UNIVERSITY OF CALGARY

Giardia duodenalis genotype frequencies in Southern Alberta

by

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Abstract

In order to better understand the genetic diversity of *Giardia duodenalis* associated with symptomatic giardiasis in Southern Alberta, clinical samples were genotyped by PCR-restriction fragment length polymorphism analysis targeting coding regions of glutamate dehydrogenase (GDH) and the small subunit ribosomal RNA (SSU) gene. Between January 2002 and March 2004 a total of 282 clinical samples from Calgary were genotyped with the GDH or SUU target loci, representing the largest published survey to date. Sub-assemblage A-I was determined to be the predominant genotype in Calgary, with a genotype frequency of 39.6%, and is possibly the predominant genotype world-wide with a frequency range of 46.7 to 53.9% recorded in travelers returning from various geographic regions of the globe. In two clinical samples genotyped a potential Assemblage E isolate was detected. If confirmed, this is the first documented occurrence of the assemblage in humans, and suggests it may have limited zoonotic potential.

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Dedication

This thesis is dedicated to the memory of Peter Forbes and his random acts of kindness.

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List of Abbreviations

Abbreviation	Description
CLS	Calgary Laboratory Services
CRHA	Calgary Regional Heath Authority
GDH	Glutamate dehydrogenase
GDH-RFLP	Glutamate dehydrogenase PCR-RFLP
GIS	Geographic Information Systems
IgA	Immunoglobin A
Mbp	Million base pairs
OR	Odds Ratio
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PGE	Pulse gel electrophoresis
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SNPs	Single nucleotide polymorphisms
SSU	Small Subunit rRNA
SSU-RFLP	Small Subunit rRNA PCR-RFLP
SWTR	Surface Water Treatment Rule
VSP	Variant-specific surface

Chapter One: Introduction

The enteric protozoan *Giardia duodenalis* is one of the top ten parasitic diseases of humans with an estimated 200 million symptomatic cases in Latin America, Asia, and Africa, with approximately 500,000 new cases each year (174). In Canada, *Giardia duodenalis* is the third most commonly reported enteric pathogen (71) and it is the number one reported waterborne disease in North America (16). The clinical signs associated with giardiasis are highly variable and can range from completely asymptomatic to chronic syndromes associated with nutritional disorders, weight loss, and failure to thrive (2)

Giardia duodenalis is one of the most common causes of non-viral diarrhoea (174) and has been recognized as a pathogen for nearly 50 years (176), yet an adequate understanding of its pathology, genetics, and epidemiology is still lacking. The following sections will discuss what is currently known with respect to: the life cycle, pathogenesis, immune response, treatment, genomics, nomenclature, and transmission of this under-rated pathogen.

1.1 Life Cycle

Giardia duodenalis (syn *G. lamblia, G. intestinalis*) is a flagellated unicellular primitive eukaryotic microorganism with a simple direct life cycle, consisting of an environmentally resistant cyst stage and a motile trophozoite stage. Ingestion of large numbers of trophozoites can result in disease, but most infections are a result of ingestion of cysts, hence cysts have been traditionally considered the infectious stage of the

parasite (2). As ingested cysts transit through the acidic environment of the stomach, excystation is initiated. To prevent destruction of the parasite, due to its premature release within the highly acidic environment of the stomach, emergence within the duodenum is triggered by the slightly alkaline pH and pancreatic proteolytic enzymes associated with the duodenal fluids (65). Once released, the parasite quickly divides into two trophozoites that utilize their flagella and specialized adhesive disk to adhere to intestinal epithelium, initiating colonization. Within the small intestine, trophozoites feed through pinocytosis, utilizing digestive vacuoles located on its dorsal surface and the Embden-Meyerhof pathway (anaerobic respiration) is the primary biochemical pathway for the generation of energy and redox potential (2).

The peristaltic movement of fluid through the intestines flushes trophozoites into the large intestine, where the mildly alkaline pH of 7.8, along with the presence of bile salts and fatty acids, stimulate encystation. During encystation, a quadrinucleate environmentally resistant cyst is formed that is voided in the feces(2). The ingestion of the cyst through direct or indirect fecal-oral transmission thus completes the parasite's life cycle.

1.2 Pathogenesis

Upon ingestion, the low pH of the stomach triggers excystation, which is completed within the duodenum, releasing trophozoites that multiply by binary fission(2). Due to their motile nature and the ability to attach to the microvillous surface of the duodenum and jejunum enterocytes via a ventral disc, trophozoites are able to resist the downward peristaltic momentum of the small intestines (151). The majority of the pathology

associated with giardiasis is restricted to the enterocyte microvilli resulting in malabsorptive diarrhoea, though limited enterocyte invasion is possible resulting in a low-grade fever (47).

The underlying mechanism of pathogenesis is not well understood, but microvilli pathology appears to be immune mediated, with shortening and/or destruction of the brush border microvillus structure due to increased infiltration of CD8 T-lymphocytes (145). T-cell mediated microvillus pathology appears to be a result of myosin light chain kinase-dependent induction of enterocyte apoptosis, inducing reorganization of cytoskeletal F-actin and tight junctional zona occludens-1, causing an increase in epithelial permeability (27, 35, 146). This epithelial permeability results in net secretion of chloride into the lumen (66) producing the characteristic diarrhoea associated with infections. Microvilli atrophy and the corresponding inhibition of several digestive disaccharidase enzymes, including sucrase and maltase, is the likely cause of the characteristic malabsorption.(47)

1.3 Immune Response

The poor immunogenic nature of most *Giardia spp.* proteins (60) and the parasite's ability to undergo antigenic variation by altering the expression of its repertoire of immunogenic variant-specific surface proteins (VSP) (118, 147) suggests *Giardia spp.* is capable of some level of immune evasion within the lumen. Despite this ability, clinical and experimental data suggest adaptive immunity in most cases is effective at clearing infections, since most patients spontaneously eliminate the parasite without treatment (117).

In respect to protective immunity, animal studies have found that infected gerbils were protected against re-infection with the same isolate for up to eight months after primary infection (19). While no direct evidence for protective immunity in humans has been established, epidemiological evidence does support protective immunity. The lower prevalence of giardiasis in adults in endemic areas when compared to newly arrived individuals suggests some level of protective immunity (60). Other epidemiological evidence suggests this protective immunity, though not complete, can last up to five years (85). It is unclear whether differences in levels of protective immunity are due to host immunological responses or are a result of variations in the infecting parasite.

In regard to the class of immune response (cellular or humoral) primarily responsible for parasite eradication, studies have produced mixed results. Numerous studies have suggested the humoral immune response, and more specifically Immunoglobin A (IgA), plays a central role in the eradication of trophozoites within the lumen (31, 46, 74, 94, 152). In contrast, a recent study suggests an antibody independent T-lymphocyte response is essential (149). While the reason for the apparent discrepancies between previous work and this recent study is unclear, the predominant belief is that the humoral response is essential for parasite clearing and that B-cell independent effector mechanisms play a varying role in host defence.

The mechanism by which IgA exerts its anti-giardial activity is not well understood, although it appears that direct antibody-dependent mechanisms of trophozoite killing do not play a role (76). Recovery of *in vivo* IgA coated trophozoites (75) suggest the possibility that attachment of antibodies to the ventral disk may block attachment and colonization. By blocking colonization, the humoral response may

inhibit clonal expansion, allowing B-cell independent antigiardial host defences the ability to clear the remaining parasites. This theory may explain the aforementioned discrepancy regarding the role of B-cells (94) and T-cells (149) in parasite clearing. In Langford et al. (94), mice were treated with oral antibiotics before inoculation to control for differences in normal microbial flora which have previously been reported to influence infections (148, 159). This likely increased the number of colonization niches for the parasite, combined with the use of a higher inoculum may account for the 10 to 100-times greater peak infectious load reported in Langford et al. Under these conditions of heavier parasite burden, B-cells may play a more central role than under lighter parasite loads observed in Singer and Nash (142).

1.4 Treatment

Despite its recognition as a pathogen for 30 years there have been few reviews of therapeutic treatments for *G. duodenalis.* and no definitive treatment regime has been published (38, 64, 99, 101, 180) Drugs used in the treatment of giardiasis include furazolidone, paramycin, albendazole, secnidazole, ormodazole, tinidazole, and metronidazole (64). Due to its availability and physicians' familiarity and not due to any increased efficacy or tolerance, metronidazole is the treatment of choice in North America (77). A treatment regime of 250 mg three times a day for 5 to 7 days has an efficacy of 60 to 95% with a median efficacy of 88% (64). Common side effects associated with metronidazole include: dry mouth, headaches, vertigo, nausea, and a metallic taste in the mouth (91).

Metronidazole exploits an anaerobic pathway present in *Giardia spp.* Once it has entered the cell, a nitro group is reduced by pyruvate:ferredoxin oxidoreductase, activating the drug (160, 161). This reduction allows for the formation of a gradient, allowing for additional transport of metronidazole into the trophozoite (25). Within the trophozoites, the reduced metronidazole binds covalently to DNA macromolecules causing DNA damage (49, 116) resulting in cell death. In addition to its nucleotoxic activity, metronidazole inhibits trophozoite respiration (129) and its reductive potential may produce toxic radicals that disrupt critical cell functions (166). Encysted trophozoites may be less susceptible to the drug due to the decreased internalization of the drug across the cyst cell wall (158). Resistance to metronidazole has been shown *in vitro* and correlates with decreased activity of pyruvate:ferredoxin oxidoreductase (161, 167). While a 16,000 fold variation in metronidazole susceptibility was been observed in the laboratory (59), a comparison of clinical isolates to laboratory reference strains suggests resistance to metronidazole is not a significant problem (37).

1.5 Genomics

Giardia spp. belongs to a group of binucleated organisms called diplomonads, and has been of great interest to evolutionary scientists due to its mixture of prokaryotic and eukaryotic features (70, 103, 106, 150). While distinctly eukaryotic *Giardia spp.* lacks mitochondria, nucleoli, and peroxisome (3) and possesses a prokaryotic-like small subunit rRNA (20, 24, 48, 150). Both nuclei are transcriptionally active and contain a full complement of chromosomes (90, 179).

During binary fission it is unclear whether daughter cells receive copies of one or both of the parental nuclei (179). The high mutation rate (1% per cell per division) and low observed level of allelic heterozygosity (15) suggests daughter cells receive only one of the parent nuclei. This is supported by the equatorial partitioning of nuclei observed during cytokinesis (179).

Giardia spp. is polyploidic with an estimated four to eight copies of each of its five chromosomes, ranging in size from 1.6 to 3.8 Mbp, and has an estimated genome size of 1.34×10^7 bp. The discrepancy between ploidy estimates is likely a function of varying levels of ploidy at different times in the cell cycle. A recent study shows that trophozoites were 4N during G_1, but spent most of their time in the G_2 phase of DNA replication with a DNA complement of 8N (20). The suggestion trophozoites are functionally tetraploid is supported by the discovery of up to four different allelic types of single VSP gene within a single clone (177).

Studies examining chromosomal size variants among isolates and even amongst cloned lines of the same isolate have observed an extreme level of plasticity within the genome (32, 96), with up to 30% of size variation in chromosome 1. This appears to be due to variations in the rRNA gene copy numbers, which are organized in tandem arrays of a 5.6 kb repeat (32). The genome is estimated to contain 60 copies of rRNA tandem repeats, located at the telomeres of some but not all of *Giardia*'s chromosomes (95)

This tremendous plasticity has obvious cellular implications, in regard to gene expression, but also needs to be considered in the construction of molecular tools used to investigate genetic diversity.

1.6 Nomenclature

In order to better understand the pathogenesis and epidemiology of *Giardia spp.* it is important that a standardized classification scheme exists. Since its initial description by van Leeuwenhoek in 1681 (43), there have been numerous variations in nomenclature. Attempts to develop a species classification model for the organism are complicated by the fact that *Giardia spp.* is presumed to be asexual, thus mating ability can not be utilized for species determination.

Initial attempts to develop a standard classification model for the organism assumed that different hosts had their own *Giardia spp.* This resulted in the description of over 40 different species (93), which may have been an overestimation. Subsequent use of light microscopy and classification based on morphological differences by Filice in 1952 reduced the number of species to three(61); *Giardia agilis*, *G. muris* and *G. duodenalis.* Data from animal infectivity models suggested this was an underestimation of the number of species. Two additional species, *G. psittaci* (54) and *G. ardeae* (55), were later described based on ultra-structural features of trophozoites using scanning electron microscopy. Discrepancies between animal infectivity models and the five species model for *Giardia spp.* still remain.

Molecular tools have become invaluable in the classification of *Giardia spp.* and have led to the description of a sixth species, *G. microti* (168). The application of DNA and isoenzyme analysis in the late 1980's (7, 111, 119) suggested there was significant genetic heterozygosity between isolates within *G. duodenalis.* Initial characterization of this genetic diversity (Table 1) suggested *G. duodenalis* clustered into

at least two genetically distinct groups and as many as four (7, 80, 105, 119). These genetic clusters have been assigned different pseudonyms by various researchers. Although no general consensus has been reached regarding nomenclature, Assemblage A or B is most commonly used to describe the two genetically distinct populations of *G. duodenalis* that infect humans. Analysis of Assemblage A and B isolates reveal the genetic distance between the two assemblages is of the same magnitude observed between G. *duodenalis* and the morphologically distinct *G. muris*, suggesting that *G. duodenalis* is a species complex (7, 105).

This initial genetic clustering of *Giardia duodenalis* into two assemblages relied on the ability to axenically culture trophozoites. This technique underestimated the degree of genetic diversity among *G. duodenalis* isolates since *in-vitro* cultures supported the growth of a narrower range of genotypes than observed under *in vivo* conditions (8). To overcome the limitation of axenic cultures, conserved regions of ribosomal and housekeeping genes were amplified by polymerase chain reaction (PCR) using DNA extracted from cysts collected directly from infected hosts. Based on cyst PCR analysis, currently *G. duodenalis* is divided into seven distinct genetic clusters called assemblages (Table 1), with two known to be infectious to humans and five others that appear to be host specific (C-G)(1, 58, 82, 114, 115, 168).

Table 1: Molecular nomenclature of *Giardia duodenalis* and host range.

Comparisons of nomenclatures used to describe *G. duodenalis* by various researchers groups, as well as host range. ♣ Host range of this genotype includes humans, livestock, dog, cats, beavers, and various other mammals.

Genotype	Nash (119)	Mayrhofer(105), (Andrews (7))	Homan (80)	Host Range
A-I	Group 1	Assemblage A (Group 1)	Polish	Mammals♣
A-II	Group 2	Assemblage A (Group 2)		Humans
B-III	Group 3	Assemblage B (Group 3)	Belgium	Humans, Beavers, Dogs
B-IV		Assemblage B (Group 4)		
C	-	Monis (115)	-	Dogs
D	-	Monis (115)	-	Dogs
E	-	Ey (58)	-	Livestock
F	-	Homan (79)	-	Cats
G	-	Thompson (157)	-	Rats

Within Assemblage A and B, cluster analysis supports four sub-assemblages. Sub-assemblage or group A-I and A-II is the predominant nomenclature used to describe Assemblage A isolates (5, 33, 57, 113) and Sub-assemblage B-III and B-IV is commonly used for Assemblage B (7, 105, 162). The genetic distance between B-III and B-IV is greater than that seen between A-I and A-II, supporting the hypothesis that Assemblage B contains older genetic lineages than those found in Assemblage A (113). The genetic distance between Assemblage A and Assemblage C and D is similar to the calculated distance between Assemblages A and B, suggesting the combined Assemblage C and D may also be a distinct species. The genetic distance between the other assemblages (E,F,G) is similar to the distance calculated between Assemblage A and D, with Assemblage E being most closely related to Assemblage A (114). One author suggests the genetic separation between the various assemblages is similar or greater than that calculated between species of other genera, such as *Escherichia spp.* and *Salmonella spp.* (114)

It is important to note the *G. duodenalis* nomenclature is based mainly on sequence and isoenzyme differences. Currently, there are few epidemiological and biological markers that can reliably distinguish between them. While some metabolism (69), *in vitro* growth rate (21), RNA virus susceptibility (39) and host-specificity (58, 114) data supports some of the nomenclature, more epidemiological and biological studies are needed in order to demonstrate that these genetic groups are, in fact, distinct.

1.7 Transmission

Giardia duodenalis is the third most reported enteric pathogen in Canada behind *Campylobacter* and *Salmonella*. In 2001 the reported incidence (cases per 100,000) of giardiasis within Canada ranged from 9.44 (Nova Scotia) to 34.27 (Northwest Territories), with the reported rate in Alberta (17.24) slightly above the national average of 16.29 (71). These numbers may only represent less than 1% of actual infection rates, since asymptomatic giardiasis has been reported at approximately 1.6% of the total population (73)

Giardia duodenalis, with an infectious dose as low as 10 cysts (137), is extremely communicable and can be transmitted through direct or indirect fecal-oral transmission. Due to the low infectious dose required to establish an infection and the fact that the cysts are environmentally resistant, waterborne transmission is the most common mode of transmission worldwide (174) and the most frequently diagnosed cause of waterborne diarrhoea in United States. Waterborne transmission can result from human or animal fecal contamination of water supplies and represents the greatest potential for large scale outbreaks. Since *Giardia spp.* is more resistant to chlorine disinfection than bacterial pathogens (89), cyst-contaminated water is difficult to remediate. In addition to waterborne transmission, direct fecal-oral transmission can play an important role in disease transmission under certain circumstances. This section will discuss the roles that direct fecal-oral, waterborne and zoonotic transmission play in the epidemiology of giardiasis.

In Alberta, as well as the rest of Canada, the highest incidence of disease is associated with children under the age of 10 years (dashed lines), with the 1-4 years age

cohort accounting for the majority of disease. Historically, the elevated incidence in children has been attributed to direct fecal-oral transmission associated with day-care centres (14, 23, 92, 169). The dramatic reduction in the incidence of giardiasis in this cohort in Alberta by 82 % from 336.9 cases / 100,000 in 1986 to 62.4 cases / 100,000 in 2001 (71) is likely due to the effective implementation of legislated infection control programs (133) in daycare settings . While not as dramatic as that seen in the preschool cohort, the incidence for other age groups has also fallen over the last 15 years. In the last 5 to 6 years, however, the incidence in all age groups has remained relatively constant or is on the increase, suggesting other endemic sources of transmission within the population.

Travel or immigration account for internationally-acquired infections. Incidence of giardiasis is higher in other regions of the world, so it is not surprising that travelers and immigrants are more likely to be infected with *Giardia spp.*. Surveillance data from the Calgary Regional Health Authority (CRHA) from 1996 to 2000 suggests 36% of reported cases were most likely internationally acquired (29). This is similar to the 35% reported by Alberta's other major health region (62) (Capital Health Region, Edmonton). While the index cases may have acquired the infection internationally through either direct or indirect fecal oral transmission, secondary transmission within Canada is likely via direct fecal-oral transmission. Contact with a family member with giardiasis or person thought to have giardiasis dramatically increases the risk of acquiring the disease, illustrating the importance of identifying index cases to prevent secondary transmission (36). Due to *G. duodenalis's* status as a reportable disease in Canada and mandated public health follow-up, secondary transmission can be controlled

to a certain degree, though the high level of asymptomatic carriage ensures a certain level of endemic secondary transmission within the population goes undiagnosed.

Another documented risk factor associated with direct fecal-oral transmission is rectal intercourse. A 5-year retrospective study based in New York found that 22% of reported cases in males were associated with homosexual activity (144). This illustrates the importance of obtaining a sexual history to ensure sexual contacts can be treated and secondary transmission of disease prevented.

