO	2X daily for 5 days
	Metronidazole	10-25 mg/kg, PO	2X daily for 5 days
	Furazolidone	4 mg/kg, PO	2X daily for 7 days
	Albendazole	25 mg/kg, PO	2X daily for 3-5 days
	Fenbendazole	50 mg/kg, PO	1X daily for 3-5 days
Horses	Metronidazole	5 mg/kg,	3X daily for 10 days
	Fenbendazole	5 mg/kg	1X daily for 3-5 days

1.4. OBJECTIVES OF THIS STUDY

The overall objectives of this study were two fold. First of all, this study was carried out in order to gain an understanding of the biology of *Giardia duodenalis* infections in dairy calves. Secondly, this study was conducted in order to evaluate the efficacy and potential advantages of treating giardiasis in dairy calves with fenbendazole. To effectively accomplish these goals a number of studies were conducted with the following aims in mind.

1. To determine the infection rate, infection patterns, and immunobiology of naturally occurring *Giardia duodenalis* infections in calves, within a commercial dairy operation.
2. To assess the importance of *Giardia duodenalis* as a pathogen of calves, and understand its relationship to other pathogens of dairy calves.
3. To examine the efficacy of fenbendazole in treating *Giardia duodenalis* infections in calves.
4. To determine the clinical, physiological, and economic benefits of treating giardiasis in calves.
5. To better understand the role of dairy calves and dairy operations regarding the potential zoonotic transmission of the parasite.

2. METHODS AND MATERIALS

2.1. GENERAL METHODS

2.1.1. Calves, Housing Procedures, and Animal Care

Unless stated otherwise, all calves used in these studies were housed at the Agriculture and Agri-food Canada dairy facility located in Lethbridge, Alberta. All calves were born at the facility, were purebred Holsteins, and housed according to the following procedures. After receiving colostrum, calves were removed from their dams and placed in clean individual pens. All pens had concrete floors and solid walls, which minimized contact between calves. Calves were fed milk from the dairy (10% body weight/day) until weaned at approximately 80 kg. Initially, all calves were provided *ad libitum* access to 0.5 kg of feed concentrate per day (Table 2). The amount of feed concentrate provided was gradually increased until, at four months of age, calves were receiving 2.5 kg of concentrate per day. Calves had access to loose alfalfa hay until they reached a body weight of 120 kg, after which they were provided with hay cubes. They were also given trace minerals and had *ad libitum* access to water (flocculated, filtered and chlorinated municipal water, Lethbridge, Alberta). The calves' pens were cleaned, and fresh bedding, consisting of wood shavings, was provided daily. All workers followed Agriculture and Agri-food Canada's recommended code of practice for the care and handling of dairy cattle. All housing procedures were in accordance with recommendations of the Canadian Council on Animal Care.

Table 2: Ingredients and composition of concentrate fed to dairy calves

Ingredients*	**Percent of Ration**
Barley, rolled	76.73
Soybean meal	5.03
Molasses beet	3.14
Canola oil	3.77
Beet pulp	6.29
Trace-mineral salt**	5.03

*Monensin added at 36 grams/tonne.
**Contains 92.6% NaCl; 1.1% Zn; 0.94% Mg; 0.32% Cu; 0.005% Co; 0.0044% Se; 0.0013% I; 5% Dynamate.

2.1.2. Parasite Isolation and Enumeration

For all studies, *Giardia* cysts and, when necessary, *Cryptosporidium* oocysts were isolated from the feces of calves and enumerated according to the methods of Xiao [44] and LeChevallier [182] with some modifications. Each fecal sample collected was filtered through a surgical gauze sponge (Nu-Gauze, Johnson & Johnson, Montreal, Quebec), washed with phosphate buffered saline solution (PBSS; 0.9% NaCl, pH 7.2), and expressed from the gauze to yield approximately 7 ml of filtrate. To clarify the sample, the filtrate was layered over 5 ml of a 1 M sucrose solution (specific gravity, 1.13). The sample was then centrifuged at 800 X g for 5 minutes in a fixed rotor centrifuge to concentrate parasite cysts and oocysts at the sucrose-PBSS interface. The interface and upper layer of liquid were transferred by pipette to a clean tube and centrifuged once more at 800 X g for 5 minutes. The supernatant was decanted, and the pellet was resuspended in PBSS to a final volume of 1 ml.

To observe and enumerate *Giardia* cysts and *Cryptosporidium* oocysts, two 0.015 ml samples of the concentrate were spotted on a fluorescent microscope slide (Erie Scientific Co., Portsmouth, New Hampshire) and air dried for 30 minutes on a 37^0 C slide warmer. Next, the slide was fixed with acetone for 1 minute and left to dry. A 0.02 ml *Giardia*-specific fluorescein isothiocyanate-labeled (FITC) monoclonal antibody solution (Giardi-a-glo, Waterborne, New Orleans, Louisiana) was placed on one of the spotted samples, and if necessary, a 0.02 ml *Cryptosporidium*-specific FITC-labeled monoclonal antibody solution (Crypt-a-glo, Waterborne, New Orleans, Louisiana) was placed on the other sample. The slide was incubated in a humidity chamber at 37^0 C for 45 minutes. After incubation, any excess antibody was gently washed off with PBSS and the slide was left to air dry. Slides were mounted with a fluorescent antibody mounting fluid (Aqua-polymount, Polysciences, Warrington, Pennsylvania) and a cover slip. Cysts and

oocysts were examined and enumerated at 100 X and 400 X magnification respectively, using an epifluorescence microscope. The numbers of cysts and oocysts per gram of feces were then calculated using the following formula:

$$N = S/(vol \times wt)$$

Where N = number of cysts or oocysts per gram of feces, s = number of cysts or oocysts counted on the slide, vol = volume of sample examined (0.015 ml), and wt = weight of fecal sample. This procedure has a theoretical sensitivity of 66 *Giardia* cysts and 66 *Cryptosporidium* oocysts per gram of feces, when a 1 g fecal sample is examined.

2.1.3. Statistical Methods

Parasite counts were natural log transformed and are expressed as geometric means. Parasite counts were evaluated by analysis of variance (ANOVA) and compared using Newman-Keul's multiple comparison of means. Parasitic prevalences were compared using Fisher's exact test. All other data were expressed as mean ± SEM. Gaussian data were compared using Student's t test, and non-gaussian data were compared using the Wilcoxon test. The above analyses were performed using a statistical software package (Instat, Graphpad Inc, San Diego, California). Values of $P < 0.05$ were considered significant. Genetic sequences were analyzed using SeqEd v1.0.3. Immunoblots were scanned into a computer using a calibrated imaging densitometer (Bio-Rad, Hercules, California) and analyzed using a software package (Quantity One, Bio-Rad, Hercules, California).

2.2. SPECIFIC METHODS

2.2.1. Naturally Acquired giardiasis in Dairy Calves

Twenty calves born at the Agriculture and Agri-food Canada dairy facility between February 16 and June 3, 1996 were followed from birth until 4 months of age. Beginning when calves were 3 days old, and continuing until calves were 45 days old, fecal samples (1-5 g) were collected from each calf 3 times per week. Samples were then collected once per week until calves were 120 days old. Fecal samples were collected directly from the rectum of each calf using a disposable latex glove. Samples were immediately placed in pre-weighed centrifuge tubes containing 5 ml of 5% formalin in PBSS, and tubes were shaken vigorously to disperse the feces. Samples were then weighed and stored at 4^0 C until *Giardia* cysts and *Cryptosporidium* oocysts could be concentrated and enumerated.

During their first 45 days of life, calves were monitored daily for signs of diarrhea. The age of each calf at onset, and the duration of each episode of diarrhea were recorded. For this study, diarrhea was defined as fluid feces with a loose enough consistency to take the shape of the container in which it was collected. During each episode of diarrhea, additional fecal samples were collected and examined for parasitic (*Giardia duodenalis*, *Cryptosporidium parvum*, *Eimeria* spp, and intestinal nematodes), bacterial, and viral pathogens. The cause of each episode of diarrhea was then determined based on the specific pathogen or pathogens identified in the sample.

Standard microbiologic methods were used to isolate and identify bacterial pathogens in fecal samples collected during episodes of diarrhea [1]. *Eschericha coli* isolates were subcultured on E agar and pilus antigens typed using an indirect fluorescent antibody staining procedure [183]. Colonies from the E agar were first suspended in

PBSS and drops of the suspension were placed on microscope slides. A drop of heat aggregated whole horse serum was added to each slide, and a smear of the bacterial preparation was created. The slides were air dried for 30 minutes, fixed with acetone, and rinsed with PBSS. Monoclonal antibody solutions specific for pilus antigens of either K88a, K88b, 987P, F41, or K99 *E coli* were added to the slides. The slides were incubated in a humidity chamber at 37^0 C for 30 minutes, rinsed in PBSS, then incubated again with FITC-labeled rabbit anti-mouse IgG. Slides were rinsed in distilled water and left to dry. Coverslips were mounted with fluorescent mounting fluid, and slides were examined at 400X magnification using an epifluorescence microscope.

To identify *Eimeria* oocysts and nematode eggs, fecal floatation tests were performed using a commercially available sodium nitrate (specific gravity, 1.2) fecal analysis kit, according to the manufacturers instructions (Fecalyzer, Evsco Pharmaceuticals, Buena, New Jersey). The slides were examined using a light microscope at 100 X magnification. Rotavirus and coronavirus were identified by use of a standard procedure for negative staining, and examination by means of electron microscopy [184].

Blood samples were collected from each calf monthly by veni-puncture. Blood collection was initiated when calves were 3 days old and ended when calves were 120 days old. Following centrifugation, serum was aliquoted into triplicate sets and stored at -20^0 C. In addition to the collection of blood samples from the calves, approximately 100 ml of colostrum was collected from each calf's dam within 24 hours of birth. Also, a 100 ml sample of milk was collected from each dam approximately 30 days after the calves were born. Colostrum and milk samples were aliquoted into triplicate sets, then stored at -20^0 C.

To determine adequacy of transfer of passive immunity, a radial immunodiffusion assay (Bovine IgG_1 test kit, VMRD Inc., Pullman, Washington) was used to quantify IgG_1 concentrations from serum samples collected from calves at 3 days of age. Calves with IgG_1 concentrations between 800 and 1600 mg/dl were considered to have partial transfer of passive immunity; calves with IgG_1 concentrations greater than 1600 mg/dl were considered to have complete transfer of passive immunity [1].

Giardia-specific serum titers for each calf were determined after each month of the study by ELISA, according to the method described by Yanke *et al.* [185]. The antigen consisted of *Giardia* trophozoites (isolate S2, University of Calgary, Calgary, Alberta) axenically grown according to the method of Farthing [186]. At the late log phase of growth, trophozoites were placed on ice for 10 minutes to detach them from the wall of the roller bottle. Next, trophozoites were washed 5 times by repeated centrifugation at 500 X g, followed by resuspending in cold, sterile PBSS. The final suspension of trophozoites was then sonicated and the protein concentration determined by protein assay (BioRad, Richmond, California). Microtiter wells (Titertek, ICN Biomedicals, Costa Mesa, California) were coated with 20 μg of the *Giardia* antigen. Following an overnight incubation, wells were washed 3 times in PBSS containing 0.1% Tween 20 (Sigma Chemical, St. Louis, MO), then blocked using 10% skim milk powder in PBSS. Serum samples were then added to the wells in duplicate serial dilutions, starting with a dilution of 1:10. Following the addition of the serum, wells were incubated at 37^0 C for 1 hour and washed as before. Next, horseradish peroxidase-conjugated sheep anti-bovine IgG, diluted 1:1000 in 5% skim milk in PBSS, was added (h+l chain specific, Cedarlane Laboratories, Hornby, Ontario), and wells were incubated again at 37^0 C for 1 hour. Following another wash, plates were developed at 37^0 C for 30 minutes with the indicator, consisting of *o*-phenylenediamine dihydrochloride (Sigma Chemicals, St. Louis, MO) and substrate containing hydrogen peroxide. Immediately

after stopping the reaction with 2.5 M H_2SO_4, optical densities were read at a wavelength of 490 nm.

In addition to ELISA, Western Blot analysis was performed on the serum collected from 6 calves, as well as the colostrum and milk collected from their dams. Trophozoites were prepared as previously described to serve as the antigen for the Western Blots. Approximately 30 μg of trophozoite sonicate was added to each well of a 4% stacking gel and 10% separating gel as described by Laemmli [187]. Electrophoresis was carried out at 200 V for 4 hours. Proteins were then transferred overnight from the polyacrylimide gel to an immobulin-P membrane (Millipore, Bedford, MA) by electrophoresis in a Tris-glycine buffer at 4^0 C with a constant current of 30 V. To determine total protein following the transfer, one strip of the blot was stained with Coomassie Blue and compared to a protein standard (Sigma Chemicals, St. Louis, MO). The rest of the membrane was blocked with 10% skim milk powder in PBSS and divided into strips. Each strip was incubated overnight at 4^0 C with a separate sample of calf serum or colostrum diluted 1:100. After washing in PBSS, each strip of membrane was incubated for 2 hours at room temperature with horseradish peroxidase-conjugated sheep anti-bovine IgG diluted 1:500 in PBSS (Cedarlane Laboratories, Hornby, Ontario). Next, the blots were developed in fresh chromogen substrate containing 66 mg of 3,3'-diaminobenzadine (Sigma Chemicals, St. Louis, MO), 99 ml of 50 mM Tris buffer and 0.015 ml of 30% hydrogen peroxide. Following development, blots were removed from the substrate, washed in double distilled H_2O and air dried.

The *in vitro* anti-*Giardia* activity of bovine colostrum and milk was determined using an adherence assay. Assays were performed in triplicate using 24 well, flat bottomed tissue culture plates (Falcon Multiwell, Becton Dickson, Mississauga, Ontario). Fresh TYI-S-33 medium was added to each well, along with 5μg of pipercillin and

approximately 10,000 *Giardia* trophozoites. Serial dilutions of colostrum and milk in TYI-S-33 medium, ranging from 1:0 to 1:10, were added to test wells. To positive control wells, metronidazole was added at a concentration of 40 µg/ml. To negative control wells, 0.1 ml of sterile PBSS was added. After wells were prepared, plates were incubated in an anaerobic chamber with a CO_2 pack at 37^0 C for 24 hours. Following the incubation, plates were gently shaken for 10 seconds to suspend non-adherent trophozoites and inverted to discharge the liquid. Each well was examined at 100 X magnification using a light microscope, and the number of adherent trophozoites counted. Percent adherence was then determined by dividing the number of adhering trophozoites in test wells by the number of adhering trophozoites in negative control wells (PBSS).

2.2.2. Efficacy of Fenbendazole as a Treatment for giardiasis in Calves

Thirty-five calves (20 male, 15 female), weighing 100-180 kg were selected for this study based on confirmation of *Giardia* infection from 3 daily pre-study fecal examinations. In addition to the normal housing procedures described above, calves' pens in this study were cleaned and disinfected daily using a quaternary ammonium disinfectant (Omega, Airkem Professional Products, Mississauga, Ontario). Also, workers disinfected their footwear and shovels before entering a calf's pen.

Calves were ranked according to geometric mean fecal cyst counts from the 3 pre-study fecal examinations, blocked, and randomly allocated to 7 treatment groups (n = 5 per group). The treatment groups were as follows: group 1 – a single administration of 5 mg of fenbendazole (FBZ, Safeguard, Hoechst Roussel Vet, Regina, Saskatchewan)/kg (label dose); group 2 – a single administration of 10 mg of FBZ/kg; group 3 – 5 mg of FBZ/kg, every 24 hours for 3 days; group 4 – 10 mg of FBZ/kg, every 24 hours for 3 days; group 5 – 20 mg of FBZ/kg, every 24 hours for 3 days; group 6 – 0.833 mg of

FBZ/kg, every 24 hours for 6 days; and group 7 (placebo) – saline solution. FBZ and the saline solution were administered orally using a dosing syringe, and no other drugs were given to the calves at any time during the study.

Fecal samples were collected daily from the rectum of each calf from days 0 through 7 of the study. Samples were then collected every second day from day 9 to 13, and additional samples were collected on days 21 and 28. Samples were stored at 4^0 C until *Giardia* cysts were isolated and enumerated, within 72 hours of sample collection. After enumeration, the number of *Giardia* cysts shed by calves per gram of feces was calculated.

