MOLECULAR EPIDEMIOLOGY OF *GIARDIA* SPP. IN DIFFERENT HOSTS AND WATERSHEDS

by

NATALIE ANNE PRYSTAJECKY
B.Sc.-B.Sc. University of Calgary, 2003

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS OF THE DEGREE OF
Doctor of Philosophy

in

THE FACULTY OF GRADUATE STUDIES
(Pathology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

April, 2010

Abstract

Giardia lamblia has been problematic in British Columbia (BC) since the 1980s, having been the etiological agent in 13 of the 29 documented waterborne outbreaks in the province. Despite improvements to drinking water facilities, giardiasis continues to occur at higher rates in BC compared to the rest of Canada. This study aimed to address knowledge gaps with regard to the occurrence and molecular epidemiology of *Giardia* isolates, to help address the higher occurrence of giardiasis in British Columbia. This study was conducted in three steps. First, tools were developed and validated to genotype *G.lamblia* isolates into groups/genotypes called Assemblages. These tools were first applied to a library of archived *G.lamblia* isolates collected from patients, animals and water sources in British Columbia. These same tools were then applied to water samples collected in the Salmon River watershed in the Township of Langley, BC, the second stage of the study. The occurrence and characteristics of isolates collected in the Salmon River were then compared to isolates collected in the Grand River watershed, an intensely developed mixed-urban watershed in Ontario, for the third stage of the study.

In the first study, it was determined that 18s rRNA nested PCR with sequencing was the most appropriate molecular epidemiological tools to study *G.lamblia* in water supplies. Using a combination of molecular methods and USEPA Method 1623, it was determined that the majority of isolates in the Salmon River watershed were potentially infectious to humans (belonging to Assemblages A and B), with Assemblage A occurring most frequently. In contrast, Assemblage B was the most frequently detected genotype in the Grand River watershed, a more intensely developed watershed. Analysis of rainfall and sequence data suggests that *G.lamblia* isolates could have originated from sewage effluent or septic tanks, which was confirmed with the occurrence of *Cryptosporidium hominis*. Non-zoonotic *G.lamblia* isolates occurred more frequently in the Grand River watershed than in the Salmon River watershed but this water source still represents a threat to public health. Although challenging to incorporate molecular analyses into environmental monitoring, suggestions are made for the most effective linking of molecular analyses into Method 1623.

.

Table of Contents

List of Tables

List of Figures

Acknowledgements

I would first like to thank my supervisors, Judith Isaac-Renton and Peter Huck, for their mentorship, guidance and support during this project. I am truly grateful to have studied under two experts in drinking water quality and you have fuelled my passion in drinking water even further. I have learned so much from you both. I also thank my supervisory committee along with my committee chair for their insights and suggestions and keeping me on track.

I am indebted to the staff at the BCCDC Environmental Microbiology and Molecular Services laboratories. Thank-you for teaching me everything from pipetting to sequencing, for troubleshooting advice and for forgiving me when I broke the centrifuge! I am particularly indebted to Brian Auk, Selena Shay, Anna Li, Renata Zanchettin, Joe Fung, Diane Eisler, Kathy Adie and Alan McNabb.

Thank-you to the researchers involved both the Canadian Institute for Health Research Source-To-Tap study (Judith Isaac-Renton, Corinne Ong, Hans Schreier, Sandra Brown, Vic Gannon, Tom Edge and Paul Sockett) and the Canadian Water Network study (Peter Huck, Judith Isaac-Renton, Michelle Van Dyke, William Anderson and Corinne Ong). Thank-you to the researchers involved in archiving the *Giardia* isolates (Lorraine McIntyre).

To my parents, Maria and Walter Prystajecky, thank-you for the years of support, both financially and emotionally. I could not have done it without your confidence and encouragement. To my siblings, my grandma, and the rest of the family (including Cathy and Chris Ebbehoj) for always being there for me and for believing in me.

Finally, to Mike, you have been constant source of support, advice and encouragement during my thesis. From driving me to the lab late at night and waiting for me as I nested my PCRs to dealing with my research papers in piles around the house to proofing papers and posters, you have been my biggest supporter. Thank-you! I look forward to a lifetime of discussing water microbiology, fuel cell technology, sports and politics with you.

Co-Authorship Statement

I designed the studies presented in Chapters 2, 3 and 4 in consultation with Judith Isaac-Renton and Peter Huck.

Isolates used in Chapter 2 were collected, cultured and archived by technical staff at the BCCDC. I complete all subsequent laboratory work, data analysis and manuscript preparation, with editorial input from my co-authors.