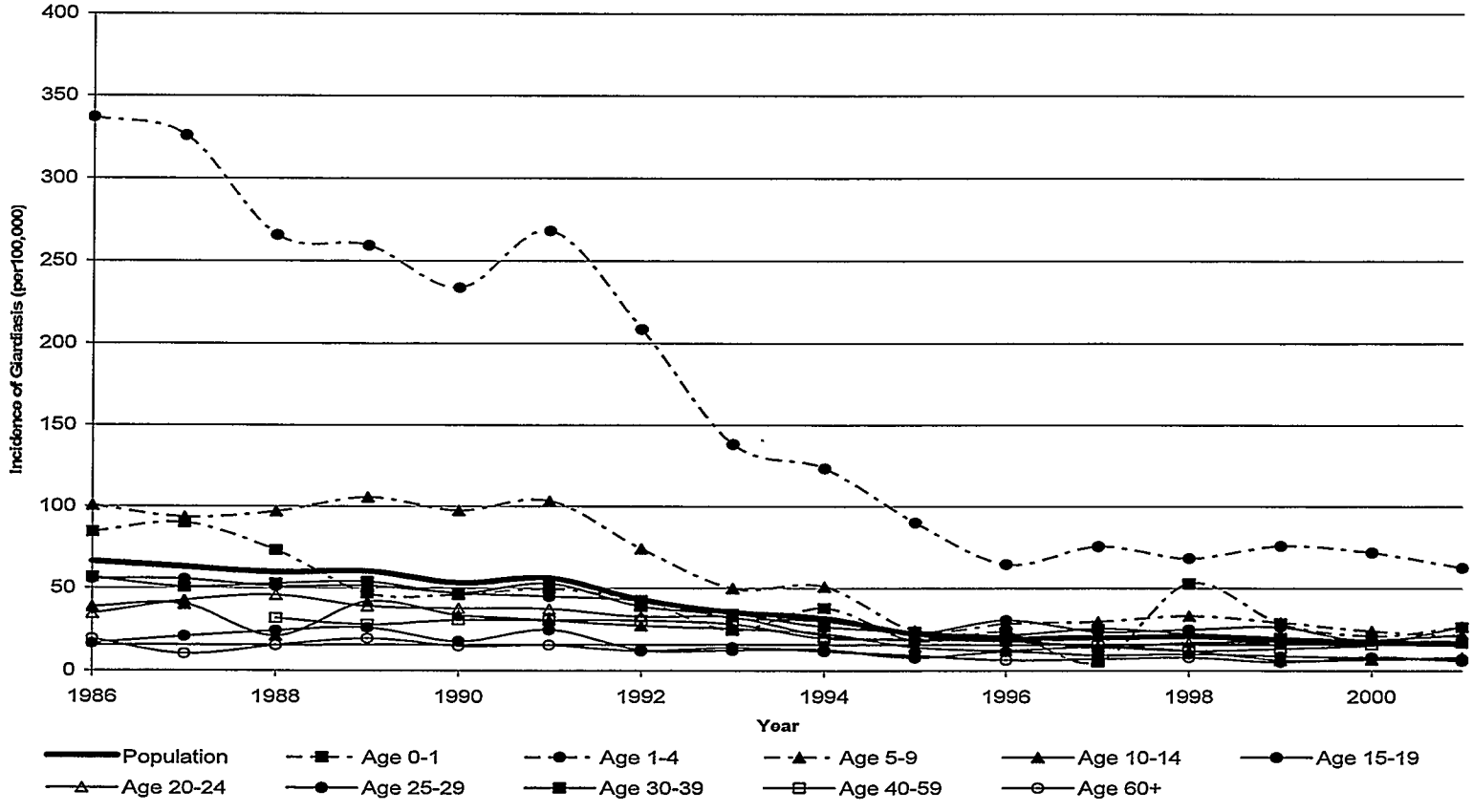


Figure 1: Reported incidence of giardiasis in Alberta from 1998-2001 (71).

1.7.1 Water

Waterborne transmission of *G. duodenalis* can be divided into two categories, those infections acquired through contaminated drinking water and those acquired through ingestion with cyst-contaminated water during recreational activities.

In respect to drinking water, the highest risk of disease is associated with private systems that do not have to conform to legislated drinking water standards. These systems commonly utilize surface water or shallow wells which are at increased risk of being contaminated by surface runoff and usually lack adequate disinfection. A case-control study found that individuals using these types of drinking water sources were 2.1 to 2.4 times more likely to become infected when compared to individuals on deep well or centralized water distribution systems(36, 40)

The probability of contacting giardiasis from public water utilities in Canada is influenced by water source, level of treatment, facility management, and seasonal change in water quality, and as a result, is not easily quantified. The Canadian Drinking Water Guidelines and the U.S. Surface Water Treatment Rule (SWTR) was set at one case per 10,000 individuals as an acceptable risk for giardiasis. To attain this acceptable level of risk, cyst concentration within the finished water should not exceed 7×10^{-6} organisms per liter (approximately one cyst per 142,000 L) (140). Compliance verification of such low levels of microbial occurrence is technically unfeasible and, as a result, regulators must rely on water utility design to obtain a set log reduction in parasite load required to meet the standard. This theoretical reduction is calculated based on filtration efficiency and disinfectant contact time. The SWTR guidelines state that water utilities must have

the capability to achieve a 3 Log (99.9%) reduction or inactivation of *Giardia spp.* cysts through filtration and disinfection and no cysts must be detected in the finished water (53).

Using either cyst detection in finished water (171), log reduction calculations (97), or statistical modelling (13), three North American surveys of 173 water utilities found that 5 to 46% of water utilities would not meet SWTR requirements for acceptable levels of risk for at least a portion of the year. The highest percentage of non-compliance was observed during the colder months (46%) when colder temperatures (5°C) negatively influenced disinfectant efficiency. Low temperatures (less than 10°C) increased *Giardia spp.* cyst survival time to up to 2 weeks in tap water (41). Data from these surveys suggest potable water, in certain communities, may contribute to a level of endemic giardiasis above the acceptable level set forth by SWTR. It is important to note that data from these water utility studies assumed that 100% of cysts within the source water were viable and capable of infecting humans. As a result, the infectious parasite load and associated risk was likely overestimated, though to what degree is uncertain. Although two population-based case-control studies (72, 104) found that drinking water did not contribute to endemic giardiasis within the population, they were site specific and were conducted on large well managed water systems. In order to better ascertain actual levels of endemic giardiasis associated with drinking water, better estimates of infectious parasite load for source water are needed.

To what degree drinking water contributes to endemic giardiasis is unclear, though failures in drinking water systems have resulted in several outbreaks in British Columbia, Alberta, Ontario, Quebec, New Brunswick, and Newfoundland (171), with

attack rates as high as 30% (172). In British Columbia, 10 water system associated outbreaks were confirmed from 1986 to 2000 , with the 1986 Penticton outbreak being the largest, consisting of 497 confirmed clinical cases and an estimated 3125 individuals infected (71). Water utility outbreaks only accounted for approximately 4% of reported cases from 1986 to 2000, suggesting utilities, historically, are normally not an important mode of transmission. The 1986 outbreak accounted for approximately 15 % of reported cases in British Columbia during that year, illustrating the enormous potential for large scale outbreaks.

Due to the presence of well-maintained centralized sewage and water systems, waterborne transmission of *G. duodenalis* within Canada is more commonly associated with water related recreational activities, where individuals are at an increased risk of contact with cyst-contaminated water (86). *Giardia duodenalis* is ubiquitous within North American surface water, with 69 to 100% of surveyed surface water having detectable parasite levels (84, 98, 128, 171). Parasite load within surface water is heavily influenced by agriculture (128) and anthropogenic activity (109, 110), with unprotected watersheds having considerable higher parasite loads than protected watersheds (no human activity) (140). Due to the presence of *Giardia spp.* in pristine watersheds and its zoonotic potential, water from protected watersheds should not be considered potable unless treated. Water-related activities such as swimming (OR = 1.7 to 6.2), and camping (OR=1.7) have been shown to significantly increase the risk of disease (36, 40, 83, 153). This is supported by the increased incidence of reported cases during the warmer summer months in North America (63, 131), coinciding with an increase in water related recreational activities.

In summary, case-control and surveillance data suggest water related recreational activities have a greater influence on the incidence of locally-acquired infection (no history of international travel) than drinking water. That said, the greatest potential for large scale outbreaks of disease is associated with water system treatment failures. Excluding outbreak scenarios, it is still not clear to what degree public water utilities contribute to endemic rates of disease within the community, though it is likely negligible when compared to other risk factors.

1.7.2 Zoonoses

The ability of *Giardia duodenalis* to infect a wide range of vertebrates including mammals, birds, reptiles, and amphibians, raises the possibility for zoonotic transmission. Of the six recognized species, only *Giardia duodenalis* is able to infect humans, along with a variety of other mammals. *Giardia duodenalis* is composed of zoonotic and host-adapted assemblages (Table 1). In respect to zoonotic potential, 3 of 4 human associated sub-assemblages appear to have some degree of infectious potential. Sub-assemblage A-I is composed of a larger number of human and animal isolates, and as a result is believed to have a greater potential than Sub-assemblage B-III and B-IV (157).

A survey of pet owners found that while a majority of owners were aware that pets could transmit parasitic diseases to humans, more than half were unaware of the possible modes of transmission (107). The prevalence of *G. duodenalis* in the domestic pet population is influenced by geographic region, animal age, and density. The highest incidences of disease are associated with juvenile animals (68), kennels and breeders (88). Based on microscopic analyses, infection rates in dogs and cats have been reported

as high as 21.5% and 12.5% respectively (Table 2). Utilizing modern detection techniques, such as enzyme-linked immunosorbence assays and PCR, carriage rates in dogs and cats have been reported at 51.6 and 80% respectively, suggesting microscopic analyses may have underestimated the extent of pathogen presence within the population. Although it appears that a large percentage of companion pets are infected, the level of risk to human health is unclear since dogs and cats are parasitized by host-adapted (Assemblage C,D,F) (1, 113, 115) and zoonotic assemblages.

Molecular studies in Australia (115), Japan (1), and India (162) found that the predominant genotypes in dogs are Assemblage C, Assemblage D, and Assemblage B respectively. Epidemiological evidence from India found that households with at least one *Giardia*-positive dog were three times more likely to have a member of the household infected, confirming potential for zoonotic transmission (162). All three studies genotyped a limited number of isolates (maximum of 13 isolates) and the latter two obtained isolates from a restricted geographic region. Data from these studies suggest the predominant canid genotype may vary considerably between geographic regions and therefore the risk of zoonotic transmission may also vary geographically. As a result, an accurate risk assessment for a region cannot be made until the prevalence of various assemblages within the domestic animal population is ascertained.

Table 2: Point Prevalence of *G. duodenalis* in various animal populations.

Host	Point Prevalence (%)		References
	Modern Detection	Microscope	
Domestic			
Dogs	7.2 - 51.6	3 – 21.5	(17, 30, 67, 87, 88, 155, 163)
Cats	51.6 – 80	8 - 12.5	(17, 108, 155)
Agriculture			
Calves	-	14 - 73	(18, 125, 142)
Cattle	-	6.5 - 80	(10, 78, 124, 134)
Pigs	-	7.6 - 9	(11, 127)
Wildlife			
Beaver	14.9 – 30	3.4 -13.7	(42, 44, 56, 170)
Deer	-	4 - 9	(127, 164)

Agriculture practices have also been a topic of concern, regarding the potential transmission of giardiasis, with the majority of the discussion directed towards cattle. Giardiasis is endemic in cattle, with studies suggesting 100% of cattle are infected with *G. duodenalis* at some point during the first 6 months of their life (125). During the infection, calves can shed up to 5000 cysts per gram of feces (125), with rates of shedding and incidence of disease decreasing with age (121), though the prevalence can remain high (Table 2)

While giardiasis is endemic in other agriculture animals, such as pigs, sheep and horses (127), cattle represent the greatest concern due to the sheer amount of intestinal waste material produced. The province of Alberta farms over five million head of cattle which produce 27.5 billion kg of feces annually (10). When this level of fecal material production is coupled with the endemic nature of the disease within the cattle population and the intensity of parasite shedding associated with infections, the enormous potential for environmental contamination is self evident. Despite this potential for environmental contamination, molecular genotyping of cattle isolates suggest cattle may not have as high a zoonotic potential as first thought. In a spring 2000 survey of beef cattle in Alberta, 98% of infected cattle were infected with Assemblage E (10), which is thought to be host-adapted to ungulates (126) and not associated with infections in humans. The high prevalence of Assemblage E in cattle, combined with its apparent host-specificity, would suggest cattle may not serve as an important reservoir for zoonotic transmission in Alberta. This may not be true for other regions of North America. A US-based study found that up to 45% of some herds of dairy cattle were infected with Assemblage A (165) and, as a result, may serve as a potential source for zoonotic transmission. Since the

prevalence of zoonotic genotypes within agriculture populations appears to vary among regions and, as a result, the potential for zoonotic transmission, an accurate risk assessment must examine the prevalence of zoonotic genotypes within the animal population rather than overall prevalence of disease.

Wildlife may also play an important role in the transmission of giardiasis through the contamination of watersheds. Most notably, beavers, due to their aquatic lifestyle, have been implicated as the most likely source of contamination in numerous waterborne outbreaks (45, 71, 157), giving rise to the nickname "Beaver Fever". Surveys of beaver populations suggest up to 30% of beavers may be infected with *G. duodenalis* (Table 2). Both Assemblage A and B have been found in beavers in Alberta, with Assemblage B being the predominant genotype (9). Beavers may act as mammalian incubators in an anthropozoonotic/zoonotic transmission cycle, amplifying zoonotic genotypes present in their aquatic environment due to human and agricultural activity increasing the number of viable cysts in the watershed and probability of transmission. While *G. duodenalis* has been detected in other wildlife populations (170), some of which have been found to harbour zoonotic genotypes (164, 175), the potential for zoonotic transmission is unclear.

In conclusion, while the potential for zoonotic transmission of *G. duodenalis* is well documented, more epidemiological evidence is needed to support the claim. The discovery of variations in the prevalence of zoonotic and host-adapted genotypes within animal populations, suggest standard cyst detection technique may provide insufficient information to perform a risk assessment. An accurate assessment must examine the prevalence of zoonotic assemblages rather than the overall prevalence of disease.

1.8 Study Hypothesis and Objectives

As alluded to in the previous section, the molecular epidemiology of giardiasis is not well investigated. This is largely due to the absence of standardized typing schemes and secondarily to the limited population that most clinical laboratories serve. Calgary Laboratory Services (CLS) provides diagnostic services for Calgary, Alberta and the surrounding communities, servicing a population of approximately one million individuals with one consolidated clinical laboratory. In most cities, such services would be provided by 3 to 10 clinical laboratories. Annually CLS performs roughly 14,000 diagnostic tests for *G. duodenalis*, with 189 and 248 positives in 2002 and 2003 respectively. The endemic nature of giardiasis in Southern Alberta, combined with the centralized diagnostic screening of clinical samples provides the unique opportunity to have access to a large number of samples from a defined geographic area. In collaboration with CLS, the objective of this study is to provide a better understanding of the epidemiology of *Giardia duodenalis* in the human population in Calgary, Alberta.

1.8.1 Genotype frequencies in Humans

In order to better comprehend the transmission dynamics of *Giardia duodenalis*, we first must have an understanding of the population diversity on both a local and global scale. A recent United Kingdom study (6) that examined 33 clinical samples found 64% were Assemblage B, 27% were Sub-assemblage A-II , and 9% were mixed A-II/B infections. Unfortunately, greater than 60% of the samples genotyped were associated with a single outbreak event and, as a result, provides limited information on the prevalence of circulating genotypes within the community. A more useful survey of day-cares in

Australia (135), though still biased by small sample size (n=23), found that Assemblage B (70%) was more prevalent than Assemblage A (305). Since this study only examined children under the age of 5 years, the results, in respect to prevalence, can only infer that Assemblage B appears to more prevalent in children. An India-based study (130), examining a larger age demographic, found that 7 of 12 infections were associated with Assemblage A, though it too, had a small sample size. In the largest study to date, an Egyptian study (52) examining 105 cases, found Sub-assemblage A-I (36.2%) accounted for the largest percentage of infections, followed by mixed assemblage infection (15.2%), A-II (12.4%), and B-III (9.5%). Clearly, larger population-based studies are required to evaluate typing techniques.

To investigate the population diversity on both a local and global scale, this study will answer some basic epidemiological questions in regards to genotype frequencies: 1) Is there a predominant genotype associated with local and international-acquired infections?; 2) Are there differences in genotype frequencies between local and internationally acquired infections?; 3) Are there differences in genotype frequencies between international-acquired infection from geographic regions? For statistical and hypothesis testing purposes, the null hypothesis that there is no statistical difference in genotype frequencies was used.

1.8.2 Age Associated Genotype Frequency Variance

A recent study of day-cares in Australia (135) found that Assemblage B was the predominant assemblage in children under the age of 5 years (70% of infection) and that approximately 78% of the Assemblage B infections were asymptomatic. While these

results may suggest Assemblage B is a less virulent strain of *Giardia duodenalis,* the study only genotyped 23 isolates and did not examine the adult population Based on this study, I hypothesised that there is a greater than expected percentage of Assemblage B in children under the age of five. To test this hypothesis, a statistical comparison of assemblage frequencies in children under the age of 5 was compared to all other age cohorts.

1.8.3 Zoonotic Potential of Assemblage E

Agriculture practices have been a topic of concern regarding the potential transmission of giardiasis. While giardiasis is endemic in other agriculture animal populations, cattle represent the greatest concern in Alberta. Surveys of beef (10) and dairy (124) cattle in Alberta found 98% and 80% of infected cattle, respectively, were infected with Assemblage E. Recent evidence suggests Assemblage E has a limited zoonotic potential and to date has not been associated with any cases of giardiasis in humans, though no concerted effort was been undertaken to confirm its absence within human population. In respect to risk assessment modelling, it is important to confirm or refute zoonotic potential of Assemblage E since it has serious implications on the role that cattle may serve as reservoirs for zoonotic transmission.

The endemic nature of Assemblage E in cattle in southern Alberta (10) and the documented negative effect that cattle management practices have on water quality (128) raises the strong possibility that southern Albertans are being exposed to the Assemblage E through waterborne transmission. If Assemblage E is capable of causing symptomatic giardiasis in humans, one would expect the assemblage would be isolated from stool

samples from symptomatic individuals in the southern Alberta. It is hypothesised that Assemblage E will not be associated with symptomatic human giardiasis.

1.8.4 Objectives Summary

In order to better understand molecular epidemiology of symptomatic giardiasis in Southern Alberta, this study will determine the overall frequency of various genotypes in the region. In addition, the study will use statistical comparisons to determine if age and international travel has an effect on genotype frequency. Finally, by determining the frequency that Assemblage E is associated with symptomatic disease, the importance of zoonotic transmission from cattle can be evaluated.

Chapter Two: Material and Methods

2.1 Patient Information

Ethics approval for the study was granted by the University of Calgary on November 7, 2002. This approval allowed access to patient's laboratory reports and permission to contact them to ask a series of approved survey questions. The laboratory report contained the following personal information: name, sex, age, history of travel, and attending physician. If contact with a patient was necessary, permission to contact the patient had to be given by the attending physician. Due to the poor response rate, there was limited amount of data, and as a result, no useful data was collected from the surveys

2.2 Standard Methods

2.2.1 Collection of fecal samples

SAF (sodium acetate, acetic acid, and formaldehyde)-fixed fecal samples collected and determined to contain *G. duodenalis* cysts via ProspecT® *Giardia* Microplate Assay (Alex-Trend, Ramsey) enzyme immunoassay, were supplied by CLS. Samples were submitted to CLS for diagnostic purposes by physicians from Calgary and the surrounding communities. Upon receiving a sample from CLS, it was rinsed three times with Phosphate Buffered Saline (PBS) by means of centrifugation to remove the fixative. Fecal samples were stored at 4°C until further processing was done.

2.2.2 *Giardia spp.* cyst concentration

The fecal sample was liquefied in PBS + 0.1% TWEEN and filtered through sterile gauze. A 10 ml aliquot of fecal filtrate was layered over 5 ml of a 1M sucrose solution

(specific gravity, 1.18). The sample was then centrifuged at 100 x g for 10 minutes in a swinging bucket rotor centrifuge to concentrate the cysts at the sucrose-PBS interface. The interface and upper layer of liquid were transferred by pipette to a clean tube and washed twice by pelleting the cyst concentrate by centrifugation at 200 x g for 5 minutes in a fixed rotor centrifuge. The supernatant was decanted, and the pellet resuspended in 10 ml of PBS. Once washed, the pellet was resuspended in PBS to a final volume of 2-3 ml.