2.2.3. Effect of Fenbendazole Treatment on Trophozoites and Intestinal Structure and Function

Twelve, 2 week old dairy calves were purchased from various commercial dairies for this study. Upon arrival at Agriculture and Agri-food Canada's dairy facility in Lethbridge, Alberta, a fecal sample was collected from each calf and examined for *Giardia* cysts. All calves not shedding fecal *Giardia* cysts at this time were orally inoculated with approximately 10^5 *Giardia* cysts obtained from a naturally infected calf (day -10). Successful establishment of *Giardia* infection was confirmed 7 days after inoculation by a second set of fecal examinations (day -3). On day 0, calves were ranked according to their day -3 cyst counts, blocked, and randomly assigned to a treatment (N = 6) and a placebo (N = 6) group. Beginning on day 0, calves in the treatment group were administered an oral dose of FBZ at 5 mg/kg once daily for 3 days. This treatment regime was selected based on the results of the previous study. Calves in the placebo group received a 5 ml oral dose of sterile saline solution, once daily for 3 days. Seven days after treatments were initiated, fecal samples were collected from each calf for

Giardia cyst enumeration. Calves were then euthanized by an intravenous injection of sodium pentobarbital. Immediately following euthanasia, samples were collected from the small intestine and prepared for the following procedures.

Trophozoite Counts

Segments measuring 10 mm^2 were removed from the duodenum, proximal jejunum, distal jejunum, and ileum. Each segment was placed in a 10 ml centrifuge tube containing 5 ml of sterile PBSS. The segments were shaken for 30 minutes at 37^0 C on a shaking incubator. After 30 minutes, a drop of the PBSS was removed from the centrifuge tubes, placed on a hemocytometer, and trophozoites were counted using a light microscope at 400 X magnification.

Light Microscopy

Intestinal sections taken from the duodenum, jejunum, and ileum were cut along the mesentery and stapled at each end to a piece of cardboard. The cardboard and attached tissue were then immersed for 24 hours in a scintillation vial containing Carnoy's fixative. Following dehydration in ethanol, the tissue was infiltrated and embedded in JB-4 plastic medium according to the manufacturer's instructions (Polysciences Inc., Warrington, Pennsylvania). Sections of approximately 1.5 μm were cut, mounted on glass slides, and stained with Lee's methylene blue. Villous and crypt measurements were made from 10 villous-crypt units per slide using a calibrated micrometer at 100 X magnification.

In addition to villous-crypt measurements, the number of enterocytes and intraepithelial lymphocytes (IELs) were counted along the villous from sections of the

duodenum and jejunum at 400 X magnification. At least 500 enterocytes were counted on each section, and the number of IELs counted per 100 enterocytes was calculated.

Disaccharidase assay

Segments from the duodenum, jejunum, and ileum of the small intestine were removed and placed on glass plates. The segments were opened along the mesentery and scraped using a pre-weighed microscope slide. The scraping was then weighed and placed in a scintillation vial containing 10% wt/vol of 2.5 mM ethylene diamino-tetra-acetic acid (EDTA). The sample and solution were then homogenized and 1 ml aliquots were transferred to microcentrifuge tubes. The samples were flash frozen in liquid nitrogen and stored at -70^0 C until they could be assayed. After thawing, samples were assayed for maltase and lactase activity according to the method of Dahlqvist [188], and protein was measured according to the method of Lowry [189]. Disaccharidase activity was then calculated in units per gram of protein.

Transmission Electron Microscopy

The ultrastructure of the jejunum was examined from 3 calves in the FBZ treatment group and 3 calves in the placebo group. Jejunal segments measuring approximately 10 mm^2 were removed and immediately placed in scintillation vials containing 5% glutaraldehyde in 0.1 M cacodylate buffer. The specimens were cut into 1 mm^2 cubes while immersed in the fixative, then stored overnight at 4^0 C. After washing the specimens in cacodylate buffer, they were postfixed in 1% OsO_4 and dehydrated in ethanol. Following dehydration, the specimens were cleared with propylene oxide, infiltrated, and 5 specimens per animal were embedded in Spurr's low viscosity medium (J.B. EM Services Inc., Dorval, Quebec). Approximately 20 thin sections were obtained

from 3 embedded specimens per calf. The thin sections were stained with uranyl acetate in 50% ethanol, followed by 0.4% lead citrate [190]. Sections were examined under an electron microscope at 75 kV, and mid-villous regions were identified under low magnification. After mid-villous regions were located, micrographs of the microvillous border were taken at the same magnification. Microvillous border surface area was then calculated according to the method of Phillips [191]. Measurements were made in blind fashion from 9 micrographs per animal (27 per group), which were coded to eliminate observer bias.

2.5. Efficacy of Repeat Fenbendazole Treatment in Dairy Calves with giardiasis on Clinical Signs and Animal Performance

For this 90 day study, sixty male dairy calves (1-2 weeks old) were purchased. Upon arrival at the dairy facility in Lethbridge (day −10) calves were experimentally infected with *Giardia* by oral inoculation of 10^5 cysts, obtained from a naturally infected calf. After 7 days (day −3), fecal samples were collected from each calf and examined to ensure successful establishment of *Giardia* infection. From this fecal sample, the presence of *Cryptosporidium*, *Eimeria*, and intestinal nematodes were also determined as previously described. Once infection with *Giardia* was confirmed (day 0), calves were weighed and randomly blocked by weight into treatment (N=30) and placebo (N=30) groups.

Beginning on day 0 (allocation day), calves in the treatment group were administered an oral dose of 5mg/kg of FBZ once daily for 3 consecutive days. Calves in the placebo group received a daily oral treatment of 5 ml of sterile saline on the same three days. These treatments were repeated on days 30 and 60 of the study.

Starting at the time of allocation, calves were weighed on a weekly basis for 90 days to determine average body weight and average daily gain for each treatment group. Rather than milk from the dairy, calves were fed 4 litres of a commercial milk replacer daily (Snowflakes, Nutrena Feeds, Winnipeg, Manitoba), until weaning. Calves had access to feed concentrate throughout the study. Concentrate was provided *ad libitum* until weaning, after which calves received 1.75% of their body weight in concentrate per day. Alfalfa hay was provided *ad libitum* throughout the study, and calves had unlimited access to water and loose mineral. Total feed intake (milk replacer, concentrate, and hay), calculated on a dry matter basis, was measured daily for each calf for the duration of the study.

Fecal samples were collected from each calf once per week for the duration of the study, and the number of *Giardia* cysts and *Cryptosporidium* oocysts were enumerated. In addition, beginning on day 0, fecal floatations for *Eimeria* oocysts and nematode eggs were performed every 30 days.

Clinical signs of intestinal disease were monitored daily for each calf, and episodes of diarrhea were recorded. Again, diarrhea was defined as fluid feces with a loose enough consistency to take the shape of the container in which it was collected. From this information the mean number of diarrhea episodes per calf, the mean duration of each episode, and the total number of days calves in each treatment group had diarrhea were calculated.

2.6. Genotypic Characterization of *Giardia* from Western Australian and Western Canadian Dairy Calves

Fecal samples were collected from 28 randomly selected Holstein calves located at Agriculture and Agri-food Canada's dairy facility near Lethbridge, Alberta. In addition, 36 fecal samples were collected from male and female Holstein calves located on two commercial dairies near Perth, Western Australia. Australian calves from one farm (N=16) were group housed in pens with slatted wooden floors raised approximately 1 metre above the floor of the cattle shed. Calves from the other farm (N=20) were pastured outside. Both the Canadian and Australian calves were between 2 and 10 weeks of age at the time of sampling. The fecal samples collected from the calves located at Lethbridge, Alberta were placed in 15 ml tubes containing approximately 10 ml of PBSS without fixative and transported to the University of Calgary. After arriving at the University of Calgary, *Giardia* cysts were isolated from the fecal samples. Following isolation of the *Giardia* cysts, samples were placed in sterile 1.5 ml tubes and shipped overnight at 4^0 C to Murdoch University in Perth, Western Australia where they were examined and the *Giardia* cysts enumerated. Samples collected from calves located near Perth, Western Australia were shipped immediately to Murdoch University where *Giardia* cysts were isolated and enumerated employing the procedures described previously.

Approximately 100 µl of sucrose-purified cyst suspension from each *Giardia* positive calf sample was used for DNA purification. DNA was purified from the cyst suspension according to the method of Morgan *et al.* [192], with some modifications. Samples were freeze-thawed 6 times in liquid nitrogen, then 100 µl of tissue lysis buffer (Qiagen, Hilden, Germany) was added to each sample. Next, samples were placed in a boiling water bath for 10 minutes and centrifuged at 14000 rpm for 15 seconds to spin

down any large particulate matter. The supernatant was transferred to a clean tube, and 180 µl of *Al* buffer (Qiagen, Hilden, Germany) was added, followed by 10 µl of glassmilk (Bio-Rad, Richmond, California). After vortexing, samples were incubated at 72° C for 10 minutes, and centrifuged at 14000 rpm for 1 minute. The supernatant was discarded, and the pellet was washed twice in 700 µl of *AW* wash buffer (Qiagen, Hilden, Germany). After the pellet was vacuum dried, DNA was eluted by first resuspending the pellet in 30 µl of *AE* elution buffer (Qiagen, Hilden, Germany), incubating at 72° C for 5 minutes, then centrifugation for 1 minute at 14000 rpm. The supernatant was transferred to a clean tube and 1 µl of the eluate was used for PCR amplification.

A 292 bp region of the 16S-rRNA gene was amplified by PCR according to the method of Hopkins *et al.* [119], using RH 11 forward primer and RH 4 reverse primer. PCR amplification was performed in 25 µl volumes, each containing the following: H_2O, 12.5 pmol of the RH 11 and RH 4 primers, 67 mM Tris-HCL (pH 8.8), 16.6mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg/ml gelatin, 2 mM $MgCl_2$, 5% dimethyl sulfoxide, 200 µM of each dNTP, and 1 unit of tTh plus DNA polymerase (Fisher Biotech, Perth, Australia). Reactions were heated to 96° C for 2 minutes, followed by 50 cycles of 96° C for 20 seconds, 59° C for 20 seconds, and 72° C for 20 seconds. This was followed by 1 cycle of 72° C for 7 minutes. Amplification was carried out using a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer, Foster City, California). The amplified product was separated by electrophoresis on an ethidium bromide stained agarose gel and visualized under ultra violet light. Bands were excised from agarose gels and purified using PCR spin columns (Qiagen, Hilden, Germany). Sequencing reactions were performed in both directions using either the RH 4 or RH 11 primers and a Dye Terminator Cycle Sequencing Core kit (Applied Biosystems, Foster City, California).

3. RESULTS

3.1. NATURALLY ACQUIRED GIARDIASIS IN CALVES

All 20 calves shed *Giardia* cysts and *Cryptosporidium* oocysts at some time during the study (Table 3). *Giardia* cysts first appeared in the feces of calves between 6 and 70 days of age, while *Cryptosporidium* oocysts first appeared in the feces of calves between 6 and 42 days of age. The mean age at which *Giardia* cysts were first detected (31.5 ± 4.1 days) was significantly greater than the mean age at which *Cryptosporidium* oocysts were first detected (16.3 ± 2.0 days).

The geometric mean number of *Giardia* cysts in the feces of calves increased from 0 at 12 days of age to a maximum of 628 cysts per gram of feces at 89 days of age (Figure 2A). After 89 days of age, the geometric mean number of *Giardia* cysts began to decline, but was still 98 cysts per gram of feces at the end of the study (day 120). The percentage of calves shedding *Giardia* cysts also increased as the calves grew older (Figure 2B). By 96 days of age, 17 (85%) calves were shedding *Giardia* cysts in their feces, and by the end of the study, 15 calves (75%) were positive for *Giardia*. Over the 120-day observation period only 1 calf (# 626) appeared to clear the infection as cysts were not detected in its final 3 fecal samples. Based on this, the duration of *Giardia* cyst shedding could not be determined, but the calf that cleared its infection shed cysts for 89 days (Table 3).

With respect to *Cryptosporidium*, the geometric mean number of oocysts detected in the feces was highest when the calves were between 10 and 19 days of age (Figure 2A), then decreased and remained low for the duration of the study. At 28 days of age, the percentage of calves shedding *Cryptosporidium* oocysts was at its highest level with 7 calves (35%) shedding oocysts (Figure 2B). The percentage of calves shedding oocysts

then decreased and remained less than 10% from 50 days of age through the end of the study. Calves shed *Cryptosporidium* oocysts in their feces for an average of 10.5 ± 5.9 days, significantly less than the number of days in which they shed *Giardia* cysts (Table 3).

Compared to the first day *Giardia* cysts were detected in the feces of each calf (Figure 3A), geometric mean *Giardia* cyst counts did not significantly increase or decrease during the study. Contrary to this, the geometric mean *Cryptosporidium* oocyst counts decreased rapidly after oocyst shedding was first detected. Seven days after oocysts were first detected in calves, geometric mean oocyst counts declined significantly compared to the initial day of shedding (Figure 3A). By 14 days after initial oocyst shedding, the geometric mean oocyst count reached 0 and remained low for the remainder of the study. Calves shed *Giardia* cysts intermittently during this study. Following the initial appearance of *Giardia* cysts, the number of calves shedding at the time of sampling fluctuated between 40 and 85%. With respect to *Cryptosporidium*, the percentage of calves shedding oocysts decreased rapidly after oocyst shedding was first detected. Seven days after oocysts were first detected, the percentage of calves shedding oocysts was 50%. After day 14, the percentage of calves shedding oocysts reached 0 and remained between 0 and 10% for the remainder of the study.

Eighteen calves had a total of 38 episodes of diarrhea during their first 45 days after birth. *Giardia duodenalis* was the only pathogen identified in association with 6 (16%) diarrhea episodes, and *Cryptosporidium parvum* was the only pathogen identified in association with 9 (24%) episodes (Table 4). *Giardia* and *Cryptosporidium* were identified together in 10 (26%) episodes, rotavirus and *Giardia* were identified concurrently in 2 (5%) episodes, and rotavirus and *Cryptosporidium* were identified in 2 (5%) episodes. For the remaining 9 (24%) episodes of diarrhea, no pathogens examined

for in this study were identified. *Salmonella* spp, coronavirus, *Eimeria* spp, intestinal nematodes, and pathogenic strains of *E. Coli* (K88a, K88b, 987P, F41, and K99) were not identified in association with any of the diarrhea episodes.

For the 6 episodes of diarrhea in which *Giardia* was the only pathogen identified, calves' mean age at the onset of diarrhea was 35.7 ± 2.1 days, and the mean duration of the diarrhea episode was 2.0 ± 0.9 days (Table 4). *Giardia* on its own was not associated with diarrhea in calves under 4 weeks of age. Conversely, the majority of diarrhea episodes associated with *Cryptosporidium* occurred when calves were less than two weeks of age. For the 9 episodes of diarrhea in which *Cryptosporidium* was the only pathogen identified, the average age at onset was 14.7 ± 2.7 days, and the mean duration of the episodes were 3.0 ± 0.6 days. The age of the calves at the onset of diarrhea was significantly lower for episodes associated with *Cryptosporidium* compared to episodes associated with *Giardia*, however there was no significant difference with respect to duration of these diarrhea episodes (Table 4). Diarrhea episodes associated with concurrent *Cryptosporidium*-rotavirus infections were of a significantly longer duration than all other diarrhea episodes observed in this study (Table 4). However, only 2 episodes of diarrhea occurred during this study as a result of *Cryptosporidium*-rotavirus infection.

All 20 calves had complete or partial transfer of passive immunity. Mean IgG_1 concentration was 1578 ± 152 mg/dl, and there was no relationship between IgG_1 concentration and duration of episodes of diarrhea, or number of *Giardia* cysts or *Cryptosporidium* oocysts in the feces. IgG immunoblots from colostrum and serum collected from calves at 3 days of age bound to most of the same dominant *Giardia* proteins (Figure 4). Both colostrum and serum from 3 day old calves bound to a group of dominant proteins with approximate molecular weights of 39, 35, and 33 kDa.

Colostrum and 3 day old serum also reacted with a number of the same proteins with molecular weights ranging from approximately 92.5 to 50 kDa. Lower molecular weight proteins of 21 and 27 kDa were bound by both colostrum and 3 day old serum. The only proteins bound by colostrum and not by the 3 day old serum had molecular weights of approximately 24 kDa and 54 kDa. IgG in milk collected from the dams also bound to a number of proteins, but fewer than either the colostrum or 3 day old serum (Figure 4). Milk IgG reacted with the 33 kDa protein, as well as a few proteins between 45 and 83 kDa. Milk IgG did not bind to proteins with molecular weights below 33 kDa, or the proteins with molecular weights of approximately 64 and 50 kDa.