The study site in Chapter 3 was selected by Hans Schreier and Judith Isaac-Renton as part of a larger Source-to-Tap study. Samples were collected by Hans Schreier and volunteers and I performed subsequent laboratory work, data analysis and manuscript preparation, with editorial input from my co-authors.

The study site in Chapter 3 was selected by Peter Huck and partners at the NSERC Chair in Drinking Water Treatment as part of a larger drinking water quality study. Samples were collected by NSERC Chair staff and shipped to BCCDC, where I performed subsequent laboratory work, data analysis and manuscript preparation, with editorial input from my co-authors.

Chapter 1
Thesis Introduction and Literature Review

1.1 Topic Justification

Despite advances in drinking water treatment, there continues to be a high incidence of gastrointestinal illness attributed to waterborne sources, both at endemic and outbreak scales. Waterborne outbreaks of disease have been attributed to different classes of microbial organisms (protozoa, bacteria and viruses) and a wide variety of water types (treated and untreated drinking water; groundwater, natural springs and surface water; swimming pools, water parks and lake water, to name a few). Of greatest concern are outbreaks that occur as a result of large municipal drinking water supplies, as they impact the greatest number of individuals. The incidence of endemic illness attributed to contaminated water probably has an even more significant impact on human health, but is much more difficult to investigate (Colford *et al*., 2006).

It is estimated that in the United States (USA), waterborne pathogens are responsible for 12 to 19.5 million infections annually (Colford *et al*., 2006 and Reynold *et al*., 2008). In Canada, it is estimated that contaminated water is responsible for 90,000 illnesses and 90 deaths annually (Statistics Canada, 2003). Differences in rates of illness in Canada and the USA relate to differences in estimation methods, rather than differences in disease prevalence. High profile outbreaks that have occurred in recent history in developed nations include an outbreak of cryptosporidiosis in Milwaukee, USA, in 1993 and the outbreak of *Escherichia coli* O157:H7 and *Campylobacter* in Walkerton, Ontario in 2000 (MacKenzie *et al.*, 1994; Krewski *et al.*, 2002). Reports on these events emphasized the impacts of contaminated water on a community, having health (short and long-term), economic and social impacts (Corso *et al.,* 2003). In North America, the majority of outbreaks can be explained with treatment equipment failure (such as a defective chlorinator) or inadequate water treatment (such as the failure to chlorinate) (Craun *et al.,* 2006; Hrudey and Hrudey, 2007).

Historically, British Columbia (BC) has experienced greater rates of enteric illnesses compared to the rest of Canada, with rates peaking in 1989 at over 200 cases per 100,000 BC residents. There have been 29 confirmed waterborne outbreaks in the province since 1980, the majority of which were due to *Cryptosporidium* spp. or *Giardia lamblia* (PHO, 2001). These organisms are particularly challenging for drinking water suppliers as they are ubiquitous in animal populations, spread

zoonotically, survive environmental stresses and are relatively resistant to disinfection. While there have been no documented outbreaks of waterborne illness attributed to drinking water contaminated with *Giardia* or *Cryptosporidium* since 1998, rates of giardiasis and cryptosporidiosis in British Columbia continue to exceed national averages without explanation. Furthermore, giardiasis greatly exceeds rates of cryptosporidiosis globally, necessitating the study of *G.lamblia* to better understand its biology and epidemiology. Recently, efforts have been made to introduce genetic typing of environmental *G.lamblia* isolates. Even less understood than the biology and epidemiology of *G.lamblia* is how to apply and link this new knowledge of the genetics of this parasite to environmental monitoring, public health risk assessment and government policy decisions. By developing new knowledge, along with the tools to apply this knowledge, greater efforts can be made to reduce the impact of giardiasis on global populations.

1.2 Thesis Description

This thesis aims at addressing a chronically understudied waterborne parasite, *Giardia lamblia*, which is known to have significant impacts on public health at both local and international levels. This thesis first aims at addressing knowledge gaps in the gathering and use of genetic data as it applied to *G.lamblia* in water supplies, an area that is not well researched. Secondly, this thesis aims to gather data on the prevalence and distribution of *G.lamblia* in the environment, at the local level (British Columbia) and between two jurisdictions (British Columbia and Ontario). Thirdly, this thesis aims to demonstrate the risks of consuming water, from both a monitoring and a risk assessment perspective, with respect to *G.lamblia* contamination of drinking water and recreational water. Lastly, this thesis aims to understand the impact that collecting genetic data might have on policy decisions, which up until now have had foundations based on classical microbiology.