2.3 Molecular Methods

2.3.1 DNA extraction

DNA was extracted by either a standard phenol:chloroform protocol or a modified QIAamp DNA Stool Kit (QIAGEN Inc, Valencia, CA) protocol. A phenol:chloroform protocol, previously described in Appelbee et. al., 2003, was used with minor alterations. A 200 µl aliquot of purified cysts was boiled for 40 minutes. Once lysed, 200 µl TE (10mM Tris–HCl (pH 7.5), 1mM EDTA) + 60 µl 10% Sodium Dodecyl Sulfate + 15 µl Proteinase K (20 mg/l) was added to the cyst lysate and incubated at 56°C overnight. After the overnight incubation, 100 µl 5M sodium chloride (NaCl) was added and vortexed, followed by the addition of 80 µl of warm 10% hexadecyltrimethylammonium bromide, and this was incubated at 65°C for an hour. After incubating, an equal volume of chloroform isoamyl (24:1) was added and the solution was vortexed. Once vortexed, the solution was centrifuged for 5 minutes at 10,0000 rpm, then the supernatant was transferred to a new 1.5 ml microcentrifuge tube. An equal volume of

phenol/chloroform/isoamyl (24:24:1) was added, the solution vortexed, then centrifuged for 5 minutes at 10,000 x g, and the supernatant was transferred to a new microcentrifuge tube. To the supernatant, 0.6 to 0.7 volume of isopropanol was added, vortexed, and allowed to incubate overnight at 4°C. After incubating, the sample was centrifuged for 20 minutes at 16000 x g, the supernatant discarded, the pellet washed with 1 ml of ethanol, the sample centrifuged again, and the supernatant discarded. The washed pellet was then resuspended in 50 µl of TE buffer and stored at -10°C until it was used. Since the DNA extract contained nucleic acids from both the cysts and fecal materials, DNA concentration was not determined for all samples.

A QIAamp DNA Stool Kit (QIAGEN Inc, Valencia, CA) protocol, as previous described (154), was also utilized. A 200 µl of cyst concentrate was aliquoted into a 1.5 ml centrifuge tube and boiled for 30 minutes. After boiling, the sample was treated initially with 66.7 µl of 1M KOH and 18.6 µl of 1M dithiothreitol followed by neutralization with 8.6 µl of 25% (v/v) hydrochloric acid. The DNA lysate was then extracted with phenol:chloroform:isoamyl (24:24:1), before the QIAamp DNA Stool Kit was utilized. Since the DNA extract contained nucleic acids from both the cysts and other fecal materials, DNA concentration was not determined for all samples.

2.3.2 Polymerase Chain Reaction (PCR) Amplification

2.3.2.1 Amplification of Small Subunit (SSU) rRNA gene

The highly conserved nature of protein synthesis genes, such as the small subunit rRNA gene, makes them good candidate markers for the molecular classification of

microorganisms. The primary tool for the classification of *G. duodenalis* has been small subunit rRNA gene, and as a result it was chosen as one of the two target loci utilized in this study. The nested amplification of SSU rRNA target loci utilized both an external and internal primer set, which target a coding region of SUU rRNA. The external SSU rRNA reaction utilized Gia2029 (5'-AAGTGTGGTGCAGACGGACTC-3') and Gia2150c (5'-CTGCTGCCGTCCTTGGATGT-3') primer set and thermal cycle conditions as previously described (10). Internal amplification of a 292 bp product utilized RH11 (5'- CATCCGGTCGATCCTGCC-3') and RH4 (5'-AGTCGAACCCTGATTCTCCGCCAGG-3') primers and thermal cycle conditions as previously described by Hopkins (82). Both internal and external SSU rRNA reaction mixtures were 25 µl and contained: 0.5U *Taq* polyermase enzyme, 60 mM Tris; 15 mM $(NH_4)_2SO_4$ (pH 9.0); 200 µmol of each deoxynucleoside triphosphate (dNTPs); 0.5 µM of each primer; 2 mM mangnesium chloride ($MgCl_2$); 5% dimethlysulfoxide; and template DNA. The concentration of template DNA in the external PCR reaction varied from 1/100 dilution of the genomic DNA extract to 5 µl. A 2 µl aliquot of external PCR product was utilized as a template DNA for the internal reaction. Thermal cycle conditions for the external primer set were as follows: 96°C for 4 minutes, 58 °C for 1 minute, 72 °C for 2 minutes, then up to 52 cycles of 96°C for 30 seconds, 58°C for 30 seconds, and 72 °C for 45 seconds followed by a final extension of 4 minutes at 72 °C. Thermal cycle conditions for the internal primer (RH4 and RH11) set were as follows: 96°C for 4 minutes, then 52 cycles of 96°C for 30 seconds, 52°C for 30 seconds, and 72 °C for 45 seconds followed by a final extension of 4 minutes at 72 °C.

2.3.2.2 Amplification of Glutamate dehydrogenase (GDH) gene

The nest amplification of the GDH target loci utilized the designed external primers (see Section 2.4.1) GDHeF (5'-GGACGCATCAACGTCAAC-3') and GDHeR (5'-AGGACGTTCTTACCCCTGAT-3') and internal primer set, GDHiF (5'-CAGTACAAC TCYGCTCTCGG-3') and GDHiR (5'-GTTRTCCTTGCACATCTCC-3') previously described by Read et al.(136). Both primer sets target coding regions of this house keeping gene used to incorporate ammonia in the production of glutamate. This primer set was chosen at the onset of the project since it was the only tool available for the genotyping of isolates to the various assemblage and sub-assemblages level without using DNA sequencing of PCR products. Both GDH reaction mixtures were 25 µl and contained: 0.5U *Taq* polymerase enzyme; 60 mM TRIS; 15 mM $(NH_4)_2SO_4$ (pH 9.0); 200 µmol of each deoxynucleoside triphosphate (dNTPs); 0.5 µM of each primer; 1.5 mM Mangnesium Chloride ($MgCl_2$); 5% dimethlysulfoxide; and template DNA. The concentration of template DNA in the external PCR reaction varied from 1/100 dilution of the genomic DNA extract to 5 µl. The external PCR reaction mix also contained 0.2 ng/µl of bovine serum album. A 2 µl of aliquot external PCR product was utilized as template DNA for the internal reaction. Thermal cycle conditions for the external primer set were as follows: 96°C for 4 minutes, 52 °C for 1 minute, 72 °C for 2 minutes, then up to 52 cycles of 96°C for 30 seconds, 52°C for 30 seconds, and 72 °C for 45 seconds followed by a final extension of 4 minutes at 72 °C. Thermal cycle conditions for the internal primer (GDHiF and GDHiR) set were as follows: 96°C for 4 minutes, then up to

52 cycles of 96°C for 30 seconds, 53°C for 30 seconds, and 72 °C for 45 seconds followed by a final extension of 4 minutes at 72 °C.

2.3.3 Restriction fragment length polymorphism (RFLP) analysis

2.3.3.1 PCR-RFLP SSU rRNA (RFLP-SSU) analysis

Restriction digests of the 292 bp SSU rRNA internal product exploits sequence heterozygosity between Assemblage A and B at nucleic acid 22 (G→A), 23 (C → T), 24 (G → C) resulting in the formation of an additional *HinfI* restriction site (G/ANTC). A 20 µl aliquot of internal product was digested with 5 U *HinfI* (New England Biolabs, Beverly, MA), and 2.2 µl of NE buffer 2 for three hours at 37°C. Profiles were visualized on a 2% agarose gel (Invitrogen) stained with ethidium bromide.

2.3.3.2 PCR-RFLP GDH (GDH-RFLP) analysis

Restriction digests of the 432 bp internal GDH product exploits sequence heterozygosis between Assemblages (Table 3) previously described in Read et al., 2004. A 20 µl aliquot of internal product was digested with 5 U *N1aIV* (New England Biolabs, Beverly, MA), 2.6 µl of NE buffer 4, and 0.1 µg/µl BSA for three hours at 37°C. Those samples producing an Assemblage B profile were digested with 5 U *RsaI* (New England Biolabs, Beverly, MA) and 2.3 µl of NE buffer 1, for eight hours at 37°C to ensure complete digestion. *N1aIV* and *RsaI* digests were visualized on 2% agarose gel (Invitrogen) stained with ethidium bromide (Sigma).

Table 3: Glutamate dehydrogenase Restriction Profiles.

Predicted and diagnostic profiles for various *G. duodenalis* Assemblages based on *NlaIV* and *RsaI* digests of a 432 bp glutamate dehydrogenase PCR. Note: Diagnostic restriction profiles exclude fragments less than 70 bp as they could not be reliably resolved on the gel and because all the Assemblages could be distinguished without these small bands (modified from Read et al., 2004).

Genotype	Enzyme (NO. of Restriction Sites)	Predicted Fragment Sizes (bp)	Diagnostic restriction Profile (bp)
Sub-Assemblage AI	*NlaIV* (5)	16, 18, 39, 87, 123, 149	90, 120, 150
Sub-Assemblage AII	*NlaIV* (6)	18, 16, 39, 72, 77, 87, 123	70, 80, 90, 120
Sub-Assemblage B-III	*NlaIV* (2)	18, 123 291	120, 290
Sub-Assemblage B-IV	*NlaIV* (2)	18, 123, 291	120, 290
Assemblage C	*NlaIV* (4)	18, 31, 72, 123, 187	70, 120, 190
Assemblage D	*NlaIV* (3)	18, 39, 126, 249	120, 250
Assemblage E	*NlaIV* (4)	16, 18, 72, 106, 218	80, 100, 220
Sub-Assemblage B-III	*RsaI*(2)	2, 133, 297	130, 300
Sub-Assemblage B-IV	*RsaI*(1)	2, 430	430

2.3.4 Sequencing of PCR Products

Sequencing of internal PCR products at the SSU and GDH loci was performed by University of Calgary Core DNA Sequencing Laboratory using 3 pmol of either RH4, RH11, GDHiR, or GDHiF primers, and PCR template from the corresponding external reaction. All reactions utilized ABI PRISM Cycle Sequencing Kits (Applied Biosystems, Foster City, California).

2.4 Bioinformatics and Statistical Analysis

2.4.1 Primer Design

To increase sensitivity of glutamate dehydrogenase RFLP analysis, primers external to GDHiR and GDHiF described in Read et al., 2004 were designed. Sequence data was obtained for a representative isolate of each of the Assemblages A-I, A-II, B-III, B-IV, C, D and E from the NCBI database (GenBank accession numbers: L40509 (A-I), AF069059 (B-III), L40510 (A-II), L40508 (B-IV), U60984(C), U60986 (D), and U47632 (E)) . Sequences were aligned using Clustal W (version 1.81) and analysed for homologous regions across all genotypes and external to GDHiR and GDHiF. Primer sequences were BLAST searched to ensure that they were specific for *G. duodenalis.* Primers were synthesized by University of Calgary Core DNA Services.

2.4.2 Phylogenetic Analysis

Sequence data utilized for phylogenetic analysis was either obtained through the sequencing clinical isolates or retrieved from the GenBank database. Clustal W (version

1.81) default parameters for pairwise and global alignments were used to align sequences. Phylogenetic relationships between known *Giardia duodenalis* assemblages/sub-assemblages and clinical isolates were inferred by the neighbour-joining analysis of glutamate dehydrogenase gene (GenBank accession numbers: L40509 (A-I), AF069059 (B-III), L40510 (A-II), L40508 (B-IV), U60984 (C), U60986 (D), and U47632 (E)). Bootstrap analysis (deleted half-jackknife) was performed with 1000 resamplings of the data set. A maximum likelihood-based consensus tree was generated with Phylip (version 3.2).

2.4.3 Statistical Analysis

Nominal categorical data (genotype frequencies) was analysed using nonparametric statistics. Pearson Chi-Squared and Cramer's V values were calculated using SPSS version 12 for Windows. Relationships between variables were considered statistically significant if the *p*-value was determined to be less than 0.01. Effect size was determined using Cohen's conventions from Cramer's Phi. For the purpose of statistical analyses, genotype frequencies for SSU and GDU were combined (SSU/GDH) to increase sample size. In order to merge the genotype frequencies, GDH Sub-assemblage A-I and A-II were combined with SSU Assemblage A data and GDH Sub-assemblage B-III and B-IV were combined with SSU Assemblage B data.

Chapter Three: Development and Confirmation of GDH RFLP Technique

In any molecular-based epidemiological study, it is important to set a base unit to be diagnosed (e.g., species, assemblage, or sub-assemblage). Until recently the RH4 and RH11 primer set, first used by Hopkins et al.(82), which amplify a 292 bp section of the SSU rRNA gene, has been a popular tool in *G. duodenalis* molecular diagnostics. As discussed in the section on nomenclature (Section 1.6), the expanded use of DNA-based techniques has greatly expanded our understanding of genetic diversity with *Giardia duodenalis.* While the RH4/RH11 primer set is capable of determining species and distinguishing between Assemblage A and B without sequencing, it is unable to resolve other assemblages. With sequencing, the primers are theoretically capable of resolving Assemblage C, D, and E though problems appear to exist. In respect to Assemblage E, a single nucleotide polymorphism (SNP) at nucleotide position 92 is used to distinguish Assemblage E from A and this can be sometimes missed. It is also unclear how reliable the SSU rRNA gene is at resolving Assemblage C and D. A recent epidemiological study (162) suggests the gene may not be able to resolve Assemblage C and D as reliably as once thought.

In order to resolve the frequencies of various sub-assemblages in Southern Alberta, a modified GDH-RFLP technique, originally described by Read and colleagues (136) was used. To increase the sensitivity of the technique, an external primer set was constructed to allow for a nested-PCR reaction. Published GDH sequences for the various assemblages and sub-assemblages were aligned using Clustal W and two regions of high homology external to GDHiR and GDHiF primers were selected (Figure 2).

Purposed primer sequences were BLAST searched, using default parameters, to ensure that they were specific for *G. duodenalis.* The NCBI Blast search returned only known *G. duodenalis* GDH sequences with exact matches to either primer. With regard to sequences with close homology to external primers, no organism other than *G. duodenalis* had close homology to both primers. Predicted PCR product size for the external primers (497 bp) was verified by agarose gel electrophoresis.

To confirm the efficacy of the GDH-RFLP technique a representative clinical isolate from each of the observed restriction profiles (Figure 3) (Assemblage C and D profiles were not observed) were sequenced. Clinical isolates had nearly complete homology for AI (99%), AII (100%), B-III (99%), B-IV (100%), and E (99%) with the four reference sequences (Figure 4-6) confirming the efficacy of the technique.

In addition to the five of seven restriction profiles initially published (136), a previously undescribed restriction profile was observed in a single clinical sample (Figure 3, 390). Sequencing of the internal GDH product of Clinical isolate 390 (CI390) (Figure 7) found it had the highest degree of homology with known AI (99%) and AII (99%), with five SNPs observed among the sequences. Three of the five SNPs were unique to the isolate and one SNP matching either Sub-assemblage A-I or A-II. Clinical isolate 390 had 91 and 89% homology to Sub-assemblage B-III and B-IV GDH sequences respectively (data not shown), and 86% homology with Assemblage E. In contrast to GDH sequence analysis, sequencing of the SSU rRNA product suggests CI390 is more closely related to Assemblage B with 100% homology observed between CI390 and a known Assemblage B sequence (Figure 8). Based on GDH phylogenetic analysis (Figure 9), CI390 clustered independently of other assemblages/sub-assemblages

58.3% of the time, but was is deemed statistically significant. Until additional target loci are sequenced and phylogenetic comparisons are performed, CI390 can not accurately be assigned a genotype. The isolate has been shipped to collaborators at Murdoch University in Perth, Australia, for further molecular analysis, which will allow the determination of genotype.

To further compare the SSU and GDH RFLP genotyping techniques, 136 samples were genotyped at both target loci. A total of 116 samples (85%) were concordant and 20 were discordant. All 20 discordant results were associated with samples where at least one of the target loci had genotyped the sample as a mixed A/B assemblage infection. There were 17 samples that genotyped as mixed assemblage infections using GDH loci, and as Assemblage B by SSU loci. There were 3 samples that genotyped as mixed assemblage infections using SSU loci and as Assemblage A or B using the GDH loci. Unlike the GDH-RFLP profile, the mixed assemblage RFLP profile for SSU-RFLP technique consists of two products with a size differential of only 10 base pairs. The loading of excessive PCR product into the agarose gel can result in the masking of the larger product (Assemblage A), resulting in a misclassification of the isolate as Assemblage B. This source of error can account for 17 of the 20 mismatches. With regard to the 3 samples that genotyped as a mixed assemblage infection with the SSU loci and as either Assemblage A or B with the GDH loci, this may be a result of the increased sensitivity associated with the SSU genotyping technique due to the increase copy number of SSU rRNA genes within the cell. A comparison of the two techniques suggests while the SSU RFLP technique may have a theoretically lower detection level, the GDH RFLP technique has numerous advantages, most important being the ability to

determine sub-assemblage and most assemblages (unable to resolve Assemblage F or G) without sequencing.

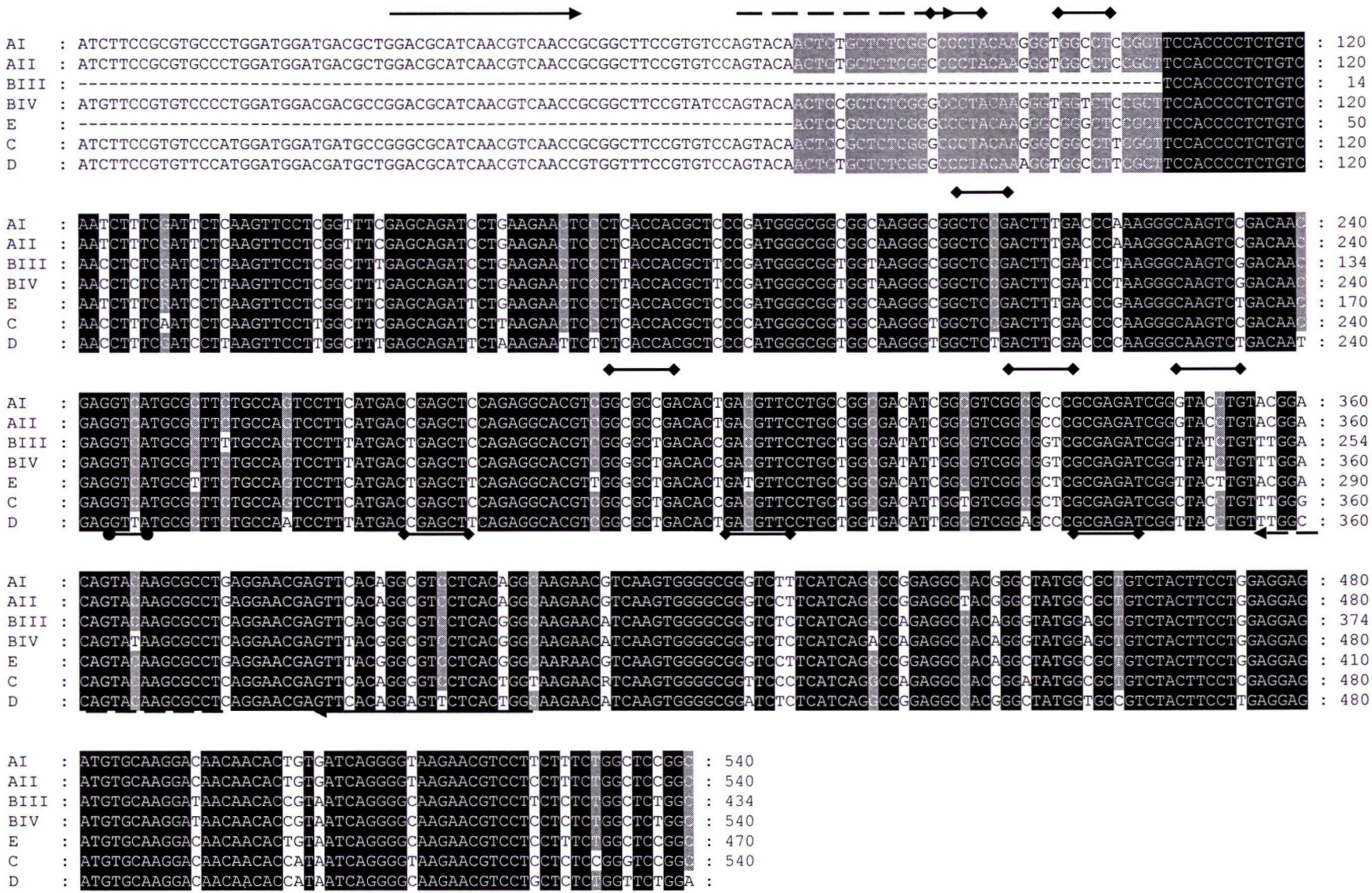
An internal verification of the reproducibility of the SSU-RFLP results found that of the 11 clinical samples genotyped more than once with the SSU-RFLP protocol, 9 were concordant. The first discordant result was genotyped independently on four occasions. On two occasions the sample genotyped as a mixed assemblage infection and the other two as an Assemblage A infection. On both occasions when the discordant sample genotyped as a mixed assemblage infection, the SSU-RFLP profile displayed a weak Assemblage B band. The discordant result may be a result of either the amount of Assemblage B DNA within the sample being close to the detection level of the assay, or possibly the Assemblage B band being masked by a more intense Assemblage A band. The second discordant result was genotyped independently on two occasions. On one occasion the sample genotyped as Assemblage A and on the other as a mixed assemblage infection. The same reasons stated above may also explain this discordant result, though for both discordant results the possibility of cross-contamination can not be discounted.