Figure 5 shows the individual and mean *Giardia*-specific serum IgG titers from calves before and after *Giardia* cysts were first detected. Mean serum IgG titers did not significantly increase in response to *Giardia* infection at any time period (Figure 5). One calf had an increased in serum IgG at every time point following initial shedding, but the titer was not significantly greater than the mean preinfection titer. Individual serum IgG titers were not significantly elevated in any calf during any time point following infection, including the calf that cleared the infection.

Colostrum and serum immunoblots from a representative calf that did not clear the *Giardia* infection (calf A) are compared to the calf that cleared the infection (calf B) in Figure 6. The approximate molecular weights of each antigen bound by IgG are displayed in Table 5. Colostrum from each calf bound to many antigens with molecular weights between 190 and 21 kDa. Colostrum from calf A bound to 29 antigens, while colostrum from calf B bound to 25 antigens. Serum IgG bound fewer antigenic proteins when calves were 30 days of age, but IgG from both calves bound to the same 13 antigens. At 60 days of age, serum IgG from calf A bound to 12 antigens, while serum from calf B bound to 16 antigens. The number of antigenic proteins bound by IgG from

calf A when 90 days of age remained at 12, and calf B IgG bound to 19 proteins. When calves were 120 days of age, serum IgG reacted with the same 13 antigens, however calf B IgG bound to additional antigens of 180 and 190 kDa.

A number of dominant antigens were bound by both colostrum and serum IgG throughout the study, such as a group of proteins with molecular weights of 33, 35 and 39 kDa. An 82 kDa antigen was also bound by IgG at every time point. Other antigens bound to consistently by colostrum and serum IgG were approximately 141, 130, 117, 101, and 21 kDa in weight, along with a group of 3 proteins from 57.5 to 52.5 kDa in weight. A 170 kDa molecular weight antigen, bound by colostrum and 30 day serum from both calves, did not reappear in blots from calves after 30 days of age. Also, IgG from both calves did not react with a 92.5 kDa protein after 60 days of age.

Immunoblots from calf A and calf B differed when calves were 60 and 90 days of age. Calf B serum IgG bound to proteins of 190, 180, and 64 kDa (Figure 6 arrow) at 60 and 90 days of age. Serum IgG from calf B also bound to additional antigens of 79 and 73 kDa at 90 days of age. Serum IgG from both calves bound a 50 kDa antigen at 90 and 120 days of age, as well as the 79, 73, and 64 kDa antigens at 120 days of age. At 120 days of age, serum IgG from both calves bound to a 150 kDa antigen.

Both the colostrum and milk obtained from the dams of these calves had anti-*Giardia* activity *in vitro* (Figure 7). Incubation in undiluted colostrum and milk prevented trophozoites from adhering to test wells. Incubation in colostrum diluted 50% (1:1 dilution) resulted in only 3.2% of trophozoites adhering compared to negative control wells. For trophozoites incubated in milk diluted 1:1, 33.8% of trophozoites were adhering compared to the negative controls (PBSS). Following serial dilutions of both the milk and colostrum, the number of adherent trophozoites increased in a linear fashion

(Figure 7). At a dilution of 1:10, 80% of trophozoites were adhering following incubation in colostrum, while 95% were adhering following incubation in milk. Colostrum exhibited significantly greater anti-*Giardia* activity than milk ($P < 0.05$) as the 50% adherence inhibition concentration (IC_{50}) for colostrum was 0.16 (dilution of 1:6.2) compared to an IC_{50} in milk of 0.72 (dilution of 1:1.4).

Table 3: *Giardia* and *Cryptosporidium* infection patterns in dairy calves.

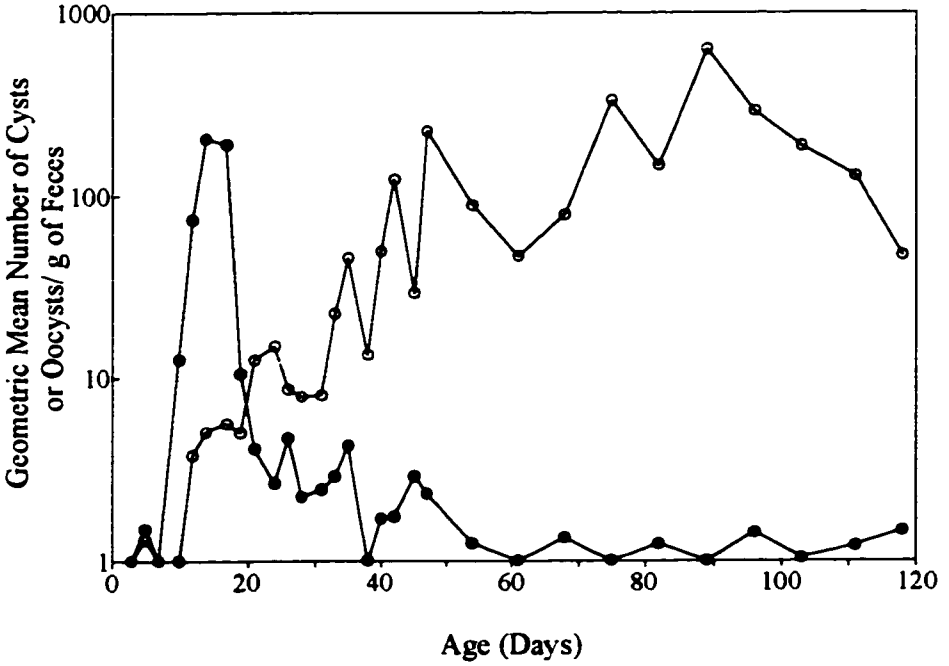
Infection Patterns	***Giardia***	***Cryptosporidium***
Number Infected (Percent)	20 (100%)	20 (100%)
Mean Age in Days of Initial Cyst/Oocyst Excretion	31.5 (± 4.1)*	16.3 (± 2.0)
Mean Duration in Days of Cyst/Oocyst Excretion	NA†	10.5 (± 5.9)
Infections Cleared (Percent)	1 (5%)	20 (100%)

*Indicates $P < 0.05$ compared to *Cryptosporidium*.
†Only one calf stopped shedding cysts during the study

Figure 2.

Geometric mean numbers of *Giardia duodenalis* cysts (○) and *Cryptosporidium parvum* oocysts (●) per gram of feces in naturally infected dairy calves, (A) and the percentage of calves shedding cysts and oocysts (B) in relation to days after birth.

A


1000
100
10
1
Geometric Mean Number of Cysts or Oocysts/ g of Feces
0
20
40
60
80
100
120
Age (Days)

B

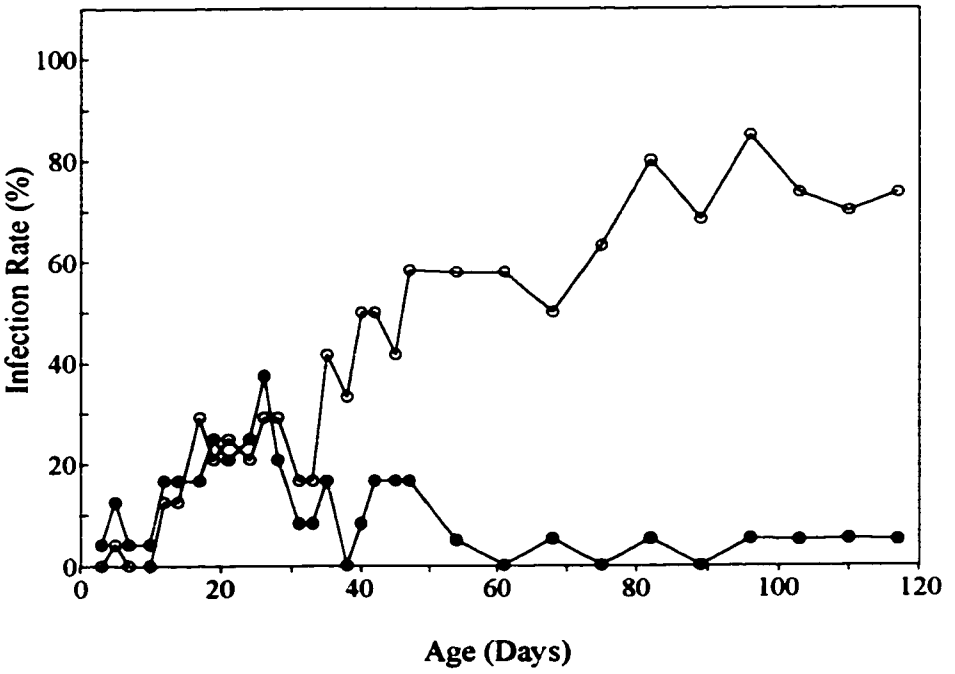
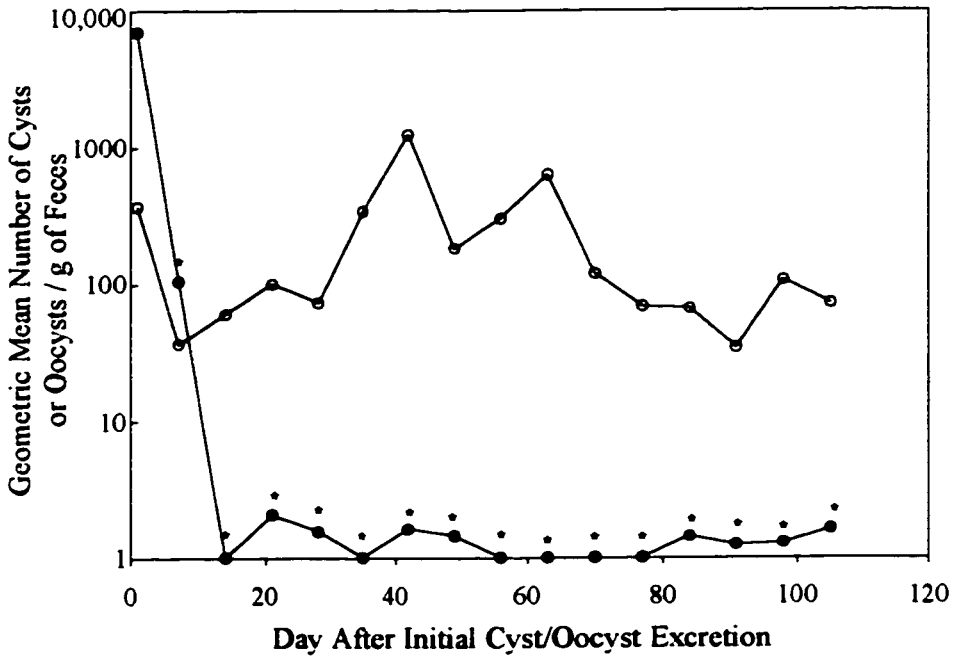

100
80
60
40
20
0
Infection Rate (%)
0
20
40
60
80
100
120
Age (Days)

Figure 3.

Geometric mean numbers of *Giardia duodenalis* cysts (❍) and *Cryptosporidium parvum* oocysts (●) per gram of feces in naturally infected calves (A), and the percentage of calves shedding cysts and oocysts (B) in relation to days after fecal shedding of cysts and oocysts was first detected. * $P < 0.05$ counts compared to value on day shedding was first detected.

A


10,000
1000
100
10
1
Geometric Mean Number of Cysts or Oocysts / g of Feces
0
20
40
60
80
100
120
Day After Initial Cyst/Oocyst Excretion

B

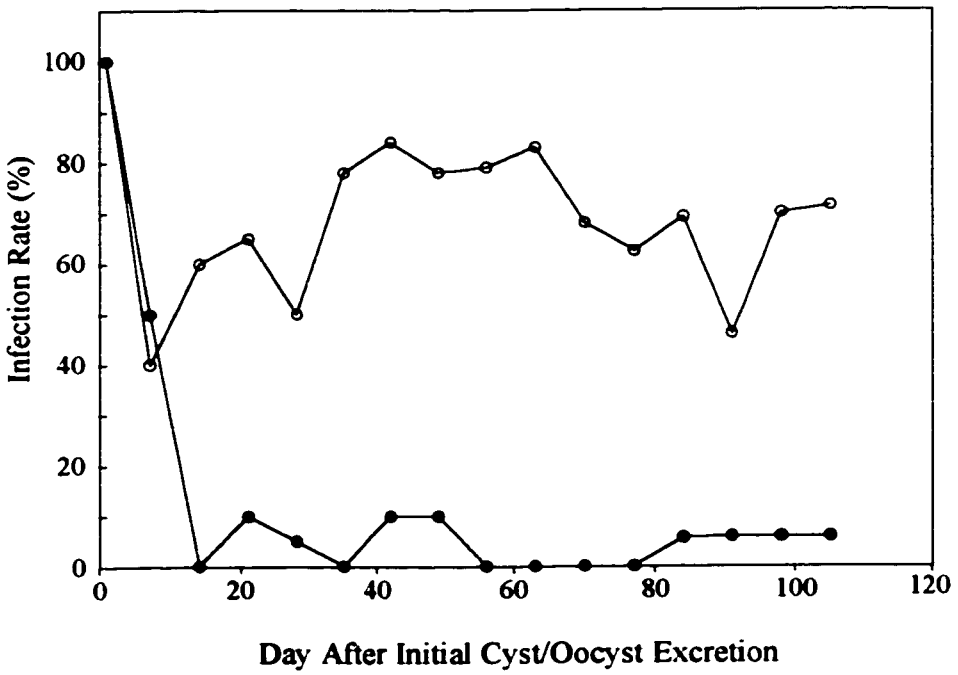

100
80
60
40
20
0
Infection Rate (%)
0
20
40
60
80
100
120
Day After Initial Cyst/Oocyst Excretion

Table 4: Etiology and duration of diarrhea in dairy calves during their first 45 days after birth.

Diarrhea	***Giardia***	***C.parvum***	***Giardia-C.parvum***	***Giardia*-Rotavirus**	***C.parvum*-Rotavirus**	**Unknown Etiology**
Number of Episodes	6	9	10	2	2	9
Mean Age at Onset (days)	35.7(±2.1)	14.7(±2.6)*	16.9(±2.9)*	35.5(±2.5)†	5.5(±5.5)*	21.0(±3.1)*
Mean Duration (Days)	2.0(± 0.4)	3.0(± 0.6)	4.6(±0.8)*	4.0(± 2.0)	9.0(± 1.0)*†	3.4(± 0.6)

*Indicates $P < 0.05$ compared to *Giardia*. †Indicates $P < 0.05$ compared to *C. parvum*.

Figure 4.

Left - Coomassie blue-stained SDS-polyacrylimide gel displaying the proteins of *Giardia duodenalis* trophozoites (S2 isolate). Center – Representative IgG immunoblot from colostrum and milk collected from calves' dams to *G. duodenalis* trophozoite antigens. Right - Representative IgG immunoblot from serum collected from calves at 3 days of age to *G. duodenalis* trophozoite antigens. Values of molecular weight markers are displayed in kilodaltons.

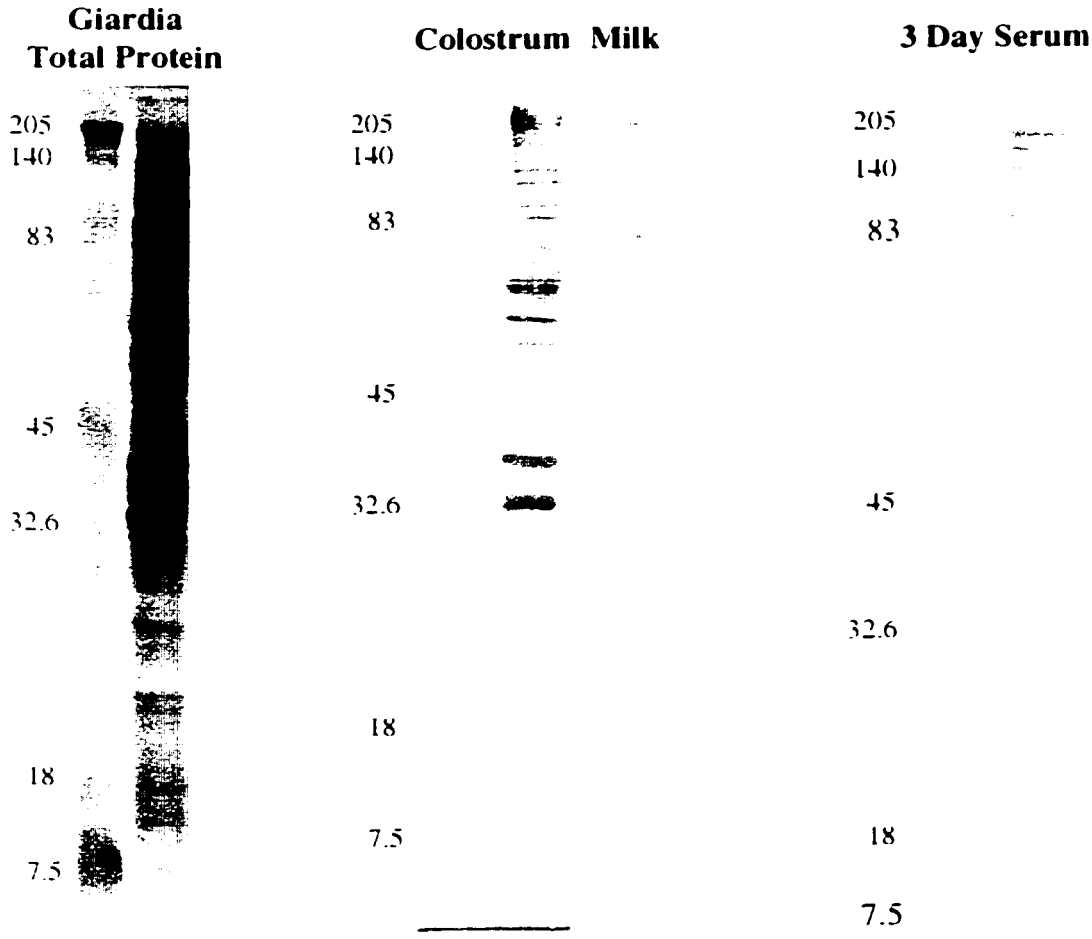

Giardia
Total Protein
205
140
83
45
32.6
18
7.5
Colostrum Milk
205
140
83
45
32.6
18
7.5
3 Day Serum
205
140
83
45
32.6
18
7.5

Figure 5.

Mean anti-*Giardia* serum IgG titers (●) and individual titers (○) from dairy calves naturally infected by *Giardia duodenalis*. Mean titers were not significantly different at any time point. Individual titers were not significantly elevated at any time point (greater than 2 standard deviations above the mean titer (–)). Titer from calf that cleared the infection shown (□).

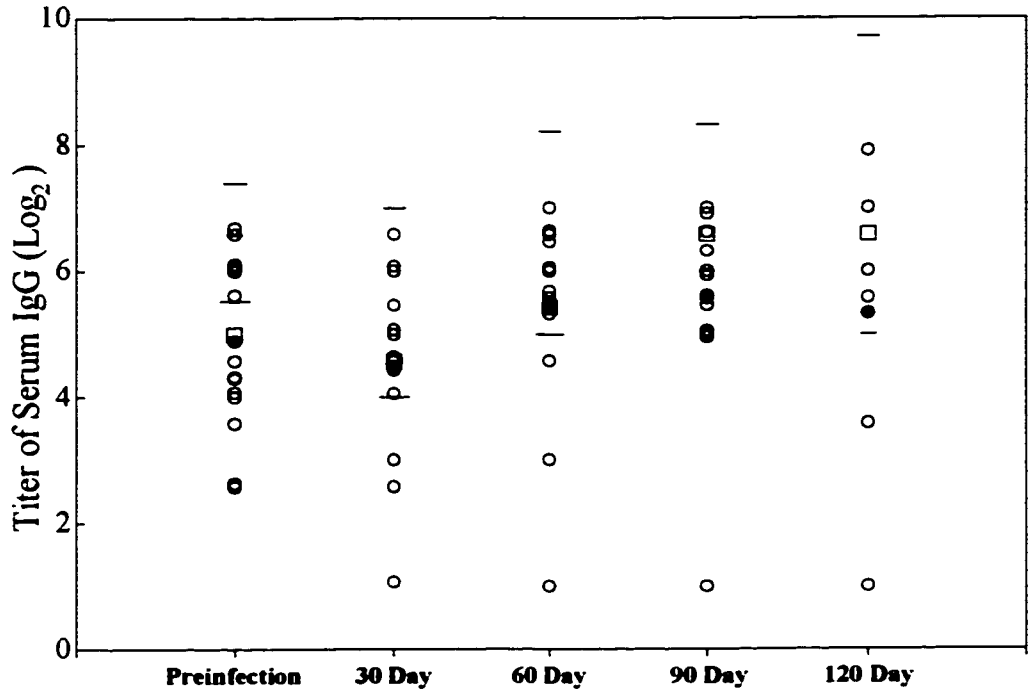

10
8
6
4
2
0
Titer of Serum IgG (Log2)
Preinfection
30 Day
60 Day
90 Day
120 Day
Period After Initial Cyst Detection

Figure 6.

Dam's colostrum (top left) and monthly serum IgG immunoblots to *Giardia duodenalis* trophozoite antigen from a chronically infected calf (A) and a calf that cleared the infection (B). Values of molecular weight markers are displayed in kilodaltons.

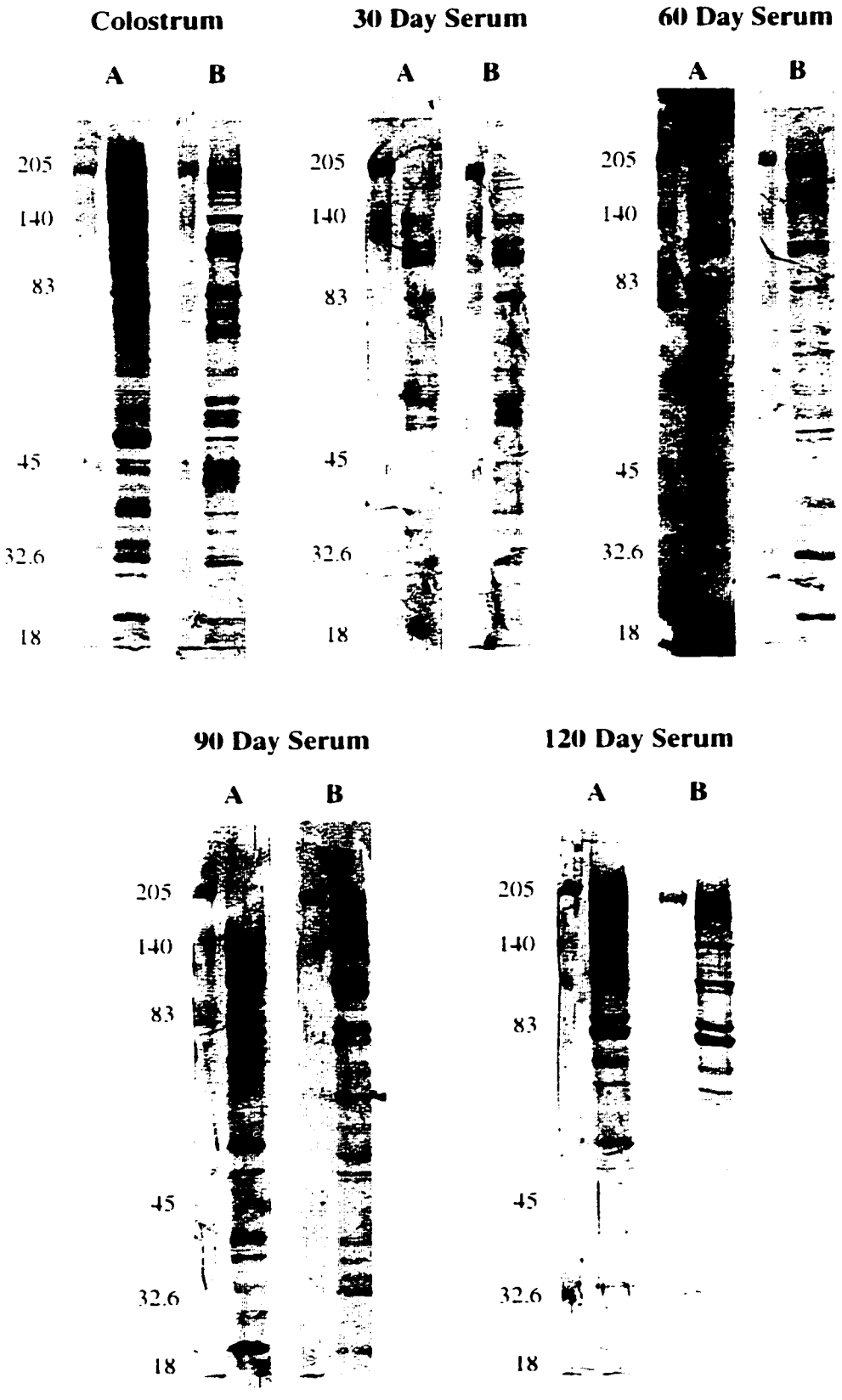
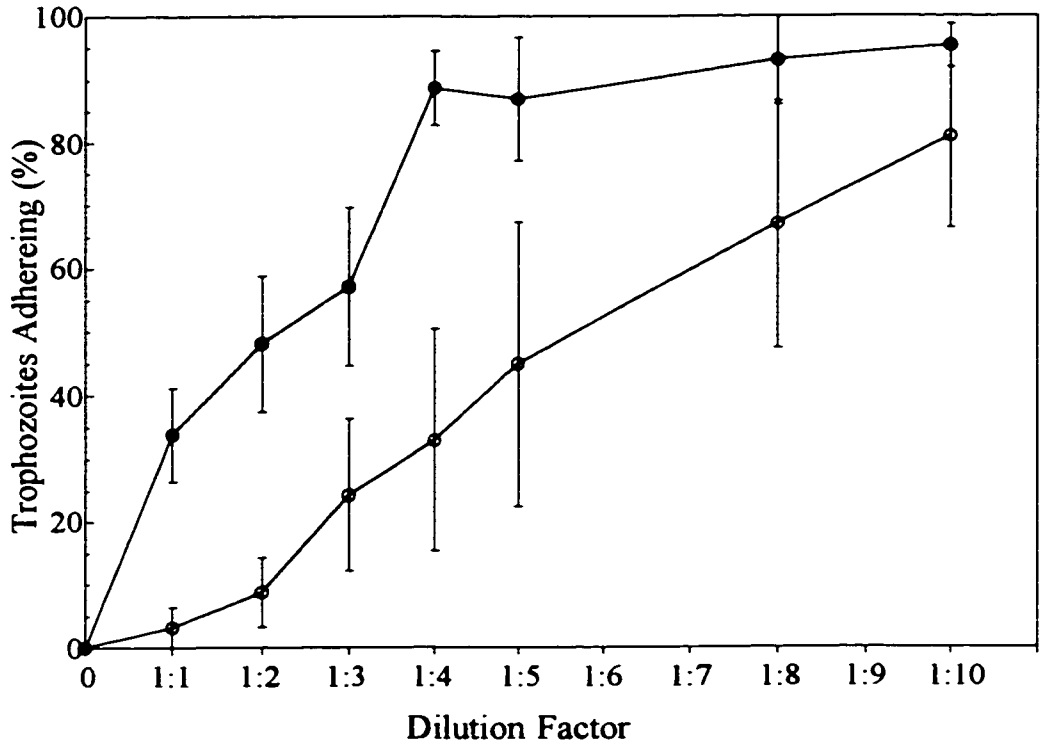

Colostrum
A
B
205
140
83
45
32.6
18
30 Day Serum
A
B
205
140
83
45
32.6
18
60 Day Serum
A
B
205
140
83
45
32.6
18
90 Day Serum
A
B
205
140
83
45
32.6
18
120 Day Serum
A
B
205
140
83
45
32.6
18

Table 5: Molecular weights (in kilodaltons) of *Giardia* antigens recognized by bovine colostrum and serum IgG from a calf that cleared the infection (B) and one that failed to clear the infection (A).

Colostrum		30 Day Serum		60 Day Serum		90 Day Serum		120 Day Serum	
A	B	A	B	A	B	A	B	A	B
190	190				190		190		190
180	180				180		180		180
170	170	170	170						
160	160								
150	150							150	150
141	141	141	141	141	141	141	141	141	141
130	130	130	130	130	130	27	130		
117	117	117	117			117	117		
108	108					108	108	108	108
101		101	101	101	101			101	101
92.5		92.5	92.5	92.5	92.5				
82	82	82	82	82	82	82	82	82	82
79	79						79	79	79
73	73						73	73	73
64	64				64		64	64	64
57.5	57.5	57.5	57.5	57.5	57.5		57.5		
54	54	54	54		54	54	54	54	54
52.5	52.5	52.5	52.5	52.5	52.5				
50	50					50	50	50	50
48	48								
45	45								
43	43								
39	39	39	39	39	39	39	39	39	39
35	35	35	35	35	35	35	35		
33	33	33	33	33	33	33	33	33	33
31	31								
27				27	27	27			
25									
21	21			21	21	21	21	21	21

Figure 7.

Number of adherent *Giardia duodenalis* trophozoites following *in vitro* incubation in bovine colostrum (❍) and milk (●). Data is expressed as a percentage ± SEM of repeated assays relative to the negative control (saline). Slope of lines was used to calculate the 50% adherence inhibition concentrations (Colostrum IC_{50} = 1:6.2, Milk IC_{50} = 1:1.4).


100
80
60
40
20
0
Trophozoites Adhereing (%)
0
1:1
1:2
1:3
1:4
1:5
1:6
1:7
1:8
1:9
1:10
Dilution Factor

3.2. Efficacy of Fenbendazole as a Treatment for Giardiasis in Calves

All 35 calves were confirmed positive for *Giardia* infection according to the pre-study fecal examinations. With the exception of treatment group 1 (single dose, 5 mg/kg) fenbendazole at all dosages and treatment schedules was 100% effective at eliminating cysts from the feces of these naturally infected calves (Table 6, Figure 8). The mean number of days to cyst elimination ranged from 1.2 days for group 5 calves (20 mg/kg, 3 days), to 5.2 days for group 6 calves (0.833 mg/kg, 6 days) (Table 6). *Giardia* cyst numbers were below detection limits in samples collected from every calf 3 days after initiating treatment in groups 2, 3, and 4 (calves receiving FBZ daily for 3 days at 5, 10, and 20 mg/kg respectively, Figure 8). Elimination of *Giardia* cysts from every calf receiving a single dose of 10 mg/kg required 4 days, whereas it required 6 days to completely eliminate cysts from group 6 calves.

Giardia cysts did reappear in fecal samples from some calves after initial elimination (Table 6). In group 2, cysts were detected in 3 of 5 calves, following treatment, within the 28 day period. In these calves, cysts were observed on day 9 (2 calves) and day 28 (1 calf). In group 4, calves also passed fecal cysts following initial elimination. Three group 4 calves passed fecal cysts on day 13 (1 calf) and day 28 (2 calves). In groups 5 and 6, one calf passed fecal cysts after elimination on day 28 of the study. Following initial elimination, fecal *Giardia* cysts were not observed in group 3 calves for the duration of the study.