An internal verification of the reproducibility of the GDH-RFLP results found that of the 19 clinical samples genotyped more than once with the GDH-RFLP protocol, 17 were concordant. The first discordant result was genotyped on three independent occasions. On two of these occasions the samples were genotyped as Sub-assemblage B-III and on the other occasion the sample was genotyped as mixed sub-assemblage B. This discordant result is likely due to an incomplete *RsaI* restriction enzyme digestion, which produces a RFLP profile the same as a mixed sub-assemblage B. The second discordant result was genotyped on three independent occasions. On two of the

occasions the samples were genotyped as mixed assemblage infections and one occasion as Sub-assemblage A-I. It is possible that this discordant result is a result of low copy numbers of Assemblage B parasite DNA associated with the sample, but the possibility of cross contamination with another sample can not be ruled out.

Figure 2: *Giardia duodenalis* glutamate dehydrogenase sequence alignment

Sequence data was obtained for each assemblage and sub-assemblage (A-I, A-II, B-III, B-IV, C, D and E) from the NCBI database (GenBank accession numbers: L40509 (A-I), L40510 (A-II), AF069059 (B-III), L40508 (B-IV), U60984(C), U60986 (D) U47632 (E)). Sequences were aligned using Clustal W. Predicted primer binding sites for the nested PCR reaction are indicated with solid line arrows (GDHeR and GDHeF) and dashed line arrows (GDHiF and GDHiR). Regions containing a predicted *NlaIV* restriction site (GGNNCC) are indicated by a line capped with diamonds. The region containing the diagnostic *RsaI* restriction site (GTAC) used to resolve Sub-assemblage B-III and B-IV is indicated by a line capped with circles.


AI : ATCTTCCGCGTGCCCTGGATGGATGACGCTGGACGCATCAACGTCAACCGCGGCTTCCGTGTCCAGTACA : 120
AII : ATCTTCCGCGTGCCCTGGATGGATGACGCTGGACGCATCAACGTCAACCGCGGCTTCCGTGTCCAGTACA : 120
BIII : ---------- : 14
BIV : ATGTTCCGTGTCCCCTGGATGGACGACGCCGGACGCATCAACGTCAACCGCGGCTTCCGTATCCAGTACA : 120
E : ---------- : 50
C : ATCTTCCGTGTCCCATGGATGGATGATGCCGGGCGCATCAACGTCAACCGCGGCTTCCGTGTCCAGTACA : 120
D : ATCTTCCGTGTTCCATGGATGGACGATGCTGGACGCATCAACGTCAACCGTGGTTTCCGTGTCCAGTACA : 120
AI : : 240
AII : : 240
BIII : : 134
BIV : : 240
E : : 170
C : : 240
D : : 240
AI : : 360
AII : : 360
BIII : : 254
BIV : : 360
E : : 290
C : : 360
D : : 360
AI : : 480
AII : : 480
BIII : : 374
BIV : : 480
E : : 410
C : : 480
D : : 480
AI : : 540
AII : : 540
BIII : : 434
BIV : : 540
E : : 470
C : : 540
D :

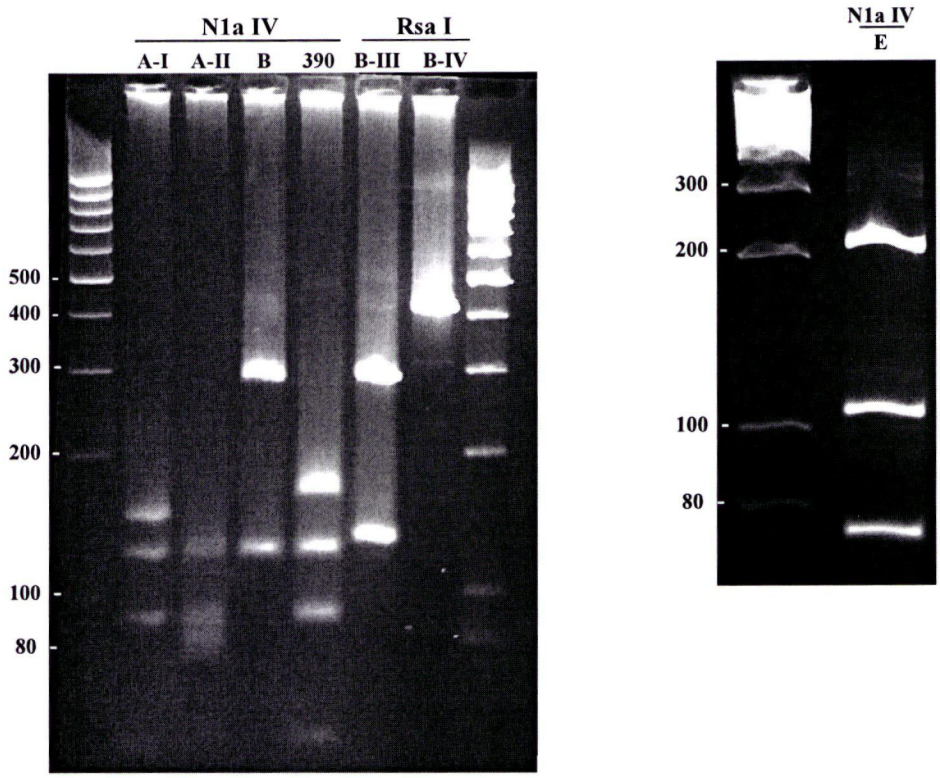


Figure 3: Glutamate dehydrogenase RFLP profiles for *G. duodenalis* for various assemblages and sub-assemblages.

Internal glutamate dehydrogenase PCR products were digested with either *NlaIV* or *RsaI* and visualized on a 2% agarose gel stained with ethidium bromide.

Figure 4: *Giardia duodenalis* Sub-assemblage AI and AII GDH sequence confirmation

Using GDH sequences retrieved from GenBank, a Clustal W sequence alignment was performed using known AI (L40509) and AII (L40510) sequences and a representative clinical isolates that were determined to be AI (CIAI) or AII (CIAII), via GDH-RFLP. Regions containing a predicted *NlaIV* restriction site (GGNNCC) are indicated by a line capped with diamonds.

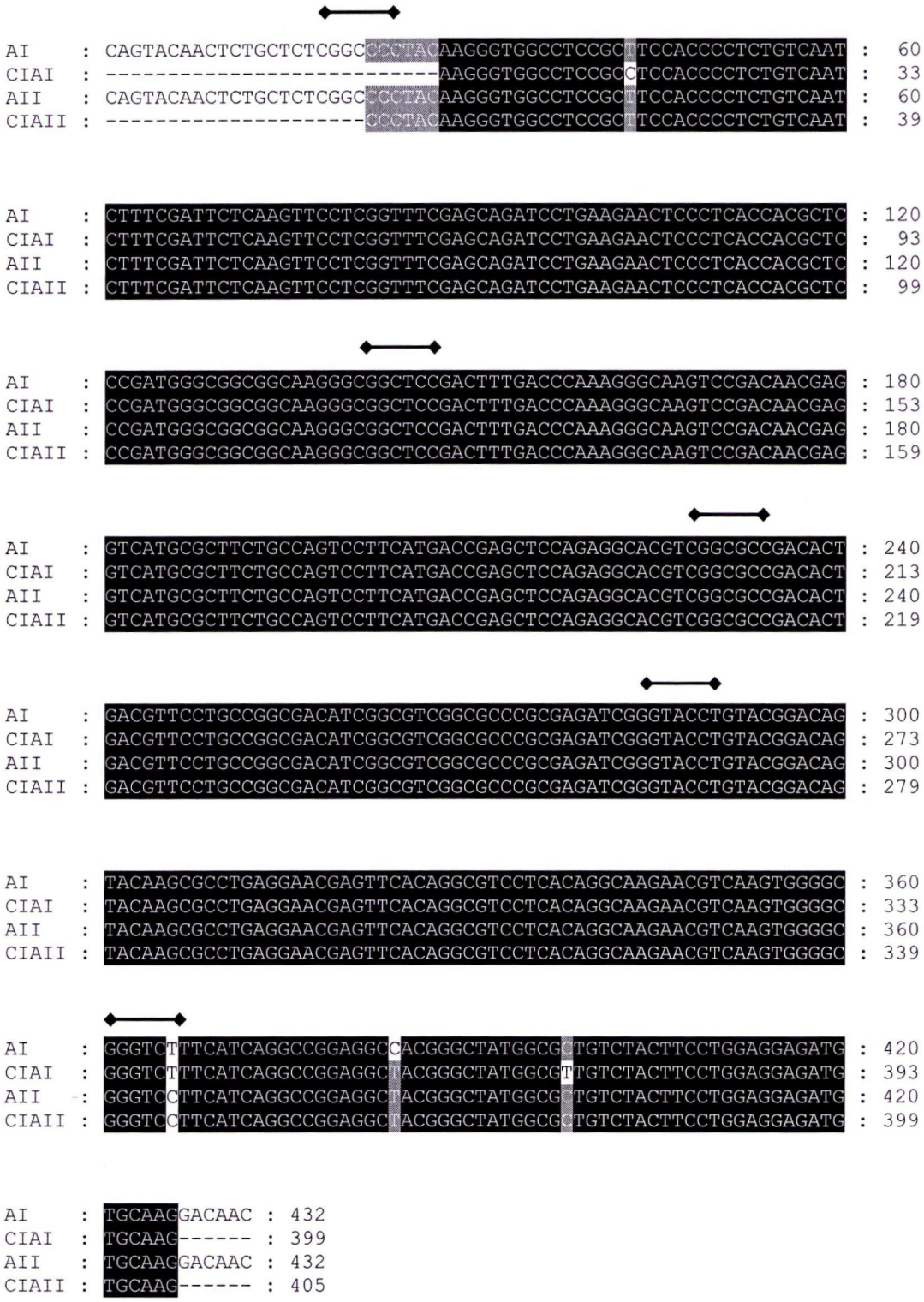

AI : CAGTACAACTCTGCTCTCGGCCCCTACAAGGGTGGCCTCCGCTTCCACCCCTCTGTCAAT : 60
CIAI : ----------AAGGGTGGCCTCCGCCTCCACCCCTCTGTCAAT : 33
AII : CAGTACAACTCTGCTCTCGGCCCCTACAAGGGTGGCCTCCGCTTCCACCCCTCTGTCAAT : 60
CIAII : ----------CCCTACAAGGGTGGCCTCCGCTTCCACCCCTCTGTCAAT : 39
AI : CTTTCGATTCTCAAGTTCCTCGGTTTCGAGCAGATCCTGAAGAACTCCCTCACCACGCTC : 120
CIAI : CTTTCGATTCTCAAGTTCCTCGGTTTCGAGCAGATCCTGAAGAACTCCCTCACCACGCTC : 93
AII : CTTTCGATTCTCAAGTTCCTCGGTTTCGAGCAGATCCTGAAGAACTCCCTCACCACGCTC : 120
CIAII : CTTTCGATTCTCAAGTTCCTCGGTTTCGAGCAGATCCTGAAGAACTCCCTCACCACGCTC : 99
AI : CCGATGGGCGGCGGCAAGGGCGGCTCCGACTTTGACCCAAAGGGCAAGTCCGACAACGAG : 180
CIAI : CCGATGGGCGGCGGCAAGGGCGGCTCCGACTTTGACCCAAAGGGCAAGTCCGACAACGAG : 153
AII : CCGATGGGCGGCGGCAAGGGCGGCTCCGACTTTGACCCAAAGGGCAAGTCCGACAACGAG : 180
CIAII : CCGATGGGCGGCGGCAAGGGCGGCTCCGACTTTGACCCAAAGGGCAAGTCCGACAACGAG : 159
AI : GTCATGCGCTTCTGCCAGTCCTTCATGACCGAGCTCCAGAGGCACGTCGGCGCCGACACT : 240
CIAI : GTCATGCGCTTCTGCCAGTCCTTCATGACCGAGCTCCAGAGGCACGTCGGCGCCGACACT : 213
AII : GTCATGCGCTTCTGCCAGTCCTTCATGACCGAGCTCCAGAGGCACGTCGGCGCCGACACT : 240
CIAII : GTCATGCGCTTCTGCCAGTCCTTCATGACCGAGCTCCAGAGGCACGTCGGCGCCGACACT : 219
AI : GACGTTCCTGCCGGCGACATCGGCGTCGGCGCCCGCGAGATCGGGTACCTGTACGGACAG : 300
CIAI : GACGTTCCTGCCGGCGACATCGGCGTCGGCGCCCGCGAGATCGGGTACCTGTACGGACAG : 273
AII : GACGTTCCTGCCGGCGACATCGGCGTCGGCGCCCGCGAGATCGGGTACCTGTACGGACAG : 300
CIAII : GACGTTCCTGCCGGCGACATCGGCGTCGGCGCCCGCGAGATCGGGTACCTGTACGGACAG : 279
AI : TACAAGCGCCTGAGGAACGAGTTCACAGGCGTCCTCACAGGCAAGAACGTCAAGTGGGGC : 360
CIAI : TACAAGCGCCTGAGGAACGAGTTCACAGGCGTCCTCACAGGCAAGAACGTCAAGTGGGGC : 333
AII : TACAAGCGCCTGAGGAACGAGTTCACAGGCGTCCTCACAGGCAAGAACGTCAAGTGGGGC : 360
CIAII : TACAAGCGCCTGAGGAACGAGTTCACAGGCGTCCTCACAGGCAAGAACGTCAAGTGGGGC : 339
AI : GGGTCTTTCATCAGGCCGGAGGCCACGGGCTATGGCGCTGTCTACTTCCTGGAGGAGATG : 420
CIAI : GGGTCTTTCATCAGGCCGGAGGCTACGGGCTATGGCGTTGTCTACTTCCTGGAGGAGATG : 393
AII : GGGTCCTTCATCAGGCCGGAGGCTACGGGCTATGGCGCTGTCTACTTCCTGGAGGAGATG : 420
CIAII : GGGTCCTTCATCAGGCCGGAGGCTACGGGCTATGGCGCTGTCTACTTCCTGGAGGAGATG : 399
AI : TGCAAGGACAAC : 432
CIAI : TGCAAG------ : 399
AII : TGCAAGGACAAC : 432
CIAII : TGCAAG------ : 405

Figure 5: *Giardia duodenalis* Sub-assemblage B-III and B-IV GDH sequence confirmation

Using GDH sequences retrieved from GenBank, a Clustal W sequence alignment was performed using known B-III (AF069059) and B-IV (L40508) sequences and a representative clinical isolates that were determined to be B-III (CIB-III) or BIV (CIBIV), via GDH-RFLP. Regions containing a predicted *NlaIV* restriction site (GGNNCC) are indicated by a line capped with diamonds. The region containing the diagnostic *RsaI* restriction site (GTAC) used resolve Sub-assemblage B-III and B-IV is indicated by a line capped with circles.

```
BIII   : CAGTACAACTCCGCTCTCGGGCCCTACAAGGGTGGTCTCCGCTTCCACCCCTCTGTCAAC :  60
CIBIII : ------------------------GCTAGGGTGGTCTCCGCTTCCACCCCTCTGTCAAC :  35
BIV    : CAGTACAACTCCGCTCTCGGGCCCTACAAGGGTGGTCTCCGCTTCCACCCCTCTGTCAAC :  60
CIBIV  : ---------------------------ACAGGTGGTCTCCGCTTCCACCCCTCTGTCAAC :  33

BIII   : CTCTCGATCCTCAAGTTCCTCGGCTTTGAGCAGATCCTGAAGAACTCCCTTACCACGCTT : 120
CIBIII : CTCTCGATCCTTAAGTTCCTCGGCTTTGAGCAGATCCTGAAGAACTCCCTTACCACGCTT :  95
BIV    : CTCTCGATCCTTAAGTTCCTCGGCTTTGAGCAGATCCTGAAGAACTCCCTTACCACGCTT : 120
CIBIV  : CTCTCGATCCTTAAGTTCCTCGGCTTTGAGCAGATCCTGAAGAACTCCCTTACCACGCTT :  93

BIII   : CCGATGGGCGGTGGTAAGGGCGGCTCCGACTTCGATCCTAAGGGCAAGTCGGACAACGAG : 180
CIBIII : CCGATGGGCGGTGGTAAGGGCGGCTCCGACTTCGATCCTAAGGGCAAGTCGGACAACGAG : 155
BIV    : CCGATGGGCGGTGGTAAGGGCGGCTCCGACTTCGATCCTAAGGGCAAGTCGGACAACGAG : 180
CIBIV  : CCGATGGGCGGTGGTAAGGGCGGCTCCGACTTCGATCCTAAGGGCAAGTCGGACAACGAG : 153

BIII   : GTCATGCGCTTTTGCCAGTCCTTTATGACTGAGCTCCAGAGGCACGTCGGGGCTGACACC : 240
CIBIII : GTCATGCGCTTTTGCCAGTCCTTTATGACTGAGCTCCAGAGGCACGTCGGGGCTGACACC : 215
BIV    : GTCATGCGCTTCTGCCAGTCCTTTATGACCGAGCTCCAGAGGCACGTCGGGGCTGACACC : 240
CIBIV  : GTCATGCGCTTCTGCCAGTCCTTTATGACCGAGCTCCAGAGGCACGTCGGGGCTGACACC : 213

BIII   : GACGTTCCTGCTGGCGATATTGGCGTCGGCGGTCGCGAGATCGGTTATCTGTTTGGACAG : 300
CIBIII : GACGTTCCTGCTGGCGATATTGGCGTCGGCGGTCGCGAGATCGGTTATCTGTTTGGACAG : 275
BIV    : GACGTTCCTGCTGGCGATATTGGCGTCGGCGGTCGCGAGATCGGTTATCTGTTTGGACAG : 300
CIBIV  : GACGTTCCTGCTGGCGATATTGGCGTCGGCGGTCGCGAGATCGGTTATCTGTTTGGACAG : 273

BIII   : TACAAGCGCCTCAGGAACGAGTTCACGGGCGTCCTCACGGGCAAGAACATCAAGTGGGGC : 360
CIBIII : TACAAGCGCCTCAGGAACGAGTTCACGGGCGTCCTCACGGGCAAGAACATCAAGTGGGGC : 335
BIV    : TATAAGCGCCTCAGGAACGAGTTTACGGGCGTCCTCACGGGCAAGAACATCAAGTGGGGC : 360
CIBIV  : TATAAGCGCCTCAGGAACGAGTTTACGGGCGTCCTCACGGGCAAGAACATCAAGTGGGGC : 333

BIII   : GGGTCTCTCATCAGGCCAGAGGCCACAGGGTATGGAGCTGTCTACTTCCTGGAGGAGATG : 420
CIBIII : GGGTCTCTCATCAGGCCAGAGGCCACAGGGTATGGAGCTGTCTACTTCCTGGAGGAGATG : 395
BIV    : GGGTCTCTCATCAGACCAGAGGCCACAGGGTATGGAGCTGTCTACTTCCTGGAGGAGATG : 420
CIBIV  : GGGTCTCTCATCAGACCAGAGGCCACAGGGTATGGAGCTGTCTACTTCCTGGAGGAGATG : 393

BIII   : TGCAAGGATAAC : 432
CIBIII : TGC--------- : 398
BIV    : TGCAAGGATAAC : 432
CIBIV  : TG---------- : 395
```