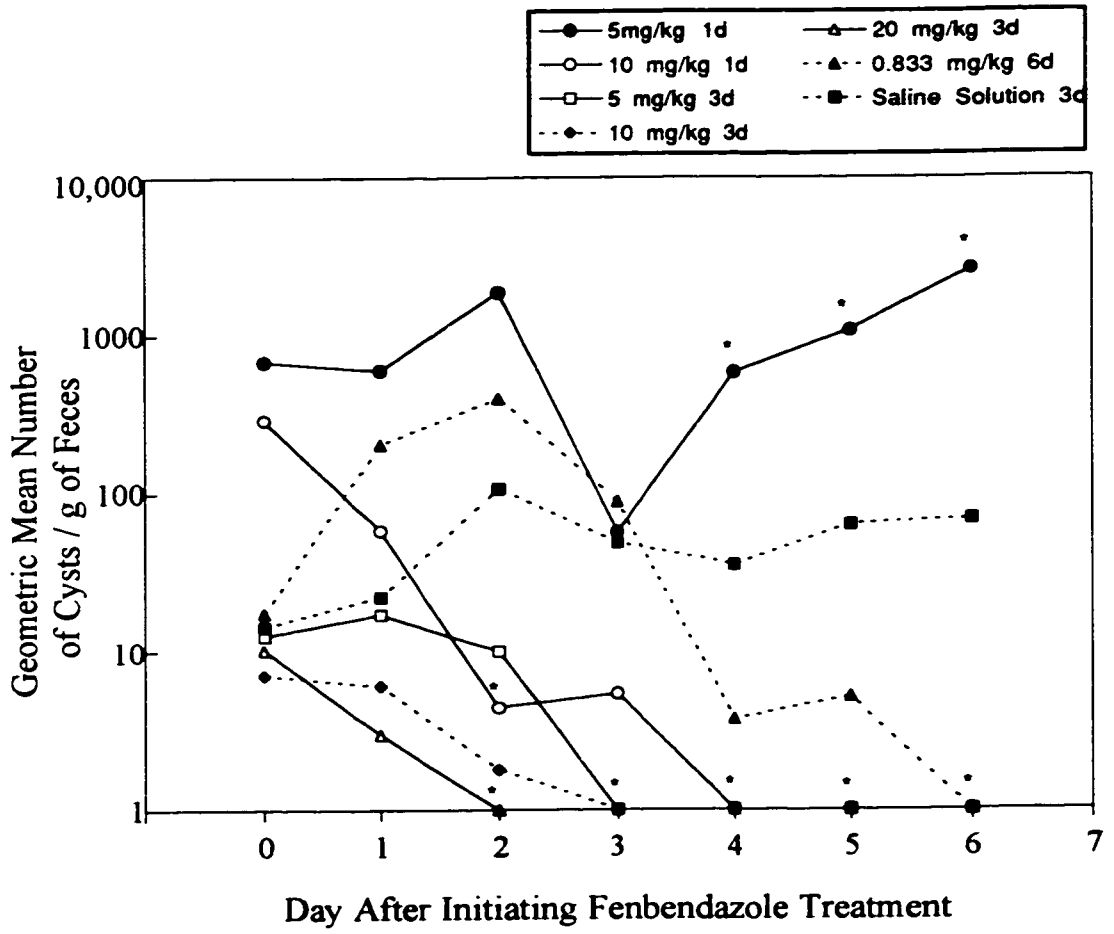
Table 6: Therapeutic efficacy of fenbendazole (FBZ) treatments for giardiasis in dairy calves

Group No.	Treatment	Clearance†	Reinfection‡	Mean days to cyst elimination
1	FBZ (5 mg/kg, 1 day)	0% (0/5)	NA	NA
2	FBZ (10 mg/kg, 1 day)	100% (5/5)*	60% (3/5)	2.6 ± 0.6
3	FBZ (5 mg/kg, 3 days)	100% (5/5)*	0% (0/5)	2.4 ± 0.4
4	FBZ (10 mg/kg, 3 days)	100% (5/5)*	60% (3/5)	1.6 ± 0.4
5	FBZ (20 mg/kg, 3 days)	100% (5/5)*	20%(1/5)	1.2 ± 0.2
6	FBZ (0.833 mg/kg, 6 days	100% (5/5)*	20% (1/5)	5.2 ± 1.5
7	Saline solution (5 ml, 3 days)	20% (1/5)	NA	NA

†Percentage of calves that cleared the infection (No. of calves not passing cysts after treatment/total No. of calves). ‡Percentage of calves that were passing cysts within 28 days after treatment. *Significantly different from saline group ($P < 0.05$).
NA = Not applicable

Figure 8.

Geometric mean numbers of *Giardia duodenalis* cysts from the feces of calves following various fenbendazole treatments and a placebo saline treatment (See legend). All treatments were initiated on day 0. *P < 0.05 fenbendazole treatment counts compared to the saline treatment.


5mg/kg 1d
20 mg/kg 3d
10 mg/kg 1d
0.833 mg/kg 6d
5 mg/kg 3d
Saline Solution 3d
10 mg/kg 3d
10,000
1000
100
10
1
Geometric Mean Number of Cysts / g of Feces
0
1
2
3
4
5
6
7
Day After Initiating Fenbendazole Treatment

3.3 Effect of Fenbendazole Treatment on Trophozoites and Intestinal Structure and Function

All 12 calves were confirmed positive for *Giardia* infection by one of the two pre-study fecal examinations. Prior to treatment, the geometric mean numbers of *Giardia* cysts were 1820 per gram of feces and 1826 per gram of feces for calves in the FBZ and placebo treatment groups respectively (Table 7). In fecal samples collected 7 days after the initiation of treatment, the geometric mean number of cysts per gram of feces was significantly reduced in the FBZ treated calves compared to calves in the placebo group (Table 7). The geometric mean numbers of cysts per gram of feces 7 days after treatment were 13,401 for calves treated with saline and 2.15 (one calf had one cyst detected on a slide) for calves in the FBZ treated group.

Coinciding with the decline in fecal cyst excretion, the number of *Giardia* trophozoites observed in the small intestine of calves was significantly reduced following FBZ treatment. After 7 days, the number of trophozoites in any intestinal segments in the FBZ treated calves were below detection limits (5×10^3 trophozoites per cm^2), while in the placebo group, trophozoites were observed in all sections of the intestine (Table 8).

Histologically, there was no difference between the two treatment groups with respect to villous height or crypt depth (Figure 9). However, the number of intraepithelial lymphocytes (IELs) observed per 100 enterocytes was significantly reduced in the duodenum and jejunum of calves that were treated with FBZ (Figure 10). Calves in the FBZ treated group had a mean count of 14.0 IELs per 100 enterocytes in the duodenum and 21.6 IELs per 100 enterocytes in the jejunum. Calves in the saline group had IEL counts of 17 per 100 enterocytes in the duodenum and 30.7 per 100 enterocytes in the jejunum. Trophozoites were observed attached to the epithelium, or within the lumen of

calves in the saline treated group. However, no trophozoites were observed during histological examination of the intestine of any calves in the FBZ treatment group.

From TEM micrographs taken from calves treated with FBZ, the microvilli appeared to be more numerous and longer compared to micrographs from placebo treated animals (Figure 11). Measurements confirmed these observations, as the microvillous surface area per μm^2 of cell surface was significantly increased in the jejunum of calves treated with FBZ compared to calves in the placebo group (Figure 12). Mean jejunal microvillous surface area in calves treated with FBZ was 32.1 μm^2 compared to 22.8 μm^2 in calves treated with saline. Brush border enzyme activity coincided with the increase in microvillous surface area in the jejunum (Figure 13). Mean maltase activity in calves treated with FBZ was 22.5 u/g of protein compared with 12.1 u/g of protein in calves treated with saline. Mean lactase activity was 3 times higher in calves treated with FBZ compared to the placebo group, mean activity being 31.1 u/g of protein and 10.4 u/g of protein respectively. However, this difference was not found to be significant due to a large standard error. In the duodenum, maltase activity was not significantly different between groups, but lactase activity was significantly increased in calves treated with saline compared to calves treated with FBZ (Figure 14). No differences with respect to maltase or lactase activity in the ileum were observed between groups (Figure 15).

Table 7: *Giardia duodenalis* cysts per gram of feces from infected calves prior to treatment and 7 days following treatment with fenbendazole and saline. Data presented as geometric mean and (mean natural log ± SEM) cysts per gram of feces.

Treatment	Pre-Treatment	Post-Treatment
Saline	1825 (7.5 ± 1.4)	13,400 (9.5 ± 0.3)
Fenbendazole	1820 (7.5 ± 1.5)	2.14* (0.8 ± 0.8)

*Significantly different from saline group ($P < 0.01$)

Table 8: *Giardia duodenalis* trophozoites per cm^2 of small intestine from infected calves treated with fenbendazole and saline. Data presented as geometric mean and (mean natural log ± SEM) trophozoites per cm^2 of intestinal segment.

Treatment	Duodenum	Proximal Jejunum	Distal Jejunum	Ileum
Saline	11,411 (9.34 ± 0.78)	18,711 (9.84 ± 2.04)	17,381 (9.75 ± 2.06)	3,873 (8.26 ± 1.79)
Fenbendazole	BD*	BD*	BD*	BD*

*Significantly different from saline group ($P < 0.05$)
BD = Below detection limits (5×10^3)

Figure 9.

Villous height and crypt depth in the duodenum, jejunum, and ileum from *Giardia duodenalis* infected calves 7 days following treatment with fenbendazole (□) or saline (■). Values presented as mean ± SEM. Significant differences were not observed between treatment groups.

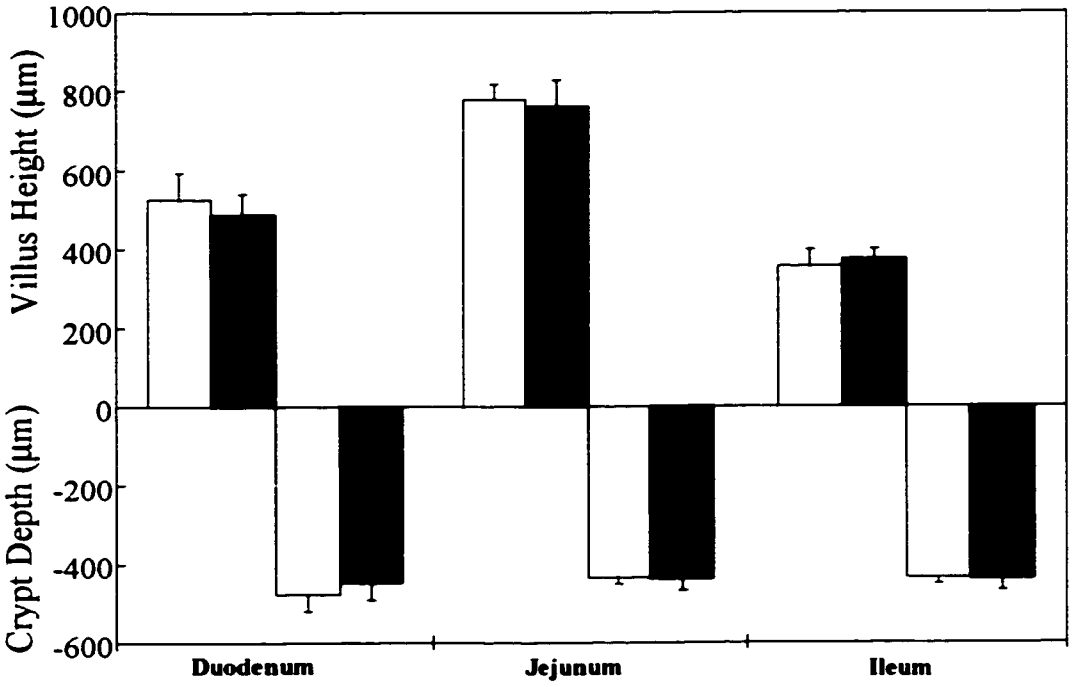

1000
800
600
400
200
0
-200
-400
-600
Villus Height (µm)
Crypt Depth (µm)
Duodenum
Jejunum
Ileum

Figure 10.

Number of intraepithelial lymphocytes (IEL) in calves infected with *Giardia duodenalis* 7 days following treatment with fenbendazole (□) or saline (■). Values presented as mean number of IEL per 100 enterocytes ± SEM. *P < 0.05, **P < 0.001 fenbendazole treated calves compared to saline treated calves.

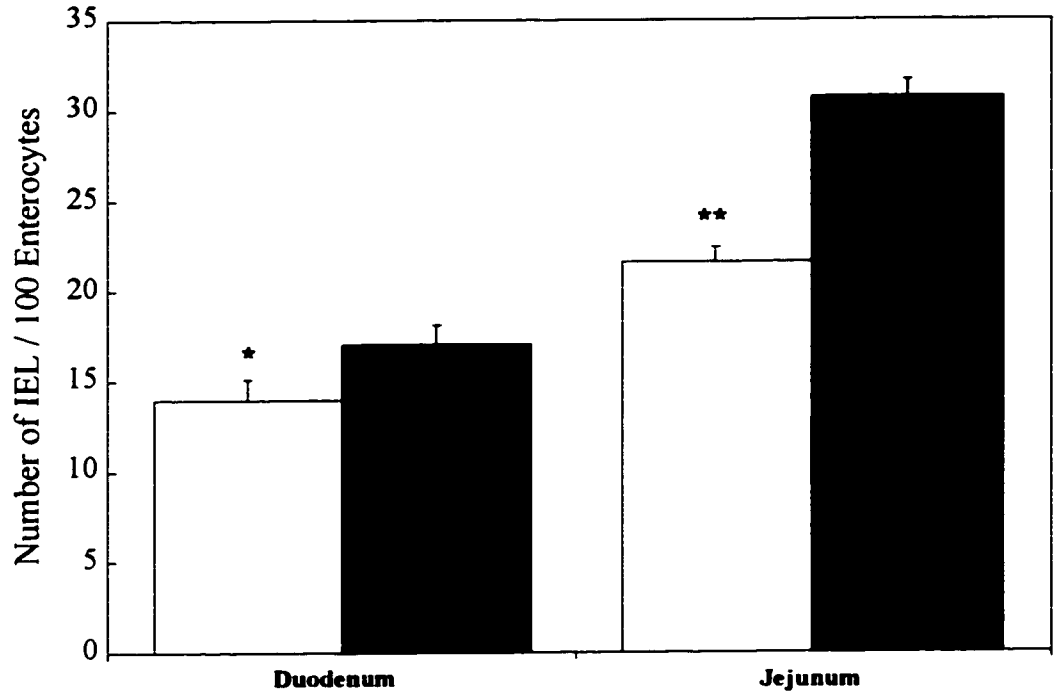

Number of IEL / 100 Enterocytes
35
30
25
20
15
10
5
0
*
**
Duodenum
Jejunum

Figure 11.

Transmission electron micrographs of microvilli from jejunal epithelia at the same magnification in *Giardia duodenalis* infected calves 7 days following treatment with fenbendazole (A) or saline (B). Bar = 1 μm.

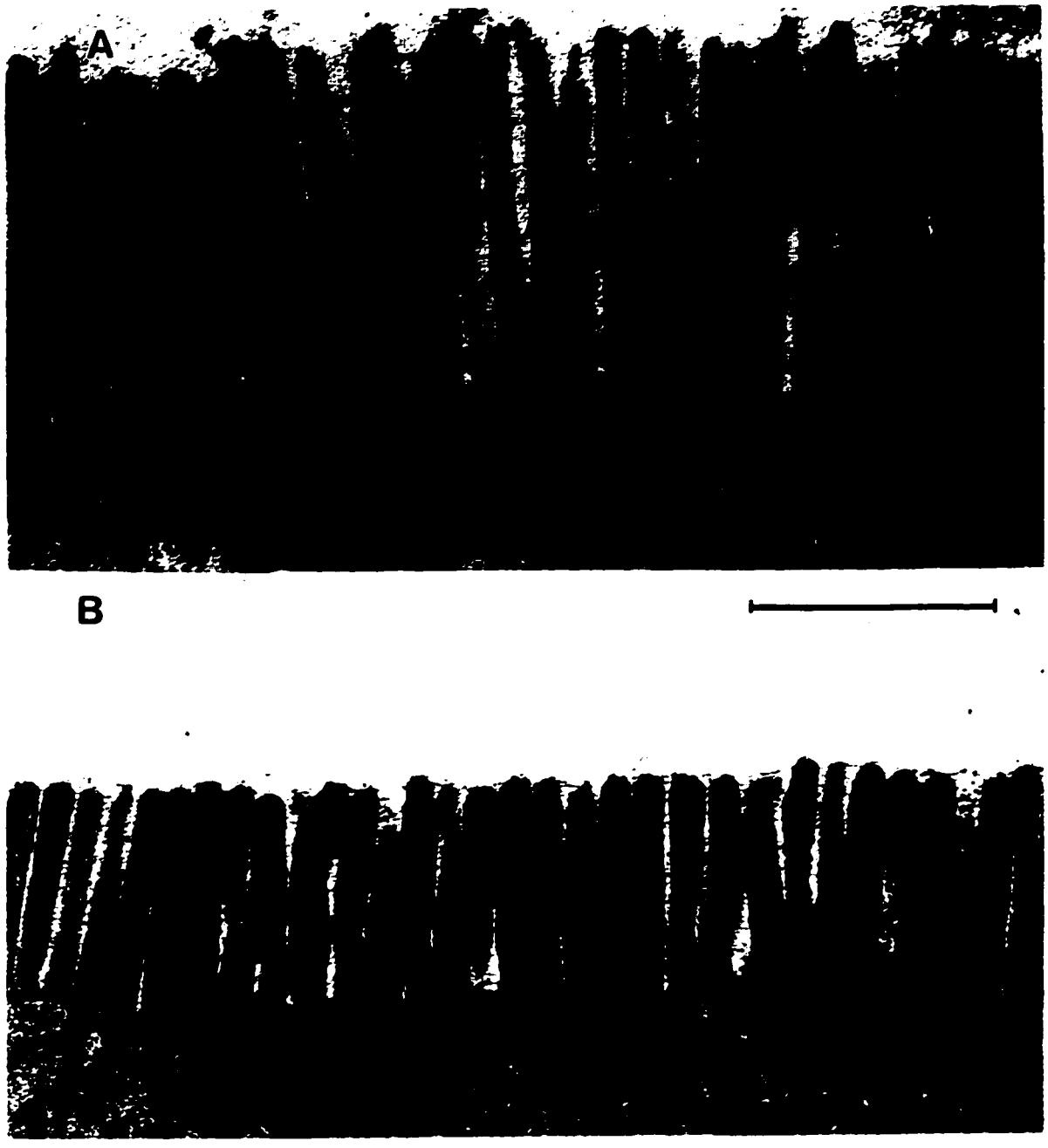

A
B

Figure 12.