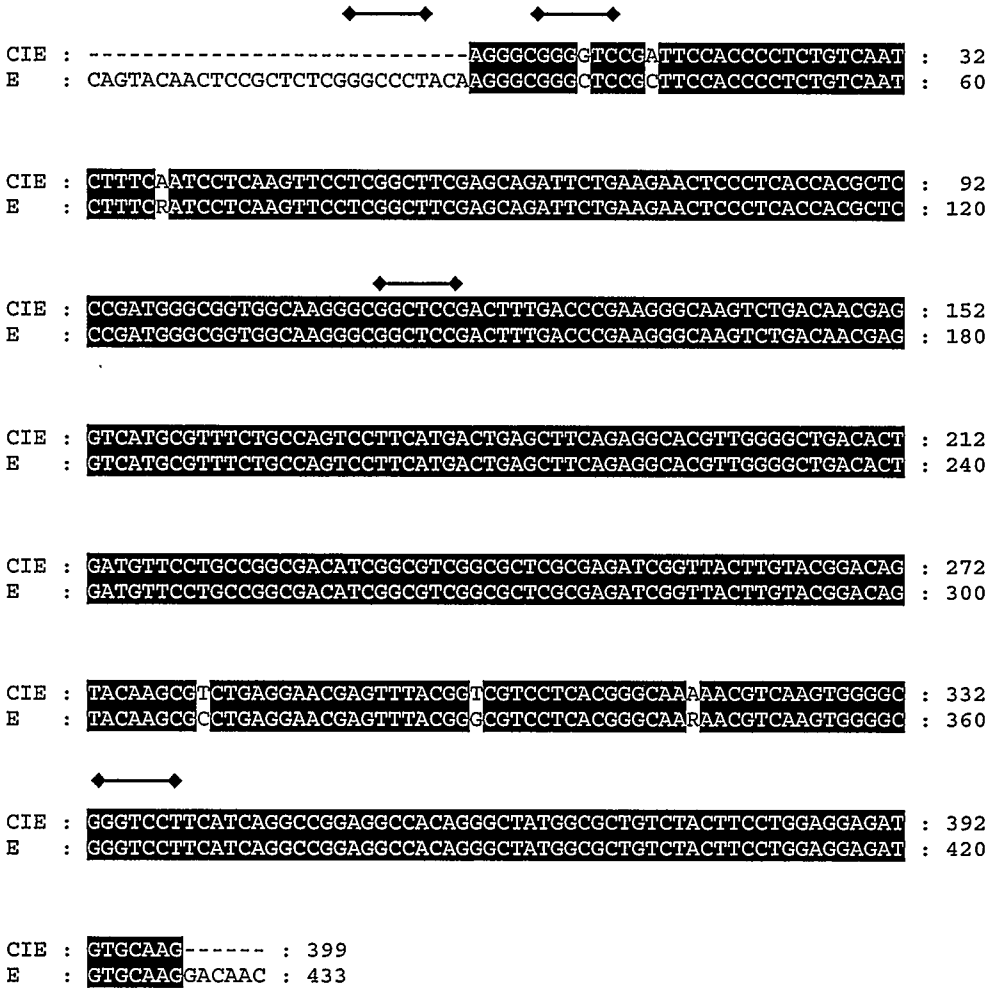
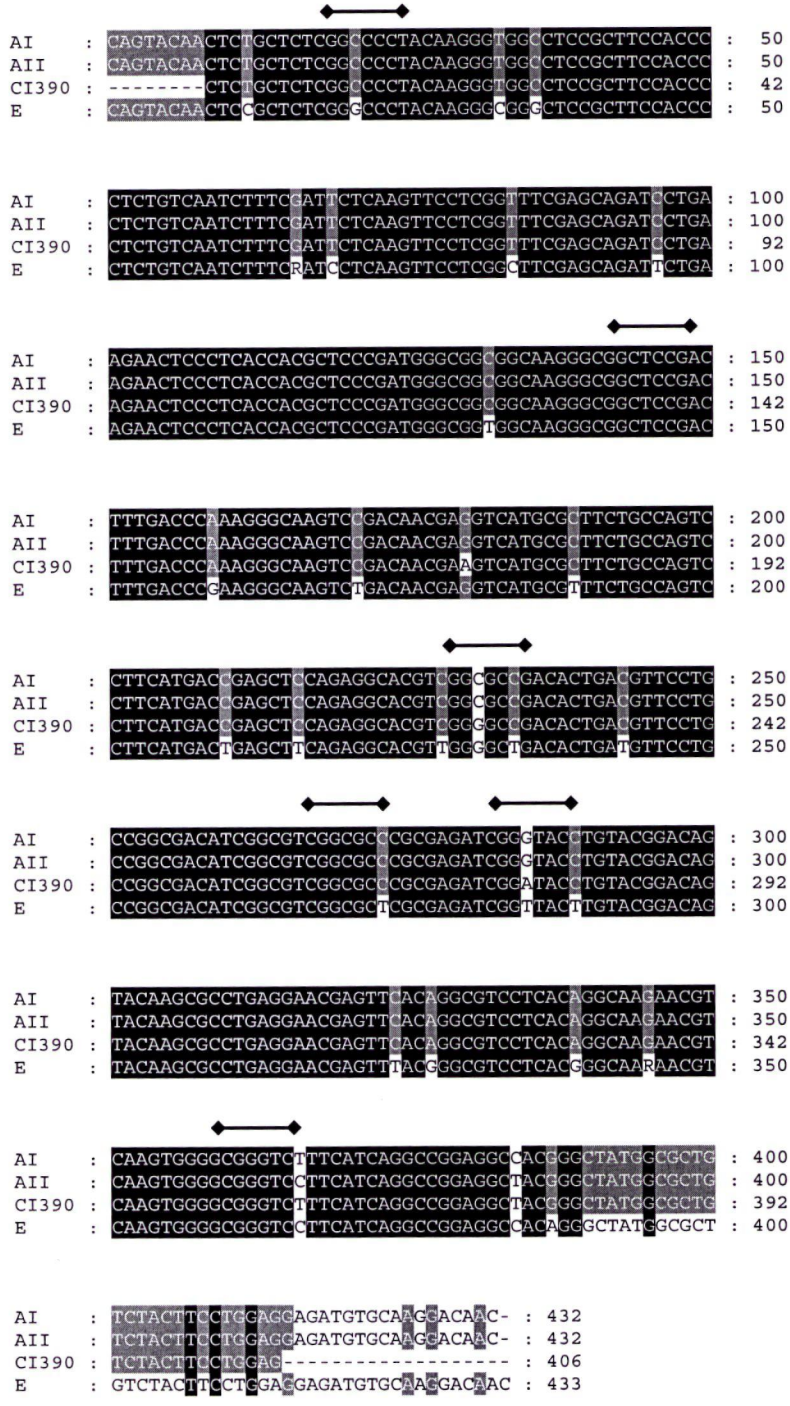


Figure 6: *Giardia duodenalis* Assemblage E GDH sequence confirmation

Using GDH sequences retrieved from GenBank, a Clustal W sequence alignment was performed using a known Assemblage E (U47632) sequence and a representative clinical isolate that was determined to be Assemblage E (CIE) via GDH-RFLP. Regions containing a predicted *NlaIV* restriction site (GGNNCC) are indicated by a line capped with diamonds.

Figure 7: GDH gene sequence alignment with a unique clinical isolate

Using GDH sequences retrieved from GenBank, a Clustal W sequence alignment was performed using a known Sub-assemblage AI (L40509), AII (L40510), and Assemblage E (U47632) sequences and a representative clinical isolate with an unpublished GDH-RFLP profile (CI390). Regions containing a predicted *NlaIV* restriction site (GGNNCC) are indicated by a line capped with diamonds


AI : CAGTACAACTCTGCTCTCGGCCCCTACAAGGGTGGCCTCCGCTTCCACCC : 50
AII : CAGTACAACTCTGCTCTCGGCCCCTACAAGGGTGGCCTCCGCTTCCACCC : 50
CI390 : --------CTCTGCTCTCGGCCCCTACAAGGGTGGCCTCCGCTTCCACCC : 42
E : CAGTACAACTCCGCTCTCGGGCCCTACAAGGGCGGGCTCCGCTTCCACCC : 50
AI : CTCTGTCAATCTTTCGATTCTCAAGTTCCTCGGTTTCGAGCAGATCCTGA : 100
AII : CTCTGTCAATCTTTCGATTCTCAAGTTCCTCGGTTTCGAGCAGATCCTGA : 100
CI390 : CTCTGTCAATCTTTCGATTCTCAAGTTCCTCGGTTTCGAGCAGATCCTGA : 92
E : CTCTGTCAATCTTTCGATCCTCAAGTTCCTCGGCTTCGAGCAGATTCTGA : 100
AI : AGAACTCCCTCACCACGCTCCCGATGGGCGGCGGCAAGGGCGGCTCCGAC : 150
AII : AGAACTCCCTCACCACGCTCCCGATGGGCGGCGGCAAGGGCGGCTCCGAC : 150
CI390 : AGAACTCCCTCACCACGCTCCCGATGGGCGGCGGCAAGGGCGGCTCCGAC : 142
E : AGAACTCCCTCACCACGCTCCCGATGGGCGGTGGCAAGGGCGGCTCCGAC : 150
AI : TTTGACCCAAAGGGCAAGTCCGACAACGAGGTCATGCGCTTCTGCCAGTC : 200
AII : TTTGACCCAAAGGGCAAGTCCGACAACGAGGTCATGCGCTTCTGCCAGTC : 200
CI390 : TTTGACCCAAAGGGCAAGTCCGACAACGAAGTCATGCGCTTCTGCCAGTC : 192
E : TTTGACCCGAAGGGCAAGTCTGACAACGAGGTCATGCGTTCTGCCAGTC : 200
AI : CTTCATGACCGAGCTCCAGAGGCACGTCGGCGCCGACACTGACGTTCCTG : 250
AII : CTTCATGACCGAGCTCCAGAGGCACGTCGGCGCCGACACTGACGTTCCTG : 250
CI390 : CTTCATGACCGAGCTCCAGAGGCACGTCGGCGGCCGACACTGACGTTCCTG : 242
E : CTTCATGACTGAGCTTCAGAGGCACGTTGGGGCTGACACTGATGTTCCTG : 250
AI : CCGGCGACATCGGCGTCGGCGCCCGCGAGATCGGGTACCTGTACGGACAG : 300
AII : CCGGCGACATCGGCGTCGGCGCCCGCGAGATCGGGTACCTGTACGGACAG : 300
CI390 : CCGGCGACATCGGCGTCGGCGCCCGCGAGATCGGATACCTGTACGGACAG : 292
E : CCGGCGACATCGGCGTCGGCGCTCGCGAGATCGGTTACTTGTACGGACAG : 300
AI : TACAAGCGCCTGAGGAACGAGTTCACAGGCGTCCTCACAGGCAAGAACGT : 350
AII : TACAAGCGCCTGAGGAACGAGTTCACAGGCGTCCTCACAGGCAAGAACGT : 350
CI390 : TACAAGCGCCTGAGGAACGAGTTCACAGGCGTCCTCACAGGCAAGAACGT : 342
E : TACAAGCGCCTGAGGAACGAGTTTACGGGCGTCCTCACGGGCAARAACGT : 350
AI : CAAGTGGGGCGGGTCTTTCATCAGGCCGGAGGCCACGGGCTATGGCGCTG : 400
AII : CAAGTGGGGCGGGTCCTTCATCAGGCCGGAGGCTACGGGCTATGGCGCTG : 400
CI390 : CAAGTGGGGCGGGTCTTTCATCAGGCCGGAGGCTACGGGCTATGGCGCTG : 392
E : CAAGTGGGGCGGGTCCTTCATCAGGCCGGAGGCCACAGGGCTATGGCGCT : 400
AI : TCTACTTCCTGGAGCAGATGTGCAAGGACAAC- : 432
AII : TCTACTTCCTGGAGCAGATGTGCAAGGACAAC- : 432
CI390 : TCTACTTCCTGGAG---------- : 406
E : GTCTACTTCCTGGAGGAGATGTGCAAGGACAAC : 433

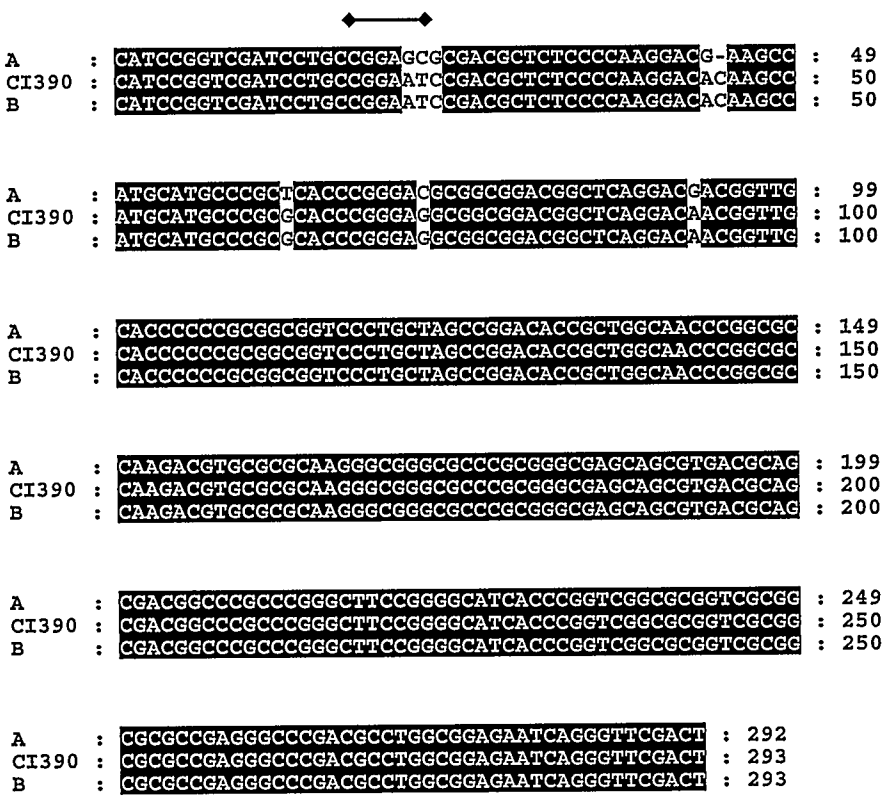


Figure 8: SSU rRNA gene sequence alignment for a unique clinical isolate

Using SSU rRNA gene sequences retrieved from GenBank, a Clustal W sequence alignment was performed using known Assemblage A (AF199448) and B (U09492) and clinical isolate 390 (CI390). Regions containing a predicted *HinFI* restriction site (GANTC) are indicated by a line capped with diamonds

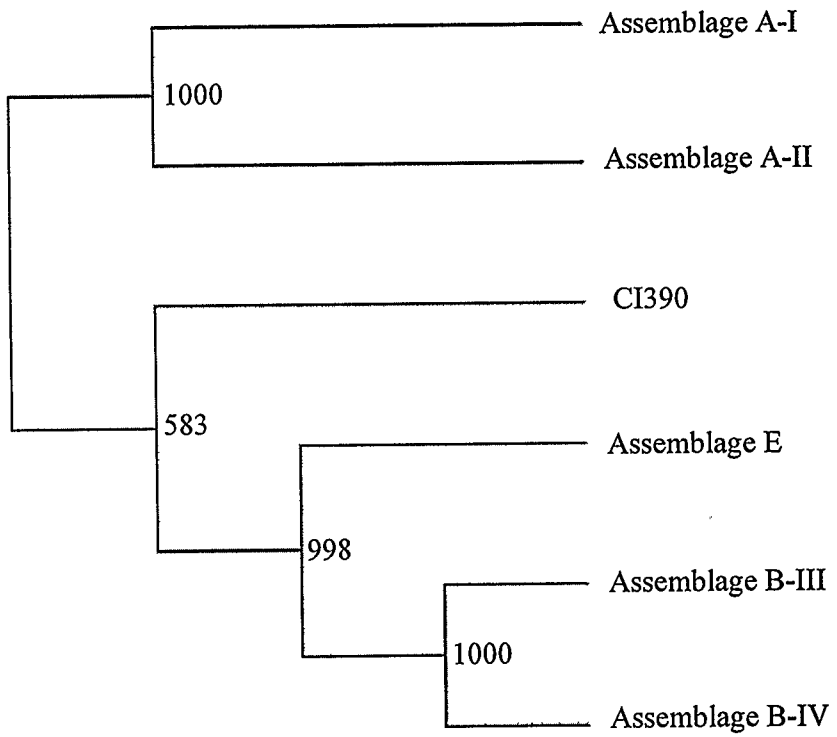


Figure 9: Glutamate dehydrogenase phylogenetic analysis of a unique clinical isolate

Phylogenetic relationships of *Giardia duodenalis* assemblages/sub-assemblages and a unique clinical isolate inferred by the neighbour-joining analysis of glutamate dehydrogenase gene.

Chapter Four: *G. duodenalis* genotypes in Southern Alberta

During the study period, January 2002 to March 2004, a total of 486 *Giardia duodenalis* positive SAF fixed stool samples were supplied by CLS. Of those received, 216 and 205 samples were genotyped with the GDH and SUU target loci respectively, with 282 samples genotyped at least one target loci. To-date this is the largest ever survey of G. duodenalis genotypes in humans. Of the 486 received, 194 samples were not genotyped due to the inability to amplify either target loci. The formalin fixing of samples and abundance of PCR inhibitors in feces are likely the two main factors contributing to this failure. The number of samples genotyped monthly ranged for 1 to 25 with median of 8 and an average of 11. Monthly genotyping efficiency (Figure 10) ranged from 33% (Feb) to 70% (Dec), with no month accounting for more than 15% of samples genotyped. Genotyping efficiency was increased by reducing the amount of time fecal samples were in contact with fixing agent and by changing to a QIAamp DNA Stool Kit for DNA extractions. For the duration of the study, routine surveillance by the Calgary Regional Health Authority detected no major outbreaks of giardiasis. Participants ranged from less than 1 year to 91 years, with 26.4% participants under 5 years of age. Due to low frequency of Assemblage E (2 clinical samples), mixed assemblage A (1 clinical sample), infections, and a unique GDH-RFLP profile (1 clinical sample: 390) these samples are discussed separately and not included in the overall frequency calculations.

4.1 Overall *G. duodenalis* Genotype Frequencies in Humans

During the study period, as expected and previously reported in other regions (6), the observed frequency of mixed assemblage infection (11.5%) was significantly less (χ^2 =

68.3, df=2,p>0.01) than single assemblage infections (Table 4). In respect to single assemblage infections, a significant difference (χ^2 = 6.504, df=1, p=0.01) in the observed frequency of Assemblage A (51.4%) and B (37.1%) infections was detected, with a greater than expected frequency of Assemblage A observed. An examination of observed sub-assemblages (Table 4) found greater than expected (χ^2 = 80.9, df=5, p>0.01) frequency of Sub-assemblage A-I (39.6%). Excluding Sub-assemblage A-I there was not significant difference in the overall frequency of the other observed genotypes (χ^2 = 0.7, df=4, p=0.95). Genotype frequencies observed in Calgary were nearly identical to those observed in Egypt (51), possibly suggesting that Sub-assemblage A-I is the predominant genotype world-wide.

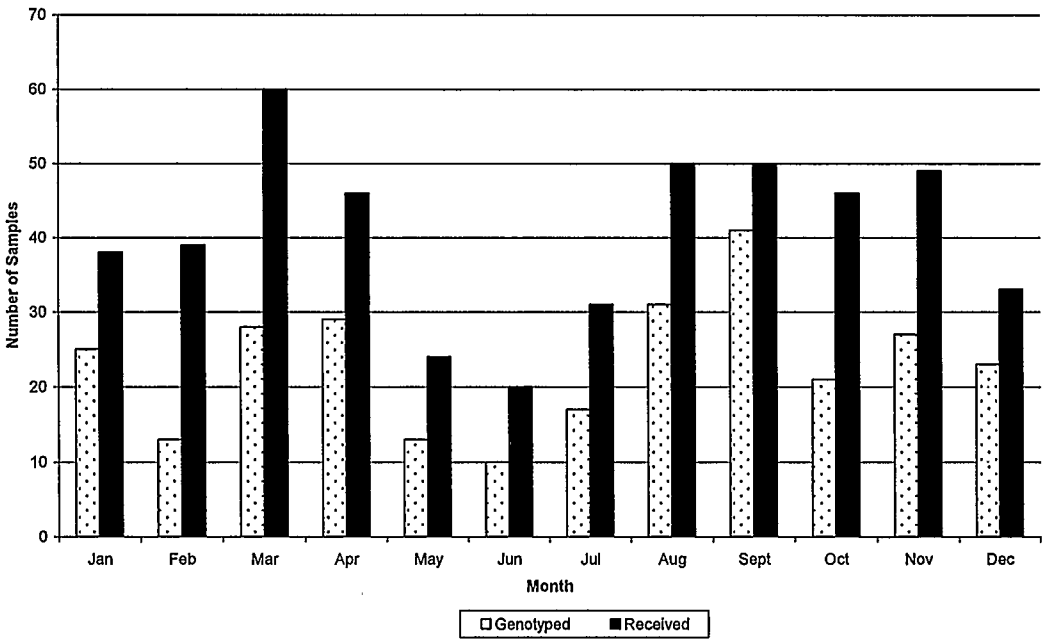


Figure 10: Monthly Distribution of *Giardia duodenalis* positive stool samples.