Microvillous surface area per μm^2 of cell surface in jejunum of *Giardia duodenalis* infected calves 7 days following treatment with fenbendazole (□) or saline (■). *P < 0.001 fenbendazole treated calves compared to saline treated calves.

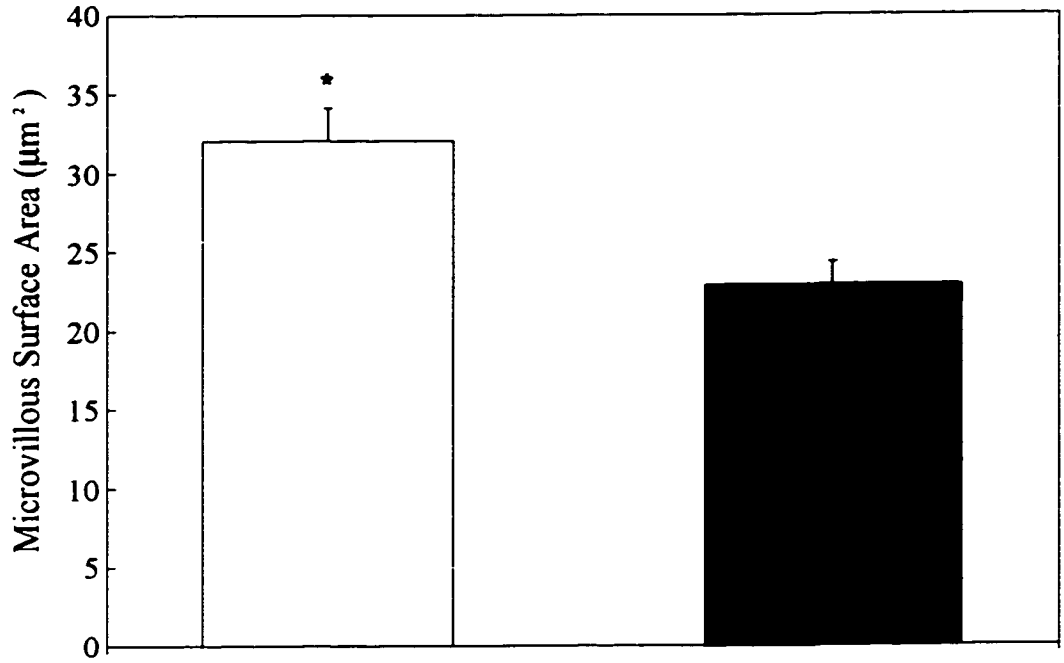

Microvillous Surface Area (µm²)
40
35
30
25
20
15
10
5
0
*

Figure 13.

Mucosal maltase and lactase activity in the jejunum of calves infected with *Giardia duodenalis* 7 days following treatment with fenbendazole (□) or saline (■). Data is expressed as mean ± SEM units per gram of protein. *P < 0.05 fenbendazole treated calves compared to saline treated calves.

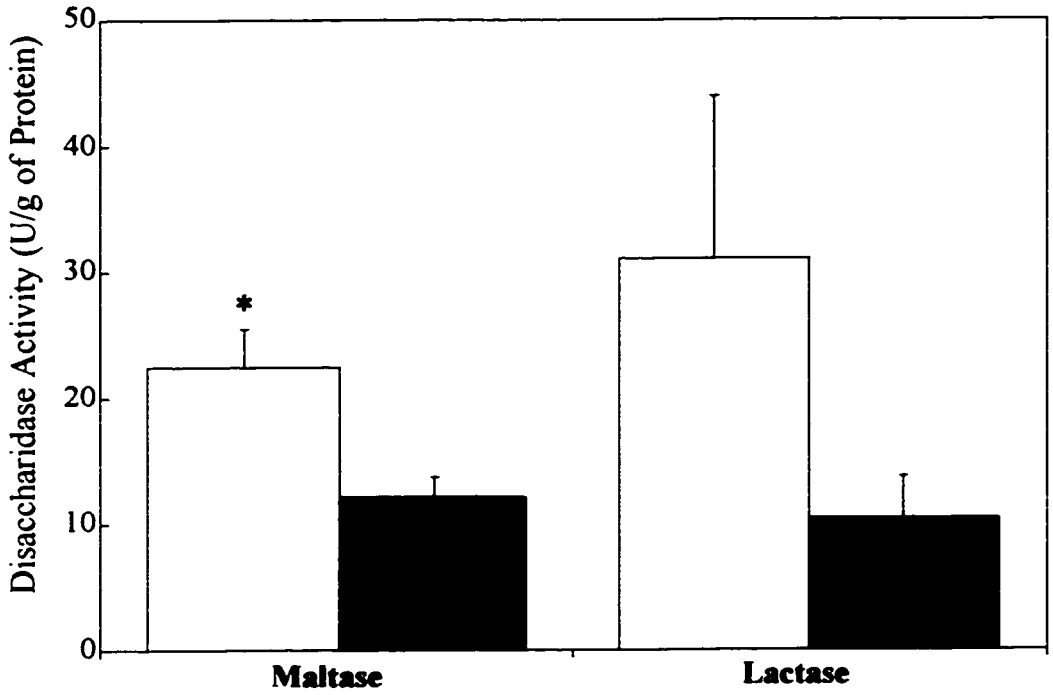

Disaccharidase Activity (U/g of Protein)
50
40
30
20
10
0
*
Maltase
Lactase

Figure 14.

Mucosal maltase and lactase activity in the duodenum of calves infected with *Giardia duodenalis* 7 days following treatment with fenbendazole (□) or saline (■). Data is expressed as mean ± SEM units per gram of protein. *P < 0.05 fenbendazole treated calves compared to saline treated calves.

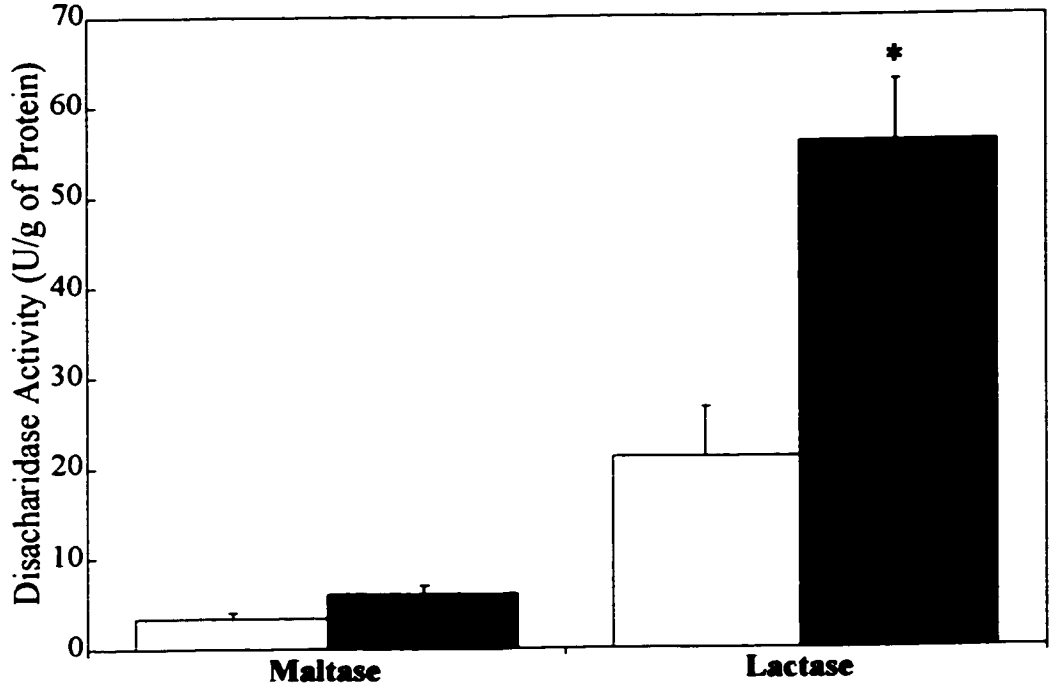
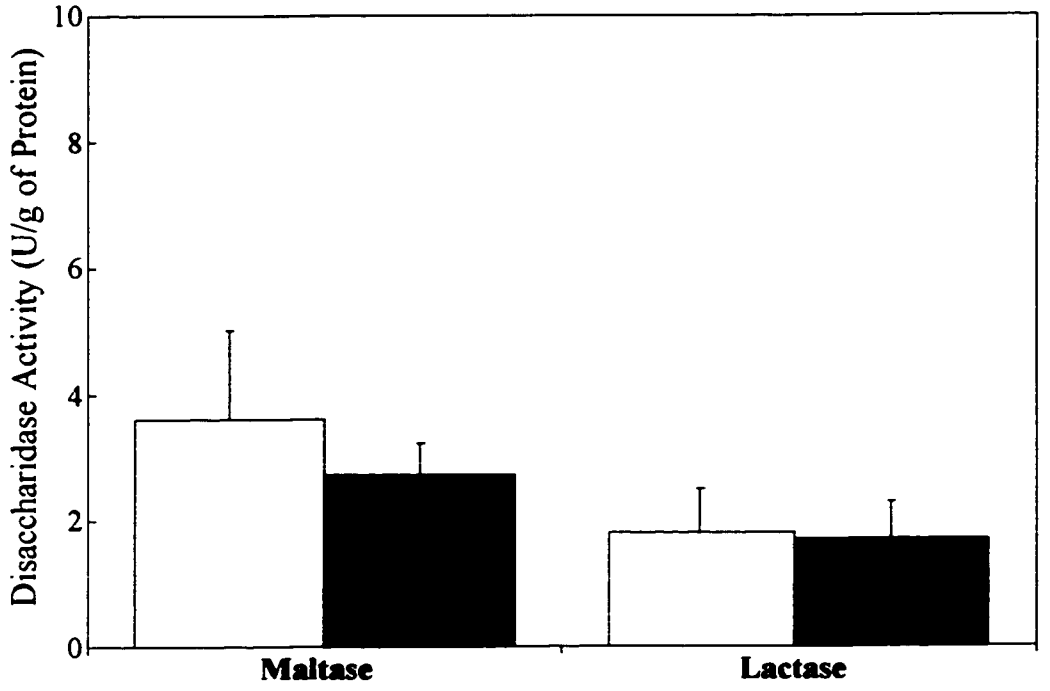

Disacharidase Activity (U/g of Protein)
70
60
50
40
30
20
10
0
*
Maltase
Lactase

Figure 15.

Mucosal maltase and lactase activity in the ileum of calves infected with *Giardia duodenalis* 7 days following treatment with fenbendazole (□) or saline (■). Data is expressed as mean ± SEM units per gram of protein. No significant differences were detected between groups.


Disaccharidase Activity (U/g of Protein)
10
8
6
4
2
0
Maltase
Lactase

3.4. Efficacy of Repeat Fenbendazole Treatment in Dairy Calves with Giardiasis on Clinical Signs and Animal Performance

The number of calves infected with *Giardia* and the geometric mean number of *Giardia* cysts shed by calves differed between groups following each treatment (Figure 16A, 16B). Seven days after treatment with fenbendazole the number of calves shedding *Giardia* cysts was significantly reduced. Also, calves had fewer cysts detected in their feces following treatment compared with calves that received saline. This reduction in the number of calves infected and fecal cyst output lasted a maximum of two weeks post treatment, after which the number of infected animals and fecal *Giardia* cysts returned to control levels. This pattern of reinfection was consistent after every treatment period.

Calves from both groups gained weight at a constant rate throughout the study, and the mean body weight from each group did not differ (Figure 17). Also, there was no significant difference in average daily gain between the fenbendazole treated and saline treated groups (Table 9), and differences could not be detected between groups with respect to feed intake (Table 9).

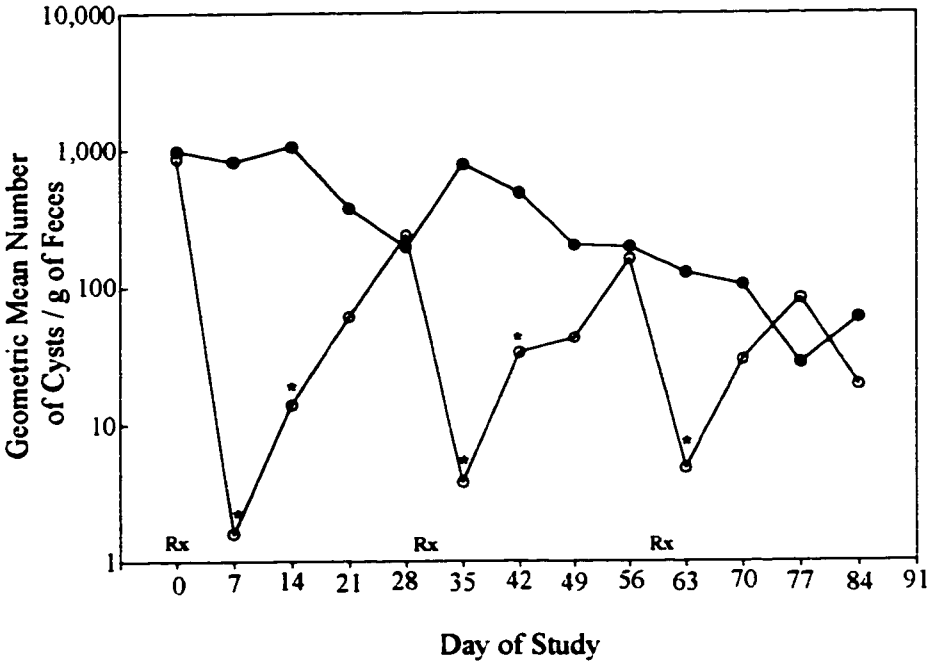
Clinically, differences were observed between the 2 treatment groups. As a group, fenbendazole treated calves had a total of 56 days in which diarrhea was observed during the study, while saline treated calves had diarrhea for a total of 82 days (Table 10). Calves in the FBZ treated group had an average of 0.83 episodes of diarrhea per animal during the study, while calves in the saline treated group had 0.80 episodes of diarrhea per animal. However, the mean duration of each episode differed significantly between groups. The duration of diarrhea episodes occurring in the FBZ treated group was 4.12 days, significantly less than an average duration of 6.21 days for diarrhea episodes in the saline treated group (Table 10).

Cryptosporidium parvum and *Eimeria* spp. were the only other parasites to be observed in the feces of calves during this study. The number of *Cryptosporidium parvum* and *Eimeria* oocysts shed by calves did not differ between each group and oocysts were only observed during the first three weeks of the study.

Figure 16.

Geometric mean number of *Giardia duodenalis* cysts from the feces of calves (A) and the percentage of calves shedding cysts (B) per week following repeated treatments with fenbendazole (❍) or saline (●). Rx indicates initiation of each treatment. *$P < 0.05$, ** $P < 0.01$ fenbendazole treatment compared to saline treatment.