Diagrammatic illustration of the number of stool samples received from CLS (Black) and the number of samples actually genotyped.

Table 4: Overall Frequency of *Giardia duodenalis* genotypes

The observed number and frequency (%) of genotypes in Calgary determined from clinical samples collected between January 2002 and March 2004. Clinical samples were genotyped using SSU-RFLP and GDH-RFLP techniques. The abbreviation BB and AB refer to mixed sub-assemblage B infections and mixed assemblage infections respectively.

SSU/GDH (%)			GDH (%)					
A	B	AB	A-I	A-II	B-III	B-IV	BB	AB
143 (51.4)	103 (37.15)	32 (11.5)	84 (39.6)	26 (12.3)	27 (12.7)	24 (11.3)	23 (10.8)	28 (13.2)

4.2 Effect of International Travel on *G. duodenalis* Genotype Frequency

To investigate the international distribution of the various genotypes, the genotype frequency of individuals with a recent history of international travel were examined. In the context of this study, newly arrived immigrants and international travelers are grouped together, since both are more likely to have acquired giardiasis while outside of Canada.

To evaluate the impact of travel on genotype frequency, a statistical comparison of genotype frequencies associated with local (no history of international travel) and internationally acquired infections was performed. No statistically significant variation in assemblage frequencies (Table 5) were observed between the two groups ($\chi^2 = 2.0$, df=2, p=0.36). At the sub-assemblage level statistically significant unequal distribution of genotypes associated with travel ($\chi^2 = 17.4$, df=5, $p<0.01$) was observed, with Sub-assemblage B-IV (5.3), mixed sub-assemblage B (7.2), and mixed assemblage AB (4.7) infections accounting for the majority of the standardized chi-squared value. No significant difference ($\chi^2 = 1.1$, df=2, p=0.58) was observed in the distribution of Sub-assemblages A-I, A-II, and B-III. International travel accounted for a statistically significant ($\chi^2 = 16.1$, df=2, $p<0.01$) greater than expected proportion of mixed Sub-assemblage B infections (78.3%), while local activities accounted for a greater proportion of Sub-assemblage B-IV (75%) and mixed assemblage (67.9%) infections . International travel was found to have a medium effect on (Cramer V = 0.46, $p<0.01$) observed frequencies of Sub-assemblage B-IV, mixed Sub-assemblage B. and mixed Assemblage infections in Calgary. Temporally, August through October accounts for the largest

proportion of Sub-assemblage B-IV (54.2%) and mixed Assemblage (46.4%) infections with the frequency of both peaking in September (data not shown). This portion of the year also corresponds with a period of warmer weather for the region and increased water related recreation, an important risk factor for giardiasis. It is possible that Sub-assemblage B-IV and mixed assemblage infections are more commonly associated with waterborne transmission of giardiasis. A positive correlation between an increase in mixed assemblage infections and an increase in water associated activities is possible since parasite composition of water is likely influenced by multiple sources and as a result there is a greater probability of multiple assemblage exposure.

As discussed earlier (Section 1.7.1), beaver are major contributors to parasite levels in watershed. While the predominant Sub-assemblage in beavers is not currently known, at the assemblage level, Assemblage B (9, 154) appears to be the predominant genotype in beavers. It is possible that the increased frequency of Sub-assemblage B-IV may be a result of an increased waterborne zoonotic transmission during warmer months. The apparent association between mixed sub-assemblage B infections and a history of travel is unclear.

In order to examine world-wide distribution of *G. duodenalis* genotypes internationally acquired infections were divided into 4 major geographic regions (Table 6), and a mixed geographic region. The mixed geographic region is composed of regions with limited numbers of travel associated clinical samples. (e.g. Europe, Australia, and the Middle East). A statistical comparison of assemblage frequencies found no significant difference in the frequencies between geographic regions ($\chi^2 = 3.9$, df=4, p=0.417). In respect to sub-assemblage frequencies, variations in Sub-assemblage A-I were

determined to be statistically insignificant (χ^2= 8.7, df=4, p=0.07), and no statistical analysis could be conducted on other *G. duodenalis* sub-assemblages due to insufficient sample size. In all geographic regions (excluding mixed geographic regions) Assemblage A was the predominant assemblage, and more specifically Sub-assemblage A-I. This further supports the suggestion that Sub-assemblage A-I is the predominant genotype world-wide. The international frequency of Sub-assemblage A-II associated infections also appear similar, though could not be confirmed statistically due to the small sample size.

Table 5: Overall effect of travel on *G. duodenalis* genotype frequency in Calgary

A comparison of the observed number and frequency (%) of genotypes associated with travel. Clinical samples were genotyped using SSU-RFLP and GDH-RFLP techniques. The abbreviation BB and AB refer to mixed sub-assemblage B infections and mixed assemblage infections respectively

	SSU/GDH (%)			GDH (%)					
	A	B	AB	A-I	A-II	B-III	B-IV	BB	AB
No History of Travel	80 (51.9)	53 (34.4)	21 (13.6)	40 (35.7)	15 (13.4)	15 (13.4)	18 (16.1)	5 (4.5)	19 (17.0)
Travel	63 (50.8)	50 (40.3)	11 (8.9)	44 (44.0)	11 (11.0)	12 (12.0)	6 (6.0)	18 (18.0)	9 (9.0)

Table 6: Geographic variations in *G. duodenalis* genotypes frequency.

The observed number and frequency (%) of genotypes of travellers and immigrants from various regions of the world. Clinical samples were genotyped using SSU-RFLP and GDH-RFLP techniques. The abbreviations BB and AB refer to mixed sub-assemblage B infections and mixed assemblage infections respectively. * The mixed geographic regions refer to a compilation of various geographic regions (i.e. Europe, Australia, Middle East) were associated with a limited numbers of *G. duodenalis* positive results.

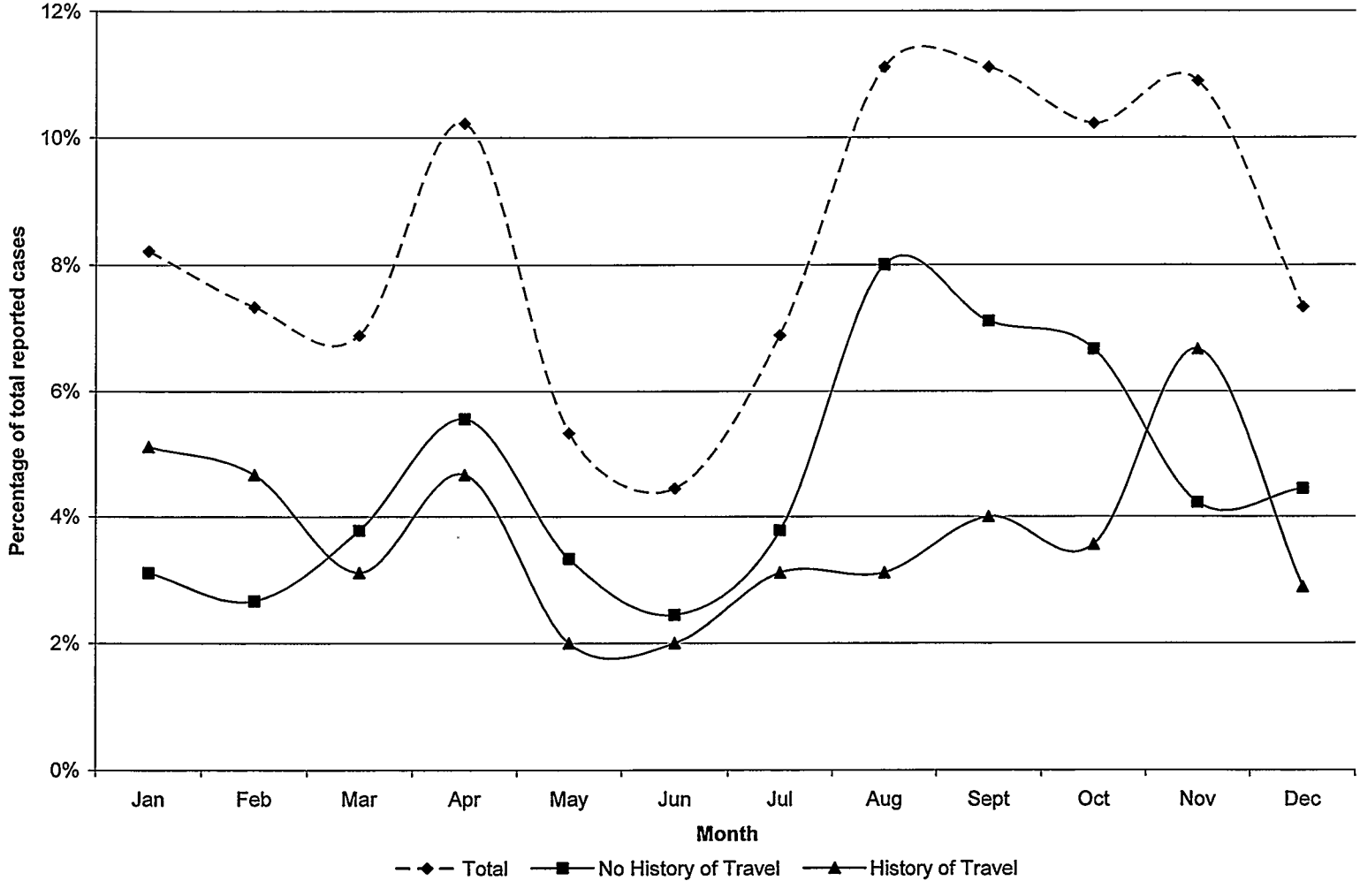
Geographic Region	SSU/GDH (%)			GDH (%)					
	A	B	AB	A-I	A-II	B-III	B-IV	BB	AB
Latin America and Caribbean	12 (60.0)	6 (30.0)	2 (10.0)	9 (52.9)	2 (11.8)	1 (5.9)	0 (0.0)	3 (17.6)	2 (11.8)
Africa	22 (52.4)	18 (42.9)	2 (4.8)	15 (46.9)	4 (12.5)	5 (15.6)	2 (6.3)	5 (15.6)	1 (3.1)
India/Pakistan/Afghanistan	14 (48.3)	12 (41.4)	3 (10.3)	10 (41.7)	2 (8.3)	3 (12.5)	0 (0.0)	7 (29.2)	2 (8.3)
South East Asia	10 (58.8)	5 (29.4)	2 (11.8)	7 (46.7)	2 (13.3)	1 (6.7)	1 (6.7)	2 (13.3)	2 (13.3)
Mixed geographic regions*	5 (31.3)	9 (56.3)	2 (12.5)	3 (25.0)	1 (8.3)	2 (16.7)	3 (25.0)	1 (8.3)	2 (16.7)

4.3 Seasonal Variation in *G. duodenalis* Genotype Frequency

Seasonal variations in the incidence of giardiasis is well documented (22, 63, 100, 122, 131) with the incidence peaking during late summer or early fall. This same trend was also observed during the study period, with a season peak in the number of reported cases (Figure 11) recorded during period of August through October (32% of reported cases). A closer examination of this period reveals that local activities (no history of travel) appear to account for the majority of the cases (67%). This period is associated with warmer weather and an increase in water related activities. As a result, it is likely this peak in locally acquired infections is a function of an increase in waterborne transmission of the parasite. In addition to the major peak in reported cases, a small secondary peak was also observed in April (10% of reported cases). The composition of this secondary peak is not dominated by neither local nor travel-related cases and the reason for this second peak is unclear. The travel-related peak in April may be a result of an increase in travel in March, which is a popular travel period for both families and university students (spring break). Statistically significant seasonal variations in genotype frequencies were observed (no shown), but these observations were trend driven rather than hypothesis driven. The sized and duration of the data set is insufficient data for trend driven research.

Figure 11: Monthly distribution of reported *G. duodenalis* cases

Graphic illustration of travel history of reported *G. duodenalis* cases in the Calgary Regional Health Authority from February 2002 to February 2004. Monthly values reported as a percentage of the total number of cases the during time period (n=450).


12%
10%
8%
6%
4%
2%
0%
Percentage of total reported cases
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sept
Oct
Nov
Dec
Month
Total
No History of Travel
History of Travel

4.4 Age Associated Variation in *G. duodenalis* Genotype Frequency

A comparison of genotype frequencies in children under 5 years of age(Table 7) to all other ages found a statistically significantly greater (χ^2 = 14.5, df=2, p<0.01) than expected frequency of Assemblage B (48.8%) and mixed Assemblage infections (17.5%) associated with children under 5 years of age. When the comparison was expanded to all pediatrics (children under the age of 16 years) the same statistically significant trend was still observed (χ^2 = 16.2, df=2, p<0.01). There was no significant difference (χ^2 = 6.5, df=5, p=0.2692) in the observed frequency of sub-assemblage genotypes. A comparison of individuals 15 to 45 years of age to individuals greater than 45 years of age found no statistical difference (χ^2 = 3.5, df=2, p=0.169) in genotype frequency at the assemblage level. At the assemblage level, a history of travel did not have a statistically significant affect on pediatric (χ^2 = 0.298, df=1, p=0.59) or adult (χ^2 = 0.767, df=1, p=0.38) genotype frequencies.

The greater than expected frequency of Assemblage B and mixed assemblage infections in children may suggest some factor may make children more prone to symptomatic giardiasis due to these infections. To better understand the magnitude of this phenomenon, assemblage level genotyping data was converted into age-grouped incidence values using population data collected by Alberta Health and Wellness for CRHA for 2003 (4). Genotyping efficiency ranged from 50 to 68% depending on the age group (Figure 12). A comparison of genotype incidences of children 5 years in age or less to other age groups reveals that Assemblage A incidence was 2.1 to 6.1 (2.2 to 2.6 excluding small group of 3 samples in the 75+ age group) time greater and Assemblage B

was 3.1 to 17.7 (3.1 to 9.4 times excluding small group of 3 samples in the 75+ age group) times greater in children. A comparison of mixed assemblage infections found that the incidence of mixed assemblage infections in children under the age of 5 was 3.2 to 14.8 times greater than that observed in other age groups. Possible factors that may explain the higher observed incidence of Assemblage B and mixed assemblage associated symptomatic giardiasis in children may be differences in humoral responses, stomach pH, or a child specific Assemblage B tropisms.

The ability to elicit an effective IgA humoral response against *Giardia duodenalis* appears to be essential for parasite clearing. Since a significant percentage of children under 5 years of age may not have a fully developed humoral response (173), there may be a greater probability of colonization by less virulent strains of *G. duodenalis*. As stated previously, data from a day-care study in Australia found evidence to suggest that Assemblage B is less virulent (135). If Assemblage B is less virulent, one would possibly expect that the observed genotype frequencies in children would be different than those in adults, with a greater than expected frequency of a less virulent genotype in children. In addition, one may also expect to observe a greater frequency of mixed genotype infections associated with children since the humoral response is likely an important selection factor *in vivo*.

Another factor that may account for the higher incidence of Assemblage B and mixed assemblage infections in children may be due differences in assemblage pathogenicity. The gastrointestinal physiology of children is different from that of adults, with gastrointestinal secretory capacity not reaching maturity until 11 years of age (26). If Assemblage B is less acid tolerant, the higher pH in the stomachs of children may

increase the probability of the parasite surviving the transit through the stomach and thus translating into higher rates of infection.

A final possible explanation for the higher incidence of Assemblage B is a child specific Assemblage B tropisms. To prevent being flushed from the small intestine, trophozoites must attach to enterocytes using their adhesive disk. If some factor provided Assemblage B an increased ability to attach to enterocytes in children, it may also account for the higher incidence.

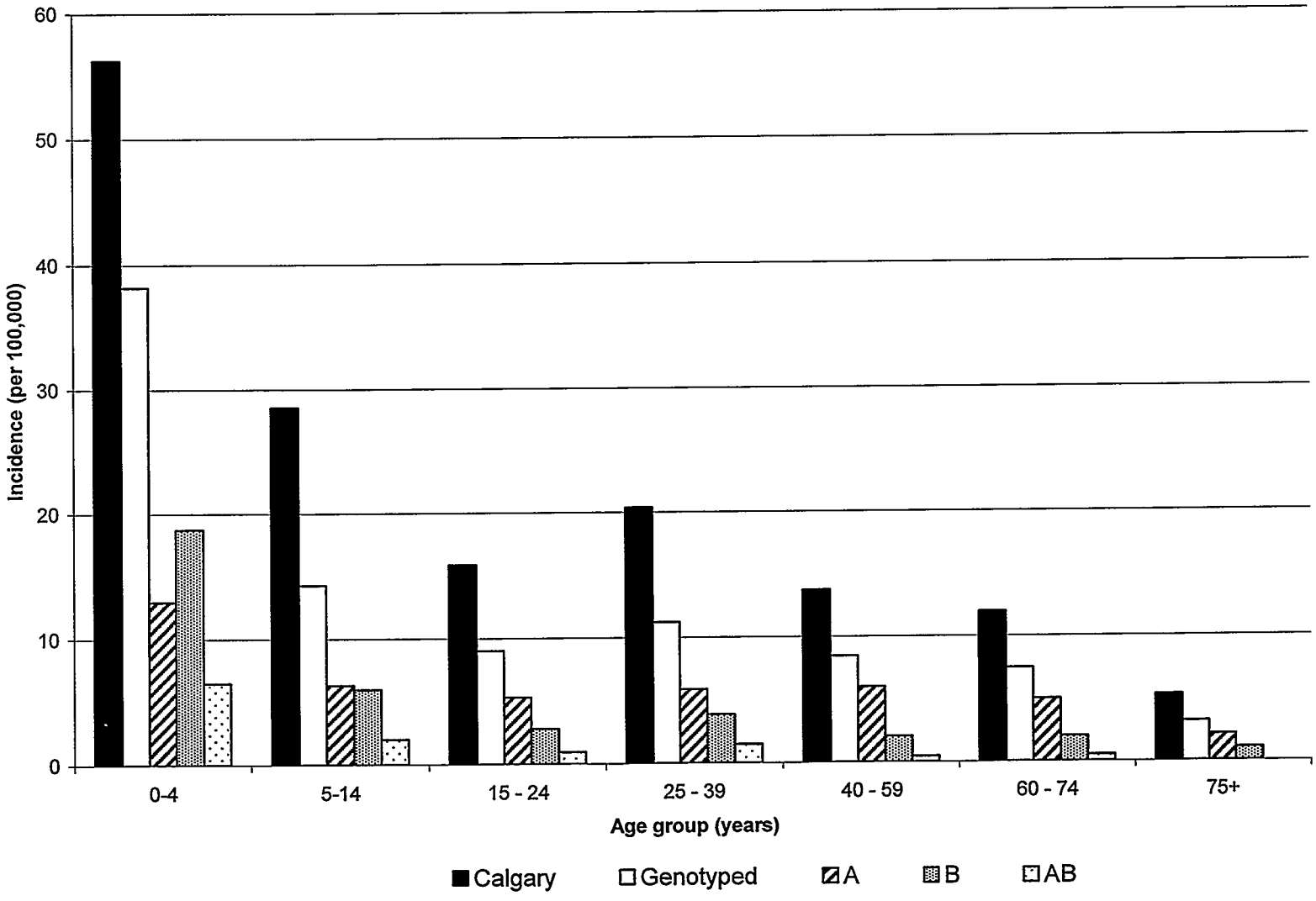
Table 7: Age associated genotype frequency variance

A comparison of the observed number and frequency (%) of genotypes associated with children less than 5 years of age to all other ages. Clinical samples were genotyped using SSU-RFLP and GDH-RFLP techniques. The abbreviations BB and AB refer to mixed sub-assemblage B infections and mixed assemblage infections respectively.