A


10,000
1,000
100
10
1
Geometric Mean Number of Cysts / g of Feces
Rx
Rx
Rx
0 7 14 21 28 35 42 49 56 63 70 77 84 91
Day of Study

B

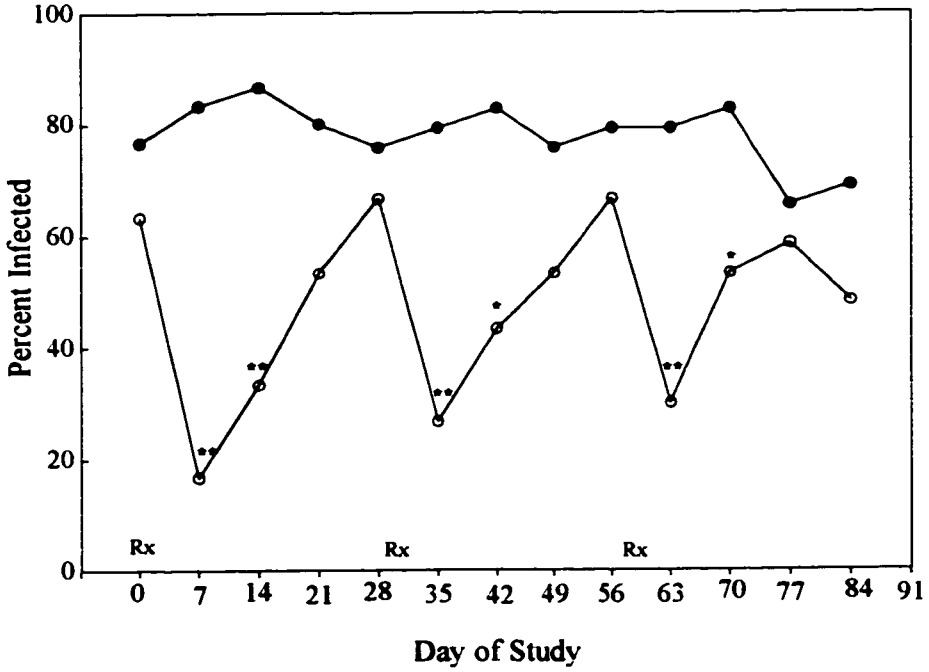

100
80
60
40
20
0
Percent Infected
Rx
Rx
Rx
0 7 14 21 28 35 42 49 56 63 70 77 84 91
Day of Study

Figure 17.

Mean weekly body weights of calves infected with *Giardia duodenalis* following repeated fenbendazole (❍) or saline (●) treatments. Data is expressed as mean ± SEM. Rx indicates initiation of each treatment. Significant differences were not observed between the two groups at any time point.

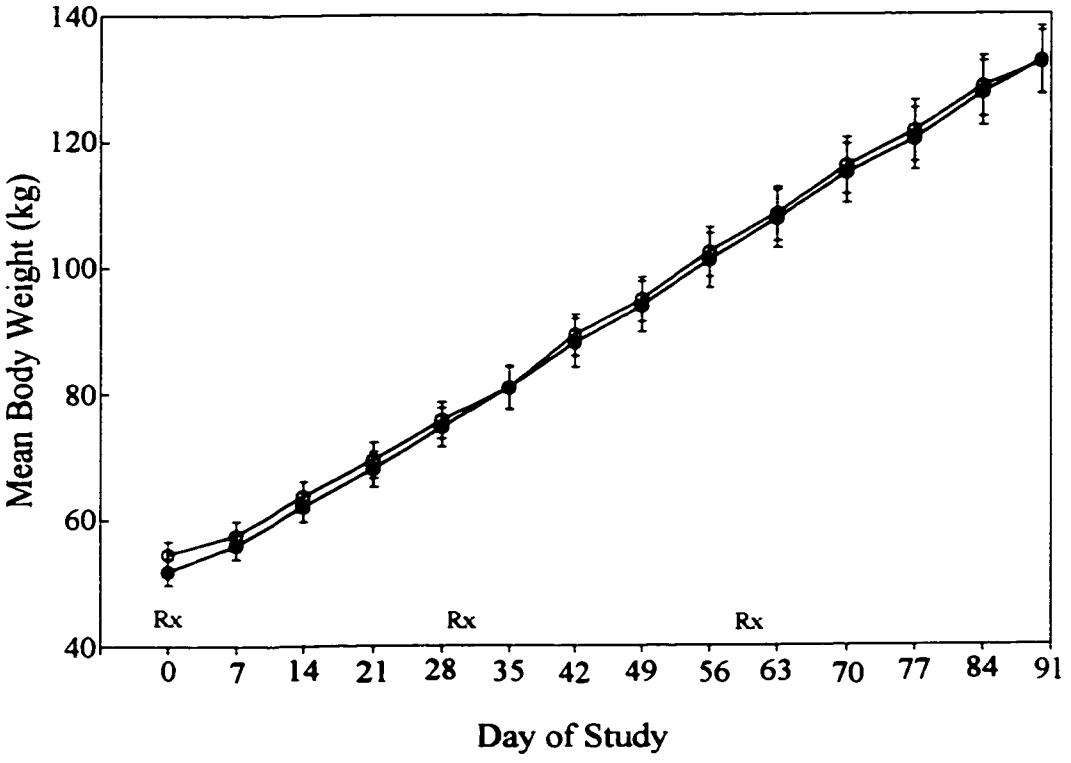

Mean Body Weight (kg)
140
120
100
80
60
40
Rx
Rx
Rx
0
7
14
21
28
35
42
49
56
63
70
77
84
91
Day of Study

Table 9: Effect of repeat fenbendazole treatment for giardiasis on dairy calf performance

Treatment Group	Mean Initial Weight (kg)	Mean Final Weight (kg)	Average Daily Gain (kg)	Mean Feed Intake (kg)[a]
Fenbendazole	54.5 ± 2.1	132.3 ± 4.9	0.862 ± 0.03	214.01 ± 11.1
Saline	51.8 ± 2.0	132.6 ± 5.3	0.898 ± 0.04	216.86 ± 11.6

[a] Feed intake on a dry matter basis (milk replacer, feed concentrate, and alfalfa hay)

Table 10: Number and duration of diarrhea episodes and total days with diarrhea in calves treated with fenbendazole and saline.

Treatment	Mean Number of Diarrhea Episodes	Mean Duration of Diarrhea Episodes	Total Calf Days with Diarrhea
Fenbendazole	.083 (± 0.22)	4.12 (± 0.47)*	56*
Saline	0.80 (± 0.18)	6.21 (± 0.81)	82

*P < 0.05 compared to saline treated group

3.5. GENOTYPIC CHARACTERIZATION OF *GIARDIA* FROM WESTERN AUSTRALIAN AND WESTERN CANADIAN DAIRY CALVES

Giardia duodenalis was identified in dairy calves from both Western Canada and Western Australia. The prevalence of *Giardia,* and number of cysts shed by positive animals, did not differ between calves from Western Australia and calves from Western Canada (Table 11). In Western Australia, 58% (21/36) of dairy calves had *Giardia* cysts in their feces, and 57% (16/28) of Western Canadian dairy calves were positive at the time of sampling. The geometric mean cysts shed per gram of feces for Western Canadian calves was 3475 compared to 839 for Western Australian calves, these numbers did not differ significantly. No significant difference with respect to *Giardia* prevalence or cysts per gram of feces was observed between groups of calves housed individually (Lethbridge, Alberta), group penned (Farm A, Western Australia) or pastured (Farm B Western Australia).

The genetic sequence for the 292-bp region of the 5' end of the 16S-rRNA gene was obtained from *Giardia* isolates from 10 Western Canadian calves, while 6 sequences were obtained from isolates from Western Australian calves (Table 12). For the Western Canadian isolates, 8 had similar sequences to the previously described Genotype (Assemblage) A sequence [119], but had a single nucleotide difference at position 92. Of the 5 isolates obtained from Western Australian calves, 4 sequences had the same nucleotide substitution as the 8 Western Canadian isolates. Two isolates from the Western Canadian calves and 1 isolate from the Western Australian calves had the identical genetic sequence to the Genotype (Assemblage) A sequence.

Table 11: Prevalence and geometric mean cysts per gram of feces of *Giardia* in dairy calves from Western Australia and Western Canada.

Location	Number of Calves	Number Positive (%)	Geometric Mean Cysts / g Feces
Lethbridge, Alberta	28	16 (57)	3475
Perth, Western Australia	36	21 (58)	840

Table 12: Genetic sequence of the 292 bp fragment of the 16S rRNA gene from Group 1 *Giardia* isolates and *Giardia* isoloates found in livestock. Isolates obtained from 12 calves were identicle to the Livestock sequence. Isolates obtained from 3 calves were identicle to the Genotype A sequence.

Group	bp	Sequence	bp
Genotype A	1	CATCCGGTCGATCCTGCCGGAGCGCGACGCTCTCCCCAAGGAC-GAAGCC	49
Livestock	1	·····	49
Genotype A	50	ATGCATGCCCGCTCACCCGGGACGCGGCGGACGGCTCAGGACAACGGTTG	99
Livestock	50	·····G·····	99
Genotype A	100	CACCCCCCGCGGCGGTCCCTGCTAGCCGGACACCGCTGGCAACCCGGCGC	149
Livestock	100	·····	149
Genotype A	150	CAAGACGTGCGCGCAAGGGCGGGCGCCCGCGGGCGAGCAGCGTGACGCAG	199
Livestock	150	·····	199
Genotype A	200	CGACGGCCCGCCCGGGCTTCCGGGGCATCACCCGGTCGGCGCGGTCGCGG	249
Livestock	200	·····	249
Genotype A	250	CGCGCCGAGGGCCCGACGCCTGGCGGAGAATCAGGGTTCGACT	292
Livestock	250	·····	292

4. DISCUSSION

This is the first study to fully examine the biology of giardiasis in dairy calves and the impact the disease has within a normal dairy operation. Within this dairy operation, the cumulative infection rate for both naturally occurring *Giardia* and *Cryptosporidium* infections was 100% in these calves. An infection rate of 100% for *Giardia* and *Cryptosporidium* infections in dairy calves was previously reported [24], however most previous cross sectional studies have underestimated the infection rates of these two parasites [23, 25, 43, 193]. Cross-sectional studies may underestimate these infection rates because cyst and oocyst excretion can be intermittent [46, 129, 194], as was observed in this study. Furthermore, *Cryptosporidium* oocysts are only shed for a few days in immunologically normal calves, and positive calves can easily remain undetected [44, 194]. In this study, the same group of calves was examined for a 4 month period, and fecal samples were collected frequently, and at regular intervals, reducing the possibility of missing calves that were infected.

The 100% infection rate observed in this study suggests that both parasites are easily transmitted between calves in confinement. It is likely that transmission results from calves coming in contact with fecal material from other infected calves. In general, the transmission of infective agents among calves can be prevented if nose-to-nose contact is avoided [195]. The calves in this study did not have direct contact with neighboring animals. However, transmission may have resulted from the transport of infective cysts and oocysts from pen to pen by dairy workers. It is also possible that calves' pens were previously contaminated by infected animals, despite cleaning efforts. As many as 10^6 *Giardia* cysts or *Cryptosporidium* oocysts were shed per gram of feces, and because these cysts and oocysts are environmentally resistant, they can remain

infective for weeks [26, 43, 50]. It is evident that the high concentration of animals in this dairy provided ideal conditions for transmission of these parasites to occur.

In addition to a high infection rate and high rate of transmission, both *Giardia* and *Cryptosporidium* were associated with diarrhea in these calves. It is well established that *Cryptosporidium* is an important pathogen of calves [26]. However, the significance of *Giardia* as a pathogen of calves has often been questioned. Only a few previous studies have examined calves with diarrhea for both *Giardia* and *Cryptosporidium* [1, 23, 44]. Also, most studies have failed to examine calves for both *Giardia* and *Cryptosporidium*, as well as bacterial, and viral pathogens. For example, in a recent study 218 fecal samples from diarrheic calves were examined for *Cryptosporidium,* bacterial, and viral pathogens, but the authors did not examine calves for *Giardia* [12]. By examining fecal samples in this study for bacterial, viral, and parasitic pathogens, a more definite diagnosis of the causes of diarrhea could be made. Confirming the results of previous studies, *Cryptosporidium parvum* was the most prevalent infection associated with diarrhea in these calves [1, 12, 26]. However, identifying *Giardia* in association with many diarrhea episodes in these calves suggests it is also an important pathogen.

Concurrent infections with *Giardia* and *Cryptosporidium* were also associated with many episodes of diarrhea in this study (10/38 episodes). Concurrent *Giardia*-rotavirus infections and *Cryptosporidium*-rotavirus infections were also detected. Concurrent infections with *Giardia* and *Cryptosporidium* were previously reported in calves with diarrhea and in clinically normal calves [25, 44, 196]. Earlier studies have also identified coinfection with *Cryptosporidium* and rotavirus [1, 12, 18, 194]. However, combined *Giardia*-rotavirus infections are not previously reported. It is likely that concurrent *Giardia* and viral or bacterial infections may occur more frequently than thought, however, as mentioned previously, many studies overlook *Giardia* as a pathogen

of calves. Although only 2 episodes of diarrhea were associated with concurrent *Cryptosporidium*-rotavirus infections, these episodes were of longer duration than episodes in which *Cryptosporidium* was the only pathogen identified. In previous studies, where concurrent *Cryptosporidium*-rotavirus infections were associated with diarrhea, duration of the diarrhea episodes were not determined [1, 12]. It is possible that by harboring both pathogens, the immune system may be placed under stress. As a result, the duration of the diarrhea was increased. However, as only two animals in this study had concurrent *Cryptosporidium*-rotavirus infections further study is required.

The only episodes of diarrhea examined during the initial part of this study occurred during the first 45 days after birth. However, the long duration of *Giardia* cyst excretion and a high mean number of cysts in the feces of calves over 45 days of age suggests that giardiasis is likely an important cause of diarrhea in older calves. The mean age at which *Giardia* cysts were first detected in the feces of calves was significantly higher than the mean age at which *Cryptosporidium* oocysts were first detected. Also, calves were of a significantly older age at the onset of diarrhea associated with *Giardia* compared to diarrhea associated with *Cryptosporidium*. Previous studies found the highest prevalence of cryptosporidiosis occurred in calves less than 2 weeks of age, and the highest prevalence of giardiasis in 2 to 7 week old calves [25, 43]. Results of the present study suggest that within this dairy, cryptosporidiosis is the most probable cause of diarrhea in calves less than 4 weeks old, and giardiasis is the most likely cause of diarrhea in calves over 4 weeks of age. On its own, *Giardia* was not associated with diarrhea in calves less than 29 days old. Conversely, *Cryptosporidium* was not associated with diarrhea in calves more than 28 days of age. A similar pattern was observed in beef calves, where the percentage of calves with diarrhea associated with *Giardia* was highest in calves over a month of age [23]. Understanding this relationship between age and parasitic infection may be important for effectively treating diarrhea in calves.

The results of this study clearly demonstrate that *Giardia* infections are chronic in dairy calves, whereas *Cryptosporidium* infections appear to be self-limiting. Most humans and experimental animals, such as the gerbil and the mouse, can eliminate *Giardia* infections through humoral immunity, and chronic giardiasis typically develops only if the host is immunosuppressed [34]. Serum IgG_1 concentrations in these calves indicate they were immunologically normal, and they were able to resolve cryptosporidiosis, as reported in previous studies [24-26]. Only low numbers of *Cryptosporidium* oocysts were shed periodically throughout this study, and periodic shedding by clinically normal calves and adult animals is often reported [24, 194]. With regard to *Giardia*, this study supports previous evidence that it is a chronic infection in calves; when calves were 96 days old, 85% (17/20) were still shedding *Giardia* cysts. Giardiasis has been reported in calves greater than 7 weeks of age [23, 25, 43], with a prevalence as high as 81% [25]. However, the high prevalence in older calves has often been associated with a low number of cysts in the feces [23, 25, 43]. This relationship was not observed in this study. The geometric mean number of cysts per gram of feces did not decrease when calves were older than 7 weeks. In the present study, geometric mean cyst numbers peaked when calves were 89 days old. Also, many calves shed high numbers of *Giardia* cysts intermittently for more than 100 days after shedding was first detected. These results suggest that giardiasis is a chronic infection in normal calves, unlike cryptosporidiosis which is a self-limiting infection of significantly shorter duration.

The infection patterns and chronicity of naturally occurring giardiasis in these calves may be related to the calves' immune response. Mean post infection serum IgG titers were not significantly elevated at any point during the study. The calf that cleared the infection demonstrated an increase in serum IgG titer following infection, however it

was never significantly higher than the mean preinfection IgG titer. Humoral immunity is considered to be important for eliminating *Giardia* infections, as humans with hypogammaglobulinemia experience prolonged symptomatic giardiasis [90]. In humans and mice, specific IgM antibodies are elevated approximately 10 days following infection, with IgG and IgA elevations occurring approximately one week later [91, 92]. The failure of these calves to mount an increased IgG response may explain the chronicity of their *Giardia* infections. In previous studies, lambs, dogs, and cats failed to develop an elevated IgG or IgA response following infection [185, 197, 198]. Giardiasis in lambs also appears to be chronic [46, 185]. It has been proposed that a difficulty in class switching from IgM to IgG may be responsible for the lack of an elevated serum IgG titer during *Giardia* infections in ruminants [185].