	SSU/GDH (%)			GDH (%)					
	A	B	AB	A-I	A-II	B-III	B-IV	BB	AB
< 5 years of age	27 (33.8)	39 (48.8)	14 (17.5)	19 (30.6)	5 (8.1)	10 (16.1)	8 (12.9)	9 (14.5)	11 (17.7)
> 5 years of age	116 (58.6)	64 (32.3)	18 (9.1)	65 (43.3)	21 (14.0)	17 (11.3)	16 (10.7)	14 (9.3)	17 (11.3)

Figure 12: *G. duodenalis* incidence in Calgary, Alberta

The average yearly incidence for the study period was constructed from clinical samples collected from February 2002 to February 2004 and Calgary population data published by Alberta Heath and wellness for 2003. Black columns represent all clinical samples received from CLS (Calgary) during the time period and white bars (Genotyped) represent the clinical samples genotyped.


60
50
40
30
20
10
0
Incidence (per 100,000)
0-4
5-14
15 - 24
25 - 39
40 - 59
60 - 74
75+
Age group (years)
Calgary
Genotyped
A
B
AB

Chapter Five: Zoonotic Potential of Assemblage E

During the study period two clinical samples produced GDH-RFLP profiles indicative of an Assemblage E isolate. The GDH-RFLP profile Clinical sample 365 (CS365) indicated a single assemblage infection with Assemblage E (Figure 13A) Subsequent sequence analysis determined the clinical isolate had a high degree of homology (98%) to a known Assemblage E sequences (Figure 14). The CS365 sequence has four unique SNPs (nucleotide position 9, 14, 301, and 362) not observed in other Assemblage E sequences. Considering that Sub-assemblage A-I and Sub-assemblage A-II only have two SNPs over this same region and there appears to be significant differences in their host-specificity, CS365 should only be considered a possible Assemblage E isolate until genetic diversity between Assemblage E isolates over this region can be better resolved. The patient was a 25 year old male, who had recently emigrated from Sudan, where it was likely he acquired the disease. Although the patient was constipated, a symptom sometimes attributed to *G. duodenalis,* he was also suffering from malaria, suspected renal colic, and chronic Hepatitis B. Considering these other infirmities, and it is likely the giardiasis was asymptomatic. Although considered immunocompetent by his physician, the individual had spent the last 5 years in an Ethiopian refugee camp and, as a result, it is plausible that he was suffering from some degree of immunosuppression.

The GDH-RFLP profile in clinical sample 386 (CS386) indicated the patient had a multiple assemblage infection, with Sub-assemblage A-I, Assemblage B, and Assemblage E (Figure 13B) present. The mixture of DNA from multiple assemblages made sequence analysis difficult, though the partial (71% of predicted PCR product)

GDH sequence obtained from the sample had a high degree of homology (99-100%) with numerous known Assemblage E sequences and contains no unique SNPs (Figure 14). Sequencing analysis of a SSU amplicon associated with CS386 revealed the presence of Assemblage B isolate in the sample (100% homology data not shown), which was detected also by the GDH RFLP profile for the clinical sample. The patient in this case was a 19 year old male, with no apparent history of travel. While it could not be confirmed, it is believed that the individual was likely symptomatic, since requests for *G. duodenalis* screens are rarely done on asymptomatic individuals. The presence of multiple genotypes may suggest the individual acquired the infection through waterborne transmission, since contaminated water is more likely to contain multiple assemblages.

The possible detection of Assemblage E in two clinical samples raises the possibility that Assemblage E may have a limited zoonotic potential. Considering higher prevalence of Assemblage E in cattle in Alberta this finding may have important public health implications. This said, assuming a potential route of transmission between cattle and humans exists, the low frequency of Assemblage E associated symptomatic giardiasis possibly suggests it may not play an important role in symptomatic giardiasis in the region.

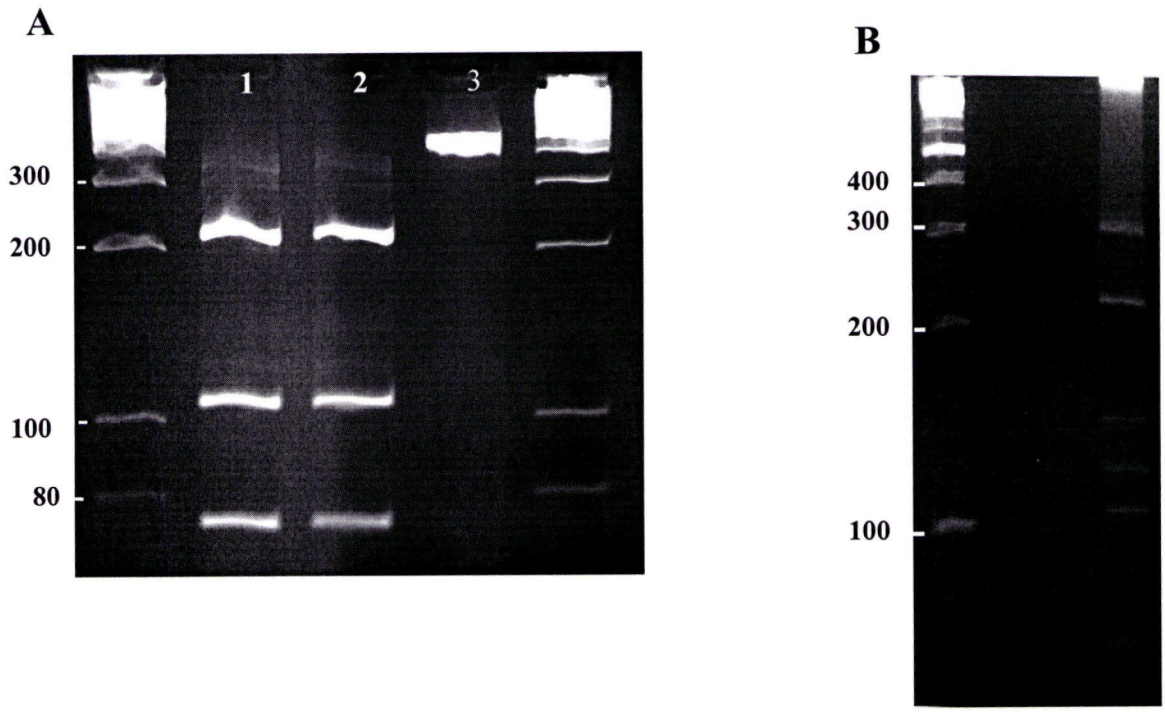
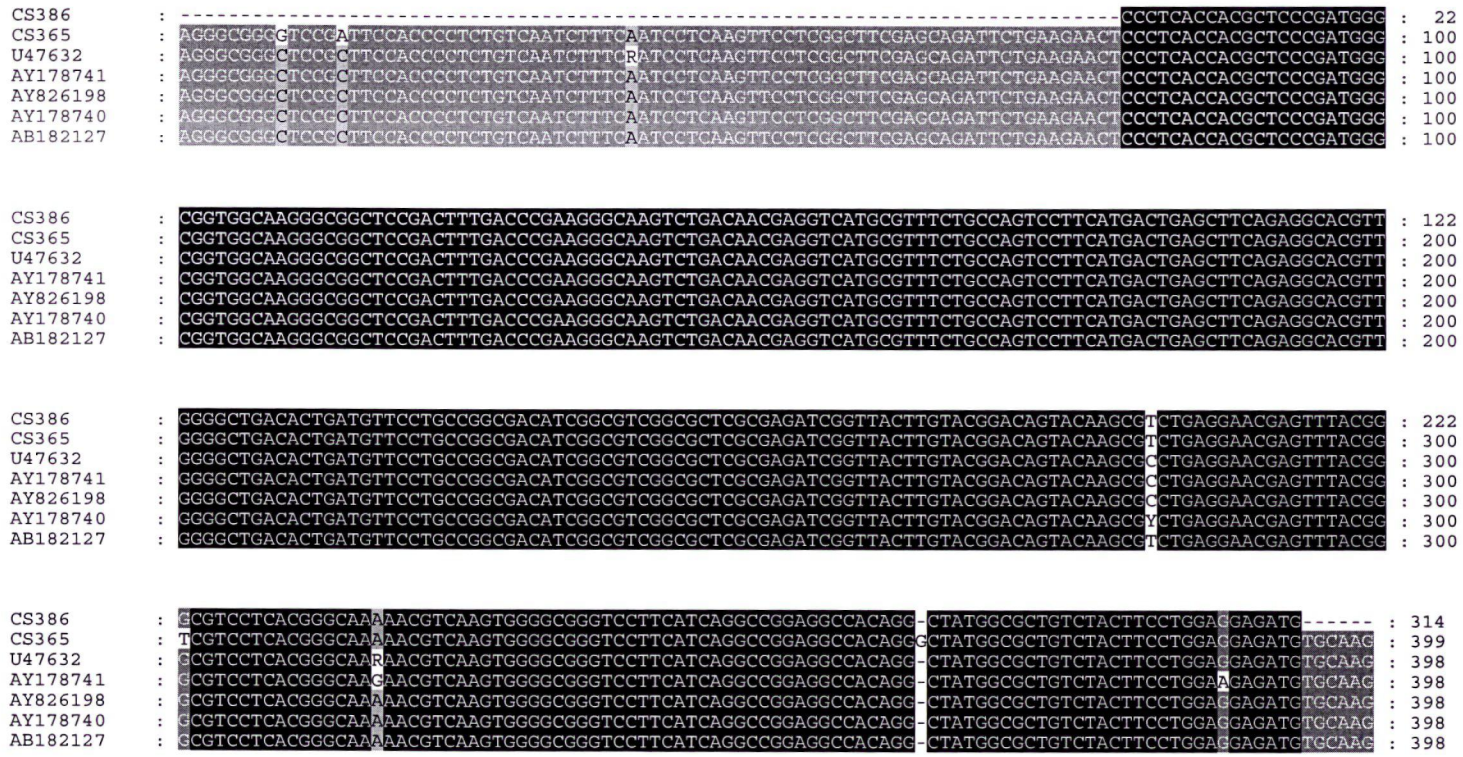


Figure 13: Glutamate dehydrogenase RFLP profiles of Assemblage E positive clinical samples.

Glutamate dehyodrogenase PCR product was digested with N1A IV and visualized on a 2% agarose gel stained with ethidium bromide. Gel A: 1) Assemblage E control 2) Clinical sample 365 (CS365) 3) Undigested GDH product. Gel B: 1) Clinical sample 386 (CS386)

Figure 14: Glutamate dehydrogenase sequence alignment of suspected clinical Assemblage E isolates

Suspected Assemblage E GDH amplicons from two clinical samples were aligned with published Assemblage E sequences using Clustal W sequence alignment. Assemblage E sequences retrieved from GenBank included (Accession#/host/Country): U47632/Ungulates/Australia; AY178741/Pig/Australia; AY826198/Goat/Netherlands AY178740/Cattle/Australia; and AB182127/Cattle/Japan.


CS386 : ---------- : 22
CS365 : AGGGCGGGGTCCCATTCCACCCCTCTGTCAATCTTTCAATCCTCAAGTTCCTCGGCTTCGAGCAGATTCTGAAGAACTCCCTCACCACGCTCCCGATGGG : 100
U47632 : AGGGCGGGCTCCCCTTCCACCCCTCTGTCAATCTTTCRATCCTCAAGTTCCTCGGCTTCGAGCAGATTCTGAAGAACTCCCTCACCACGCTCCCGATGGG : 100
AY178741 : AGGGCGGGCTCCCCTTCCACCCCTCTGTCAATCTTTCAATCCTCAAGTTCCTCGGCTTCGAGCAGATTCTGAAGAACTCCCTCACCACGCTCCCGATGGG : 100
AY826198 : AGGGCGGGCTCCCCTTCCACCCCTCTGTCAATCTTTCAATCCTCAAGTTCCTCGGCTTCGAGCAGATTCTGAAGAACTCCCTCACCACGCTCCCGATGGG : 100
AY178740 : AGGGCGGGCTCCCCTTCCACCCCTCTGTCAATCTTTCAATCCTCAAGTTCCTCGGCTTCGAGCAGATTCTGAAGAACTCCCTCACCACGCTCCCGATGGG : 100
AB182127 : AGGGCGGGCTCCCCTTCCACCCCTCTGTCAATCTTTCAATCCTCAAGTTCCTCGGCTTCGAGCAGATTCTGAAGAACTCCCTCACCACGCTCCCGATGGG : 100
CS386 : CGGTGGCAAGGGCGGCTCCGACTTTGACCCGAAGGGCAAGTCTGACAACGAGGTCATGCGTTTCTGCCAGTCCTTCATGACTGAGCTTCAGAGGCACGTT : 122
CS365 : CGGTGGCAAGGGCGGCTCCGACTTTGACCCGAAGGGCAAGTCTGACAACGAGGTCATGCGTTTCTGCCAGTCCTTCATGACTGAGCTTCAGAGGCACGTT : 200
U47632 : CGGTGGCAAGGGCGGCTCCGACTTTGACCCGAAGGGCAAGTCTGACAACGAGGTCATGCGTTTCTGCCAGTCCTTCATGACTGAGCTTCAGAGGCACGTT : 200
AY178741 : CGGTGGCAAGGGCGGCTCCGACTTTGACCCGAAGGGCAAGTCTGACAACGAGGTCATGCGTTTCTGCCAGTCCTTCATGACTGAGCTTCAGAGGCACGTT : 200
AY826198 : CGGTGGCAAGGGCGGCTCCGACTTTGACCCGAAGGGCAAGTCTGACAACGAGGTCATGCGTTTCTGCCAGTCCTTCATGACTGAGCTTCAGAGGCACGTT : 200
AY178740 : CGGTGGCAAGGGCGGCTCCGACTTTGACCCGAAGGGCAAGTCTGACAACGAGGTCATGCGTTTCTGCCAGTCCTTCATGACTGAGCTTCAGAGGCACGTT : 200
AB182127 : CGGTGGCAAGGGCGGCTCCGACTTTGACCCGAAGGGCAAGTCTGACAACGAGGTCATGCGTTTCTGCCAGTCCTTCATGACTGAGCTTCAGAGGCACGTT : 200
CS386 : GGGGCTGACACTGATGTTCCTGCCGGCGACATCGGCGTCGGCGCTCGCGAGATCGGTTACTTGTACGGACAGTACAAGCGTCTGAGGAACGAGTTTACGG : 222
CS365 : GGGGCTGACACTGATGTTCCTGCCGGCGACATCGGCGTCGGCGCTCGCGAGATCGGTTACTTGTACGGACAGTACAAGCGTCTGAGGAACGAGTTTACGG : 300
U47632 : GGGGCTGACACTGATGTTCCTGCCGGCGACATCGGCGTCGGCGCTCGCGAGATCGGTTACTTGTACGGACAGTACAAGCGCCTGAGGAACGAGTTTACGG : 300
AY178741 : GGGGCTGACACTGATGTTCCTGCCGGCGACATCGGCGTCGGCGCTCGCGAGATCGGTTACTTGTACGGACAGTACAAGCGCCTGAGGAACGAGTTTACGG : 300
AY826198 : GGGGCTGACACTGATGTTCCTGCCGGCGACATCGGCGTCGGCGCTCGCGAGATCGGTTACTTGTACGGACAGTACAAGCGCCTGAGGAACGAGTTTACGG : 300
AY178740 : GGGGCTGACACTGATGTTCCTGCCGGCGACATCGGCGTCGGCGCTCGCGAGATCGGTTACTTGTACGGACAGTACAAGCGYCTGAGGAACGAGTTTACGG : 300
AB182127 : GGGGCTGACACTGATGTTCCTGCCGGCGACATCGGCGTCGGCGCTCGCGAGATCGGTTACTTGTACGGACAGTACAAGCGTCTGAGGAACGAGTTTACGG : 300
CS386 : GCGTCCTCACGGGCAAAAACGTCAAGTGGGGCGGGTCCTTCATCAGGCCGGAGGCCACAGG-CTATGGCGCTGTCTACTTCCTGGAGGAGATG------ : 314
CS365 : TCGTCCTCACGGGCAAAAACGTCAAGTGGGGCGGGTCCTTCATCAGGCCGGAGGCCACAGGGCTATGGCGCTGTCTACTTCCTGGAGGAGATGTGCAAG : 399
U47632 : GCGTCCTCACGGGCAARAACGTCAAGTGGGGCGGGTCCTTCATCAGGCCGGAGGCCACAGG-CTATGGCGCTGTCTACTTCCTGGAGGAGATGTGCAAG : 398
AY178741 : GCGTCCTCACGGGCAAGAACGTCAAGTGGGGCGGGTCCTTCATCAGGCCGGAGGCCACAGG-CTATGGCGCTGTCTACTTCCTGGAAGAGATGTGCAAG : 398
AY826198 : GCGTCCTCACGGGCAAAAACGTCAAGTGGGGCGGGTCCTTCATCAGGCCGGAGGCCACAGG-CTATGGCGCTGTCTACTTCCTGGAGGAGATGTGCAAG : 398
AY178740 : GCGTCCTCACGGGCAAAAACGTCAAGTGGGGCGGGTCCTTCATCAGGCCGGAGGCCACAGG-CTATGGCGCTGTCTACTTCCTGGAGGAGATGTGCAAG : 398
AB182127 : GCGTCCTCACGGGCAAAAACGTCAAGTGGGGCGGGTCCTTCATCAGGCCGGAGGCCACAGG-CTATGGCGCTGTCTACTTCCTGGAGGAGATGTGCAAG : 398

Chapter Six: Discussion

Molecular epidemiology can be defined as the application of "tools, both laboratory and analytical, that can be used to obtain predictive data than can be interpreted to better define the aetiology of specific disease" (156). In recent years, numerous published molecular epidemiology papers examining *G. duodenalis* have been focused primarily on the molecular tools used to characterise the various assemblages rather than the epidemiological significance of their results. To some degree this phenomenon can be attributed to a lack of standardised molecular nomenclature for the parasite, though in some cases it appears the molecular tools are being applied in the absence of any epidemiological framework. A good example of this is the recent observation of Assemblage A in white tail deer (164). Considering that Assemblage A has been isolated from most domesticated artiodactyl mammals (157), the finding is not unexpected. Furthermore, the authors genotyped only a single isolate and, thus could not provide any data in respect to the prevalence of the various assemblages within the deer population, which would allow for some evaluation of the potential for zoonotic transmission.

Another common problem is small sample size, which can result in observed genotype frequencies that might not truly reflect those within the population. For example, two studies conducted over the same period and based on axenic culture reported contradicting results. One study reported all isolates genotyped as Sub-assemblage A-II(132) and the other all Sub-assemblage A-I (33). The lack of Assemblage B isolates within their samples may be partly due to the use of axenic cultures, which selects for Assemblage A (21), but does not account for the differences in Sub-

assemblage A-I and A-II frequencies. Both studies characterized less than 25 isolates, and it is possible that their small sample size influenced the contradicting results.

Despite the short-comings discussed above, the application of molecular tools has provided a wealth of information with respect to the evolution, biology, and epidemiology of *Giardia duodenalis.* Unfortunately, a standardized taxonomical nomenclature has not been able to keep pace with recent molecular characterization of the parasite, giving rise to the use of multiple nomenclatures. The current use of assemblages or groups to describe genetically distinct populations of *G. duodenalis* is causing confusion, hindering research, and needs to be revised. As discussed in the introductory chapter (Section 1.6) there is sufficient evidence for species designation for 6 of 7 assemblages and some researchers (Thompson and Monis, in progress) have suggested reclassification of *Giardia duodenalis* into six separate species bases on host-specificity and molecular genetics.