Although calves failed to produce increased serum IgG in response to the infection, the IgG immunoblots demonstrate that infection patterns of *Giardia* observed in these calves may be more complex. Colostrum immunoblots reacted with many *Giardia* antigens. Colostrum IgG from calf A bound 29 *Giardia* antigens with molecular weights ranging from 21-190 kDa, while calf B colostrum reacted with 25 antigens within in the same range. In a previous study, 28 crude soluble antigens were identified in the range of 14-200 kDa [199]. Thus, the immunoblots from this study would indicate that colostrum received by the calves bound to most, if not all, *Giardia* antigens within this range. Based on the 100% infection rate for *Giardia* in these calves, it is likely that the dams were also infected with *Giardia* at some point. Considering this, it is not surprising that IgG from the colostrum reacted with many *Giardia* antigens.

Based on radial immunodiffusion assays and immunoblots from calves at 3 days of age, antibodies from the colostrum were successfully transferred to the calf and appear to provide protection against *Giardia*. Protection from rotavirus infection through an

immune dam's colostrum has been previously demonstrated [200], and the suckling of immune milk has been shown to protect mice from *Giardia* infection [201]. Only 4 calves passed *Giardia* cysts in their feces before they were 21 days of age. Three of these calves shed low numbers of cysts for a single day, before continuous shedding was established after calves were 21 days of age. Immunoblots from calves at 30 days of age reacted with the identical antigens, however fewer *Giardia* antigens were bound by serum IgG compared to the colostrum. During their first month of life, calves must produce their own antibodies, as antibodies obtained through passive transfer become depleted within the first two weeks [202]. The onset of *Giardia* infections in these calves coincides with this switch from passive to active immunity. At 30 days of age, calves are producing their own antibodies against *Giardia*. These antibodies do not bind to as many antigens as colostral antibodies and may not offer protection against infection. This may account for the appearance of *Giardia* when calves are approximately 30 days of age.

Both colostrum and serum IgG from these calves reacted with a number of *Giardia* antigens throughout the study. IgG from calves also bound to an increased number of antigens as the infections progressed. In a previous study, IgG from lambs challenged with *Giardia* bound to two antigens with molecular weights of 32 and 49 kDa. Serum from the calves in this study reacted with antigens of comparable molecular weights (33 and 50 kDa), however the seum IgG from calves also reacted with many other antigens. The 33 kDa protein bound by calf IgG has been described in other studies [90, 125, 203]. This protein is thought to be "giardin" which is located on the ventral disk [125]. The 82 kDa protein bound to by IgG from these calves has also been described previously as a common glycoprotein [204]. Other, previously described major antigens, comparable to the ones reacted with by these calves include proteins with molecular weights of 27 and 56 kDa [92], along with a group of surface-exposed trophozoite components from 53-55 kDa [205].

Despite the antigenicity of many major *Giardia* proteins to calf serum, only a few antigens appeared to be important for clearing the infection. In the calf that cleared the infection, clearance was associated with recognition of antigens at 64, 73, and 79 kDa, as well as high molecular weight antigens of 190 and 180 kDa. It has been suggested that recognition of high molecular weight antigens may be important in clearing the infection [199, 206]. The 73 kDa protein may represent the VSP H7 surface antigen, which is thought to be antigenically distinct from other VSPs [207], and the 64 kDa antigen is likely analogous to the GSA-65 antigen previously described by Rosoff and Stibbs [208]. Serum IgG from the representative calf that did not clear the infection bound to these potentially important antigens at 120 days of age. However, fecal samples were not collected past 120 days, and it is not known if recognition of these antigens would have been associated with clearance of the infection by this calf.

Interestingly, both colostrum and milk demonstrated efficacy against *Giardia* using the in vitro adherence assay. In previous studies, milk from humans and primates was found to kill *Giardia* trophozoites in vitro [209-211], however bovine milk was not effective [209]. The killing effect of human milk is due to the presence of bile salt-stimulated lipase, which is not found in bovine milk [209]. It is possible that the colostrum and milk from cows in this study only prevented attachment of trophozoites *in vitro* through opsonization [34], as both colostrum and milk contained anti-*Giardia* antibodies based on their immunoblots. It is also possible that milk and colostrum kill trophozoites through another mechanism, such as complement [97]. Nevertheless, the larger amounts of antibodies in colostrum may have accounted for its higher efficacy against *Giardia*.

The results of the initial study demonstrate the need for developing an effective and cost-efficient method for treating giardiasis in calves. Currently, there are no therapeutic agents with label claims for treating giardiasis in food animals. Many drugs such as metronidazole are effective in treating *Giardia* infections in humans, companion animals, and cattle [43, 132, 212, 213]. However, the undesirable side effects and toxicologic data of these compounds make it unlikely that they will be approved for treating giardiasis in food animals. Metronidazole, for example, is a mutagen and teratogen [132], which can cause moderate-to-severe intestinal and neurologic disease in dogs [133, 134]. Many recent studies demonstrate several benzimidazoles to be effective in treating giardiasis [135, 137, 214, 215]. Benzimidazoles are 50 times more effective in vitro than metronidazole [141], and in a previous study albendazole and fenbendazole reduced or eliminated *Giardia* cysts from the feces of naturally infected calves [137]. The results presented in this study confirm that fenbendazole is effective at eliminating *Giardia* cysts when administered to calves, but at a much lower dose than previously reported [137]. Fenbendazole was 100% effective in eliminating *Giardia* cysts from the feces of calves using a number of different treatment regimes. Only calves receiving a single dose of 5 mg/kg were still shedding fecal *Giardia* cysts 6 days following treatment. In other studies, single doses of albendazole or fenbendazole were insufficient for treating giardiasis in dogs [135, 216]. These results suggest that duration of treatment may be more important than dose when treating giardiasis. This is clearly evident in this study as complete elimination of *Giardia* cysts in the feces was achieved by providing a 5 mg/kg dose divided over a six day period (group 6, 0.833 mg/kg 6 days), whereas a single dose of 5mg/kg was ineffective.

Although previous studies demonstrate that fenbendazole is effective at eliminating *Giardia* cysts from the feces of dogs and calves [135, 137], the efficacy of the drug against trophozoites *in vivo* was not previously examined. In addition to eliminating

Giardia cysts from the feces of these calves, fenbendazole treatment in this study completely eliminated trophozoites from the small intestine of infected calves. In addition, elimination of trophozoites following treatment resulted in a physiological benefit. Previous studies demonstrate giardiasis can be associated with a number of pathological characteristics in laboratory animals and humans [74, 75, 77, 78, 82]. However, few studies have examined the pathophysiology of giardiasis in calves. The authors of one study observed villous atrophy and an increase in IELs in calves infected with *Giardia* when compared to non-infected calves [30]. However, these authors did not examine intestinal enzyme activity or microvillous surface area following infection [30]. The study presented here can not represent a true comparison between infected and non-infected calves. However, treating calves with fenbendazole was 100% effective at eliminating *Giardia* trophozoites from the intestinal tract. Thus, the physiology of treated calves may be similar to non-infected animals.

When compared to untreated calves, it appears that treated calves had some morphological and functional recovery of the small intestine. Calves treated with FBZ had increased brush border surface area and increased maltase activity in the jejunum. FBZ treatment also resulted in a decrease in the number of IELs present within the epithelium. A diffuse shortening of the microvillous border is characteristic of giardiasis in laboratory animals [74, 82], and this reduction in brush border surface area plays a major role in contributing to the malabsorption associated with the disease. Coinciding with the increase in brush border surface area, in the present study, was an increase in maltase activity. Thus, treatment appears to result in the recovery of the microvillous and brush border enzymes. This recovery would provide calves with a greater surface area and capacity for absorption, which could translate into an economic benefit through greater production. Surface area measurements were only made in the jejunum of these calves because trophozoites most heavily colonized this section of the gut. Therefore, it

is unknown if an increase in brush border surface area resulted in other sections of the intestine following treatment.

Lactase activity was found to be almost 3 fold higher in the jejunum of FBZ treated calves compared to the saline treated calves, however this difference was not significant. Also, lactase activity was significanty increased in the duodenum of saline treated calves compared to the FBZ group. The effect of giardiasis on disaccharidase activity can be variable, and infection has been shown to result in an increase of some enzymes in laboratory animals [84]. Furthermore, lactase activity is quite variable in calves of this age as they make a transition from pre-ruminant to ruminant [217] thus, both the effects of the parasite and the calf have to be considered.

Treating calves for giardiasis also reduced the number IELs present within the epithelium of the duodenum and jejunum. The role of the IEL during *Giardia* infection is not fully understood. IELs have been implicated in the pathophysiology of giardiasis [75], however increased IELs are not always observed or found to be activated during *Giardia* infection [78, 84, 96, 104]. An increase in IELs was previously demonstrated in *Giardia* infected calves [30], and increased IELs are associated with pathology in celiac disease [104]. Thus, the reduction of IELs following treatment for giardiasis may be beneficial.

No change was observed in villous height or crypt depth following treatment. However, alterations in villous architecture during giardiasis are not always observed [75]. It is possible that calves were not given enough time between treatment and sacrifice to allow for any changes with respect to villous height and crypt depth to occur. It is also possible that *Giardia* infection in these calves had no effect on villous and crypt morphology. However, without the comparison of a noninfected control group, the

effects of *Giardia*, or treatment, on villous architecture in these calves can not be determined.

Fenbendazole-mediated improvement in intestinal morphology was associated with clinical benefits. Repeated monthly treatments led to a decreased number and reduced duration of diarrhea episodes. As the pathological characteristics associated with giardiasis result in a malabsorptive diarrhea [74, 75], recovery of intestinal enzyme activity and microvillous surface area in calves following fenbendazole treatment likely results in greater absorptive capacity in the small intestine. This may have played a role in reducing the number and severity of the diarrhea episodes in the treated calves.

Despite the clinical and physiological improvement in calves treated for giardiasis with FBZ, there was no difference between the treatment groups with respect to production parameters. In a specific pathogen free ruminant model, giardiasis had a significant impact on production parameters [129]; however, in this previous study, control animals were prevented from becoming infected with *Giardia*. In the present study, calves were initially infected with *Giardia*, treated with FBZ to clear their infections, and became reinfected following treatment. This may have prevented any improvement in performance. Reinfection occurred rapidly after calves were treated with fenbendazole regardless of the treatment regime employed. Following repeated monthly treatments, the number of *Giardia* cysts passed in the feces was significantly reduced. However, cyst excretion levels in fenbendazole treated calves returned to the same level as saline treated calves within two weeks after each treatment. The prepatent period for *Giardia* is between 7 and 10 days [129], therefore, trophozoite colonization of calves' intestinal tracts could have occurred within a week after treatment. Although the length of time calves spent free of infection was sufficient to allow for intestinal recovery and reduce clinical signs, calves may not have had sufficient time to completely recover.

It appears from this study that calves may have to be kept free from *Giardia* in order for FBZ treatment to have an impact on production. It is unlikely that producers would treat calves for giardiasis unless treatment was cost effective. Thus, in order to prevent reinfection from occurring, it may be necessary to employ a different treatment regime. A dose of 5 mg/kg of fenbendazole administered once daily for 3 days was used for repeated treatment in these calves. However, a much smaller dose of .83 mg/kg administered over 6 days was also effective at eliminating *Giardia*. The efficacy of this treatment would suggest a continuous, low dose treatment may prevent calves from becoming reinfected with *Giardia* and may result in improved performance. This type of treatment could be incorporated into feed or mineral for easy administration. Further investigation is required with respect to efficacy of such a treatment and possible development of resistance.

Although this work primarily focused on calves from Southern Alberta, it is the first known study to examine the prevalence of *Giardia duodenalis* in Australian dairy calves. The results suggest that *Giardia* must not be overlooked as an important parasite to Australian dairy producers as well as Canadian dairy producers. It was not an extensive study of Australian calves, however virtually identical results were obtained from the dairies near Perth, Western Australia and the dairy in Lethbridge, Alberta after a single fecal sample was examined from calves. These results compliment those of earlier, much larger prevalence surveys in North America where the prevalence of *Giardia* in dairy calves ranged from 50% to 100% [24, 25, 43], with geometric mean fecal cyst counts between 300 and 5600 cysts per gram after a single sampling [25]. Therefore, *Giardia* should not be overlooked as an important, potential pathogen of Australian dairy calves.

It is apparent that a cost effective method of administering a continuous low dose of fenbendazole for treating giardiasis may benefit cattle producers through healthier animals, and potentially providing increased animal production. Furthermore, treating giardiasis in calves may also provide a public health benefit, by reducing the potential for the zoonotic transmission of *Giardia*. The zoonotic potential of *Giardia* is the subject of much debate and host specificity remains unclear [113, 114]. Many studies suggest there is a potential for the zoonotic transmission of *Giardia* from infected livestock to humans. *Giardia* isolates recovered from ruminants are morphologically and antigenically identical to human isolates [46], and conform to the *G. duodenalis* morphological group as defined by Filice [60]. Dairy operations are thought to be a potential source of human infection through direct contact with infected animals, or contamination of water supplies [37, 70, 218-220]. However, the source of human outbreaks of giardiasis is rarely determined.

Despite the vast geographical distance, the majority of *Giardia* isolates sequenced from calves located at Lethbridge, Alberta and Perth, Australia conformed to the "Livestock" genotype. Isolates with this genetic sequence have yet to be identified in humans, therefore it appears that this "Livestock" genotype is host specific [221]. Based on previous studies, calves may be infected with a genotype best adapted to cattle [48, 119]. The frequency of *Giardia* transmission appears to be very high in dairy calves, and although calves may be exposed to more than one genotype, it is likely that the livestock genotype would out compete the human genotype in calves. The idea of host adapted genotypes out competing other genotypes was also proposed in a 1997 study, suggesting the dog-adapted genotype will out compete other genotypes in situations where the frequency of transmission is high [119].

Although the livestock genotype of *Giardia* appears to occur most frequently in cattle, the occurrence of Genotype A (the most common human genotype) in calves from Lethbridge and Perth is of potential public health significance. This genotype may put producers, and other members of the community, at risk. Therefore, treating giardiasis may be beneficial from a public health, as well as from an animal health and production aspect.

The results from this study indicate that *Giardia* in cattle may, for the most part, be host specific. It is important that future work be carried out to further characterize this livestock genotype and compare it to human isolates. The ability to genotype isolates within the *Giardia duodenalis* group will enable a predictive assessment to be made on the source of infection in future outbreaks, particularly those where livestock may be involved. Using this information, efficient, cost effective control methods, such as fenbendazole treatment, could be applied.

5. CONCLUSION

The importance of the intestinal parasite *Giardia duodenalis* as a pathogen of cattle has been the subject of much debate. Numerous studies demonstrate the parasite is highly prevalent in calves and associated with diarrhea. However, the parasite is often associated with calves that show no signs of intestinal disease. In addition, the potential zoonotic transmission of the parasite has raised concerns that infected livestock may act as a reservoir for outbreaks of human giardiasis.

By following the same group of calves from birth, the importance of *Giardia* as a pathogen of calves under normal conditions became clear. The parasite was associated with many episodes of diarrhea, on its own or in combination with other pathogens. *Giardia* was also highly prevalent, with an infection rate of 100% observed. Therefore, it appeared that *Giardia* may be of significance to the dairy operation from an animal health and economic aspect. In addition, calves from two geographically different locations were found to harbor *Giardia* isolates of a unique genotype. However, calves from both locations were also found to be infected with a genotype known to infect humans. Thus, the importance of giardiasis in dairy calves was expanded to include a potential public health risk.

Based on these studies, treating giardiasis in dairy calves is warranted. The efficacy and potential benefits of treating giardiasis in calves was evaluated using the anthelmintic fenbendazole. The drug proved to be very effective at eliminating the passage of cysts as well as trophozoites from infected calves. Treatment also resulted in significant benefits to the calves clinically and physiologically. However, due to the high transmission rate of the parasite an economic benefit, in terms of increased production, was not observed following treatment.

In summary, this work demonstrated that *Giardia* is an important parasite of dairy calves. It is highly prevalent, associated with diarrhea, and infections in calves may pose a risk to public health. However, giardiasis can be successfully treated in calves, resulting in a clinical benefit to calves, a potential economic benefit to producers, and a decreased threat to public health.

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