6.1 Parasite Competition

During the study period, mixed infections (Table 4) accounted for nearly one quarter of the 215 samples genotyped. While the frequency of mixed sub-assemblage B and mixed assemblage infections were approximately equal, only a single mixed Sub-assemblage A infection was observed (Table 4). The low frequency of mixed Sub-assemblage A infections may be a result of differences in intra-species competition. An *in vitro* study suggests the growth rate of Assemblage A may be nearly double that of Assemblage B (21). Based on the supposition that these same growth rate differences also occur in vivo, it could be hypothesised that intra-assemblage competition between sub-

assemblage A isolates may be greater than that between sub-assemblage B isolates, resulting in a more rapid progress to a single assemblage infection. If growth rates influence intra-assemblage competition one would predict that the frequency of mixed sub-assemblage B infections would represent the greatest proportion of mixed infections. Although the frequency of mixed sub-assemblage B (10.8%) infections appears to be similar to that of mixed assemblage infections (13.2%), the values do not account for the lower overall frequency of Assemblage B infections (24% vs. 51.9%). Examined as a percentage of Assemblage B infections (BB/ (B-III + B-IV)), mixed sub-assemblage infections account for 31% of Assemblage B infections, suggesting once the lower genotype frequency has been accounted for, mixed sub-assemblage B infections may be more common. While relative frequencies of mixed sub-assemblage and assemblage infections appear to support the hypothesis that *in vivo* growth rates may influence intra-assemblage competition, the argument is based on the large assumption that observed differences *in vitro* growth rates are reflective of *in vivo* conditions. If growth rates influence intra-assemblage it has the potential to have a dramatic effect on parasite population dynamics. If sub-assemblage A isolates can out compete host-adapted assemblages within their respective hosts, the introduction of the parasite to the host could result in a shift of the predominant assemblage within the animal population and an increase in the potential for zoonotic transmission.

6.2 Virulence

Host-parasite gene-to-gene co-evolution theory suggests there is no advantage for virulence genes against universally susceptible hosts (143). Based on the probable assumption that humans are universally susceptible to Assemblage A and Assemblage B, the theory would predict that older genetic lineages of *G. duodenalis* would be less virulent. The greater genetic divergence observed within Assemblage B isolates, when compared to Assemblage A isolates (81, 112), suggests Assemblage B is an older genetic lineage and therefore should be less virulent. The observed 3.1 to 17.7 times greater incidence of Assemblage B infections in children under 5 years of age in Calgary (Table 7) and Read et al. 2002 finding that Assemblage B was more commonly associated with asymptomatic giardiasis may also suggest that Assemblage B is less virulent. Clinically, this is also supported by a recent study that found 83% of endoscope aspirates from asymptomatic individuals were Assemblage B (12). The hypothesis that Assemblage B is less virulent appears to be supported by co-evolution theory, as well as epidemiological and clinical data.

Current infection control measures for giardiasis in daycare settings rely on the exclusion of symptomatic children. Given the higher frequency of Assemblage B in children under the age of 5 and the possibility that Assemblage B may be more commonly associated with asymptomatic infections in children (135), the exclusion of only symptomatic individuals may not be a completely effective policy for preventing transmission. Under certain circumstances, the testing of all children at a facility may be required to discover hidden reservoirs of infection.

Derived from the supposition that Assemblage B is less virulent, the potential for sampling bias arises. With the exception of samples received from the refugee clinic, all samples collected during the study period were associated with clinical cases of giardiasis and, as a result, the sampling protocol may invariably select for Assemblage A infections. Due to this sampling bias the true frequency of Assemblage B associated infections within the population may have been underestimated.

Despite the possibility of underestimation of Assemblage B infections within the population, Sub-assemblage A-I appears to be the predominant genotype world-wide (Table 6), and considering the selection bias associated with the study, it may also be the most virulent. Sub-assemblage A-I consists of a mixture of closely related animal and human isolates and is believed to have undergone a recent global dispersion (157). Sub-assemblage A-Is' recent dispersion, close relation to animal isolates, and possible increased virulence, it is possible that Sub-assemblage A-I may have more recently jumped species into humans. If so, animals likely still play a significant role in transmission of this genotype. While the importance of animal reservoirs in the transmission zoonotic assemblages can be speculated on, without the development and application of better molecular typing tools the answer will remain speculative.

6.3 Zoonotic Potential

Despite *Giardia duodenalis* designation as a zoonotic parasite by the World Health Organization in 1979 (174) there is only limited data available with regard to the prevalence of the various genotypes within domestic (pets), agricultural, and wildlife animals. The high prevalence of Assemblage E (10) and Assemblage C and D (115) in

cattle and dogs may suggest that host-adapted assemblages are the predominant genotype within their respective hosts. The near absence of these assemblages associated with clinical infections suggests host-adapted assemblages may have a low zoonotic potential. In addition to being able to harbour host-adapted assemblage, it has been observed that many animal populations can harbour Assemblage A and B, with Sub-assemblage A-I appearing to have the greatest zoonotic potential (157). If, as discussed earlier, Sub-Assemblage A-I is capable of successfully out competing host-adapted assemblages, the exposure of animals to Sub-assemblage A-I may be a mitigating factor influencing the potential for the zoonotic transmission of this parasite. It is important to note that while Sub-assemblage A-I may be able to out complete host-adapted assemblages on a short time basis, it is the belief of this researcher that without continual re-exposure to Sub-assemblage A-I, the host-adapted assemblage will eventually become the predominant genotype within their respective host(s). In order to better understand this dynamic we first must explore the possible routes of exposure that animal populations may have to zoonotic assemblages, such as Sub-assemblage A-I.

One potential route of exposure may be the use of municipal sewage by-products for agricultural irrigation and fertilization. In recent years there has been an increase in the use of these by-products in order to conserve limited water resources, to take advantage of their high nutrient content, and to provide an economically viable method for their disposal. In order to minimize public health concerns, with respect to microbial pathogens, most provinces have legislated various restrictions on the application of the reclaimed water and biosolids, based on the level of treatment, which is monitored daily or weekly and must meet specific fecal coliform requirements. Although fecal coliforms

are good indicators for bacterial pathogens, the increased resistance of cysts to common treatment options such as anaerobic digestion (34) and chlorination, reduces the efficacy of fecal coliforms as an indictor for *Giardia spp.* (138). While most legislation includes a blanket statement requiring operators to demonstrate that by-products do not contain pathogens at a level of concern to local health authorities (for unrestricted use), currently there are no standards for acceptable levels of cysts in reclaimed water or biosolids. Zoonotic assemblages have been detected in reclaimed water (28, 141, 178) and biosolids (34). In British Columbia, current legislated furloughing for pastures after the application of reclaimed water is dependent on the level of treatment, with the maximum of 6 days required. In respect to the application of biosolids, Ontario guidelines require a 2 month furlough of pastures after the application of Class B biosolids (limited restrictions on pathogen levels). In the absence of sub-zero temperatures, viable cysts can remain in the soil for at least 3 months (139), and thus current waste management practices may be providing a potential route of exposure for agriculture animals.

The majority of risk assessments for the use of sewage by-products primarily focus on the risk of exposure to chemical contaminants (102), with limited work dedicated to microbial health hazards. Current microbial risk assessment modeling is based on direct or secondary transmission within the human population (50) and does not account for the potential bioamplification by susceptible animal populations, whose levels of exposure may be much greater than humans. Since *G. duodenalis* is endemic in many agricultural animal populations, a shift in the predominant genotype within the affected population from a host-adapted assemblage to a zoonotic assemblage, due to

exposure to sewage by-products, could occur without any significant change in the overall incidence of disease within the population. The shift to, and subsequent bioamplification of zoonotic assemblages, could thus dramatically increase the potential for zoonotic transmission.

A GIS study investigating factors influencing the incidence of giardiasis (123) found a significant positive correlation between the increased use of manure, and the incidence of giardiasis in two of seven health regions in Ontario. This suggests under the right conditions the application of biosolids can influence the rate of giardiasis in the adjacent human population. In the context of the Ontario study, the applied biosolids appear to be only of animal origin, nevertheless zoonotic assemblages are present in agriculture animal populations, and thus manure of animal origin may also serve as a potential source of exposure to zoonotic assemblages. Differences in the predominant genotype in agriculture animal populations within the various health regions may examine poor correlation in five of the seven health regions.

6.4 Future directions

This study's development/validation of GDH RFLP protocol and survey of genotype frequencies in Calgary represents a first step in understanding the epidemiological factors responsible for endemic rates of giardiasis in Calgary. This section will discuss other avenues of epidemiological and methodological research that will hopefully lead to a better comprehension of the epidemiology of *G. duodenalis.*

As mentioned earlier in the discussion, the possible differences in assemblage virulence likely resulted in a selection bias that influenced genotype frequency. Since

reported cases may represent less than 1% of actual infection rates within the population (73), genotype frequencies associated with reported cases may not truly represent overall frequencies within the population. To determine population genotype frequencies, a future study should include the genotype frequencies of asymptomatic individuals through random fecal screens of individuals with no history of gastrointestinal disease. In addition to providing genotype frequencies that are more representative of the population, it would offer information with respect to true endemic rates of giardiasis within the community. A comparison of genotype frequencies between symptomatic and asymptomatic individuals could allow for further investigation into differences in assemblage virulence and pathogenesis.

The absence of Assemblage C and D in the 215 clinical samples genotyped suggests either these assemblages are host-adapted and have a limited zoonotic potential, or that Assemblage C and D are not present in domestic canids in Southern Alberta and thus there is no potential human access to the assemblages. Wildlife surveillance suggests Assemblage C is the predominant genotype in coyotes (unpublished), though the prevalence in domestic canids is unclear. During the study, two samples from *G. duodenalis* positive domestic canid were genotyped and found to be infected with Sub-assemblage A-I or Assemblage B. Though it appears that the canids were not the source of their owner's infections, it illustrates the potential for zoonotic transmission. While it is likely that Assemblage C and D represent a limited zoonotic potential, in order to confirm this hypothesis, genotype prevalence data for canids needs to be determined. The prevalence of various genotypes within the canid populations would also allow for an accurate risk assessment of the potential for canid/human transmission of *G.*

duodenalis. To determine the genotype frequencies within the domestic canid population, collaborations with local veterinary clinics, which routinely test for *G. duodenalis*, could be established to obtain fecal material. Depending on the scope of the study, samples from other domestic animals, such as cats, could also be collected to assess the overall risk that domestic animals represent to human health.

During the study period, a statistical significant greater than expected proportion of Sub-assemblage B-IV infections were associated with locally acquired infections (Table 5). The largest proportion (54.2%) were acquired during the warm summer months (July through September), a period of increased water activity. The importance of beavers in the waterborne transmission of *G. duodenalis* is well documented, though the prevalence of the various sub-assemblages is currently unknown. Assemblage B is believed to be the predominant genotype within the population, and given the finding of this study, further genotyping of beaver isolates to the sub-assemblage level may give further evidence of their impact on endemic giardiasis within the region.

As discussed earlier, the exposure of animals to zoonotic genotypes may be an important factor influencing the potential for zoonotic transmission. To further investigate this possibility, the effect of current municipal sewage practices on genotype frequencies in agriculture animal populations needs to be evaluated. By employing a case/control epidemiological design, enrolling farms using reclaimed water or biosolids, and farms that do not, the influence of municipal-sewage practices could be evaluated. Since most watershed harbour zoonotic genotypes, ideally control farms would utilise well water for pasture irrigation.

While current PCR protocols are providing a wealth of information that could not have been obtained through the use of axenic cultures, they still lack the ability of clonal resolution. This level of resolution would provide investigators a far greater understanding of parasite transmission and population dynamics. Currently, techniques capable of this resolution, such as pulse field gel electrophoresis (PFGE), and Random Amplification of Polymorphic DNA (RAPD) PCR, require the ability to culture the trophozoites axenically. The inability to successfully culture certain assemblages, and the selection bias inherent with this technique, makes PFGE and RAPD PCR impractical tools for epidemiological surveillance. In order to be an effective epidemiological tool, capable to tracking transmission dynamics of G. duodenalis in humans, the next generation of molecular genotyping tools must meet the following criteria: 1) no requirement of axenic culturing of trophozoites; 2) high degree of *Giardia duodenalis* specificity; and 3) near clonal resolution. Taking into consideration the high degree of genetic plasticity associated with rRNA genes (Discussed in Section 1.5), it is possible that analysis using variable number tandem repeat like elements, similar to that used for DNA fingerprinting in humans, would meet all three criteria. The use of non-coding intergenic rRNA spacer regions has been applied to *Giardia spp.* research in the past (81) for the genotyping of isolates, though it was deemed unreliable due to the high level of chromosome rearrangement associated with the rRNA gene. It is this high degree of heterozygosity that makes intergenic rDNA spacer regions a good target candidate for clonal resolution. The discovery of an intron within the *G. duodenalis* genome (120) suggests other potential target loci may also exist, and data from the current sequencing of the WB clone 6 (*Giardia* Genome Project) will likely reveal others.

Since *Giardia duodenalis* genotypes appear to influence symptom severity and zoonotic potential, factors influencing predominant genotypes within human and animal populations need to be investigated. Results from this study may suggest inter-assemblage and intra-assemblage competition may influence genotype frequencies within the human population.

To investigate the impact that intra-species competition has on mixed assemblage infections and the resulting succession to a predominant assemblage/sub-assemblage, animal models could be utilized. While gerbils could be used to examine mixed Assemblage A/B and Sub-assemblage A/B competition, cattle (Assemblage E), dogs (Assemblage C and D) or cats (Assemblage F) would likely have to be used to explore competition between zoonotic and host-adapted assemblages. The application of assemblage/sub-assemblage specific probes and fluorescent in situ hybridization analysis would allow a quantitative measure of the level of competition by measuring the number and genotype of cysts shedding throughout the infection. The level of competition between zoonotic and host-adapted genotypes with their respective hosts has serious implications on the potential stability and propagation of zoonotic assemblage with animal populations.

6.5 Study Limitations

All samples genotyped during the study period, with the exception of a limited number submitted by the refugee clinic, were submitted to CLS for diagnostic purposes by physicians. Since physicians do not routinely submit diagnostic sample from

asymptomatic individual, it can be concluded that the majority of samples genotyped were from symptomatic individuals, and may introduce a selection bias into the study. A population based study has suggested that symptomatic giardiasis may represent less than 1% of actual infection rates, (73) and as a result, frequencies from this study must be qualified as genotype frequencies of symptomatic giardiasis, which may not reflect overall genotype frequencies with the in general population.

The majority of samples received by Calgary Laboratory Services were from individuals residing in Calgary, with a limited number of samples from the surrounding communities. The limited geographic area from which the samples were collected limits the ability to generalize the study results and the genotype frequencies observed in this urban setting may not be reflective of adjacent rural areas or other geographic regions of the province.

The final limitation of this study, from an epidemiological perspective, is the small sample size. While this study is the largest survey of *Giardia duodenalis* genotypes undertaken, the small number of samples genotyped monthly (1 to 25 samples genotypes) limited the ability to examine seasonal variations in genotype frequency. The small sample sizes also prevented trend driven research and multivariable analysis.

6.6 Summary

The enteric protozoan *Giardia duodenalis* is one of the top ten parasitic diseases of humans, (174) the third most commonly reported enteric pathogen, (71) and is the number one reported waterborne disease in North America (16). This flagellated intestinal protozoan has a simple direct life cycle, consisting of an environmentally

resistant cyst stage and a motile trophozoite stage and is transmitted through both direct and indirect fecal-oral transmission (2).

In recent years, the application of molecular tools to the study of the parasite suggests *G. duodenalis* is a species complex (7), with isolates clustering into one of seven genetically distinct groups called assemblages or genotypes (157). Currently, Assemblage A and Assemblage B are the only two assemblages know to cause symptomatic giardiasis in humans, though both assemblages are also known to cause disease in a variety of other mammals (157). Assemblage A and B can be further divided into groups or sub-assemblages based on genetic clustering and host-specificity (7, 105). In addition to the zoonotic assemblages (Assemblage A and B) *G. duodenalis* is believed to be composed of host-adapted assemblages able to colonize dogs (Assemblage C and D), livestock (Assemblage E), cats (Assemblage F) and rats (Assemblage G) (157).

In order to comprehend the transmission dynamics of *Giardia duodenalis*, we first must have an understanding of the population diversity of genotypes on both a local and global scale. The purpose of was study was to provide a better understanding of the diversity of genotypes associated with symptomatic giardiasis in the humans from Calgary, Alberta and to examine factors that may influence this diversity. This was accomplished by genotyping clinical samples submitted to Calgary Laboratory Services as part of routine surveillance for the disease. To meet this objective, the secondary goal of this study was to evaluate the efficacy of two genotyping loci, the SSU and GDH gene, commonly used to genotype *G. duodenalis*.

A comparison of the SSU-RFLP and GDH-RFLP protocols suggest while the SSU-RFLP protocol theoretically has a lower detection, the GDH-RFLP protocol has

many advantages. This advantage was reduced by converting the GDH-RLFLP protocol, initially described by Read et al, 2004 (136), into a nested PCR. Both the SSU-RFLP and GDH-RFLP were able to resolve Assemblage A and B without sequencing, though the GDH-RFLP protocol also has the ability to resolve Assemblage C, D, E, Sub-assemblage A-I, Sub-assemblage A-II, Sub-assemblage B-III, and Sub-assemblage B-IV without sequencing. In addition to observing 5 of 7 published GDH-RFLP profiles a previously unpublished profile (CS390) was observed.

During the study period, January 2002 to March 2004, 282 samples were genotyped with at least one target loci. To-date this is the largest ever survey of *G. duodenalis* genotypes in humans. At the assemblage level, genotype frequencies of Assemblage A, Assemblage B, and mixed assemblage infections were 51.3, 37.1, and 11.5% respectively. During the study, two clinical samples produced GDH-RFLP profiles that suggested the possibility that the individuals were infected with Assemblage E or mixed assemblage infection including Assemblage E. If confirmed, this is the first documented occurrence of Assemblage E in a human clinical sample, though the low frequency suggests it may not play an important role in symptomatic giardiasis in the region. At the sub-assemblage level, Sub-assemblage A-I appears to be the predominant genotype in Calgary, accounting for 39.6% of all samples genotyped to the sub-assemblage level. A survey of international travellers and immigrants returning or arriving in Calgary, appears to suggest Sub-assemblage A-I may also be the predominate genotype world-wide. Further examination of the influence that international travel has on genotype frequency found that travel accounted for a greater than expected proportion of mixed sub-assemblage B infections (78.3%), while local activities accounted for a

greater proportion of Sub-assemblage B-IV (75%) and mixed assemblage (67.9%) infections.

Seasonal variations in the number of reported cases of giardiasis suggest a bimodal distribution, with the major peak being August through October, and a secondary peak in April. The major peak is associated with a period of warmer weather and increased water activity, and is composed mainly of locally acquired infections. The secondary peak is composed nearly equally of local and internationally acquired infections.

An examination of the influence that age has on genotype frequency found a statistical significant greater number of Assemblage B (48.8%) and mixed Assemblage infections (17.5%) associated with children less than 5 years of age. A comparison of genotype incidences of children 5 years in age or less to other age groups found Assemblage B was 3.1 to 17.7 times greater in children. A possible explanation for the higher incidence of Assemblage B in children is that Assemblage B is less virulent, which has been suggested by other studies (52, 135).

The possibility that Sub-assemblage A-I may be the predominant genotype world-wide, coupled with the suggestion that it may also have the greatest zoonotic potential (157) has serious implications on the potential for zoonotic transmission of the parasite. One potential route of exposure for animals to zoonotic assemblages may be the use of municipal sewage by-products for agricultural irrigation and fertilization. In recent years, there has been an increase in the use of these by-products in order to conserve limited water resources, to take advantage of their high nutrient content, and to provide an economically viable method for their disposal. Current microbial risk assessment

modeling is based on direct or secondary transmission within the human population (50), and does not account for the potential bioamplification by susceptible animal populations, whose levels of exposure may be much greater than humans. The exposure of susceptible animal populations to zoonotic genotypes could result in a shift in the predominant genotype within the affected population from a host-adapted assemblage to a zoonotic assemblage, without any significant change in the overall incidence of disease within the population. This shift to, and subsequent bioamplification of zoonotic assemblages, could thus dramatically increase the potential for zoonotic transmission.

Current genotyping protocols have allowed for the collection of a wealth of information, which has provided researchers with some insight into the epidemiology of *Giardia duodenalis*, though presently they lack sufficient resolution for certain epidemiological applications. The next generation of molecular tools should meet the following criteria: 1) no requirement of axenic culturing of trophozoites; 2) high degree of *Giardia duodenalis* specificity; and 3) near clonal resolution. Two potential targets include non-coding intergenic rDNA spacers and intronic regions within the genome.

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