By studying the aforementioned aims in an environmental health framework a greater understanding of the risks associated with consuming surface water will be clarified. Knowledge regarding the prevalence and distribution of the organism in the environment will be further elucidated. This research has the potential to generate not only new information of *G.lamblia* biology, but also provide data that will be useful in both environmental and health policy development.

This thesis is divided into six chapters. Chapter 2 is a literature review to provide background and framework for the research investigations. It includes an overview of water quality, pathogens that affect water resources and water treatment processes necessary to remove pathogens from water

supplies. An overview of the public health context in British Columbia, with specific reference to water quality and legislation, is also provided. Next, a detailed overview of *Giardia lamblia* is provided, describing the biology and genetic characteristics of the organism, clinical manifestations, the ecology and epidemiology of the organism. Lastly, the impacts of *Giardia* on drinking water are described, as well as the methods to detect the organism in these supplies. This information is to provide context of *G.lamblia* within three major themes of this research: public health and risk assessment; information for monitoring drinking water supplies and how policy and regulations apply in a Source-to-Tap framework.

Chapters 3, 4 and 5 are research papers, to be submitted to peer-reviewed journals. Each article is formatted according to the requirements of the journal. Chapter 3 is entitled **Retrospective Genetic Analysis of Giardiasis Specimens Collected From Clinical, Animal and Drinking Water Samples.** In this investigation, clinical specimens and related drinking water and animal samples which comprise a library of *G.lamblia* isolates were re-analyzed using current genotypic methods. These samples were collected during the height of the giardiasis problem British Columbia, during the late 1980s and 1990s, at a time when there were numerous outbreaks and elevated endemic illness occurrence. The purpose of this study was to develop, validate and analyze clinical, environmental and animal isolates by new genetic methods compared to previous gold-standard genotypic methods (PFGE and isoenzyme analysis) in order to provide state of the art molecular epidemiological tools for use in this field of work. Furthermore, this provided a baseline of *G.lamblia* sequencing data in British Columbia and other regions, for comparisons in prospective *Giardia* molecular epidemiology studies.

Chapter 4 is entitled **Occurrence of *Giardia* and *Cryptosporidium* in a Mixed Urban-Rural Watershed.** In this study, water samples were collected over a two-year period from source waters for a mixed urban-rural community in British Columbia and were analyzed for parasite occurrence and characterization (genotyping). These results were compared to classical water quality parameters and to climate characteristics. Furthermore, genotype data were compared to determine relationships between *Giardia* and *Cryptosporidium* sources and to determine variability in genotype prevalence over time.

Chapter 5 is entitled **Comparative analysis of *Giardia lamblia* Occurrence and Genotype Prevalence in Two Canadian Watersheds of Differing Land-Use Practices**. This investigation compared *Giardia lamblia* occurrence and genotype prevalence between two mixed urban-rural

communities located in southern British Columbia (Salmon River) and southern Ontario (Grand River). The communities differed significantly with respect to their potential sources of pathogens; the Grand River is both heavily impacted by sewage effluents and intense agriculture, while the Salmon River is largely impacted by less intense agriculture ventures. This study aimed to identify differences in genotype characteristics in two geographically distinct communities in Canada, with varying sources of pathogen loadings.

The final Chapter, Chapter 6, summarizes the findings of the three research papers and provides conclusions regarding the application of new tools for genetic analysis of waterborne parasite applied to the prevalence and molecular epidemiological questions regarding the impact of *Giardia lamblia* on public health risk assessment, parasite monitoring and policy development. It highlights changes in predominant genotypes from historical human specimens compared to environmental isolates collected in British Columbia, and how these isolates differ from those found in a watershed more significantly impacted by point sources and non-point source pollution. Furthermore, it provides suggestions on new approaches and future directions for analysis of *G.lamblia* and related pathogens in water supplies, including approaches for enhanced filtration and enhanced molecular characterization of this organism. This research has significant implications for the health of Canadians as well as for environmental and public health policy created for the protection of the public health.

1.3 Drinking Water and Health

Worldwide, it is estimated that one in five individuals do not have access to safe drinking water (Payment and Hunter, 2001). Diarrheal disease is ranked by the World Health Organization as the most common cause of morbidity and the sixth most common cause of mortality worldwide (WHO, 1999, Ford, 1997). It is estimated that contaminated water is responsible for 28% of deaths in the under-five age group. The Millennium Development Goal (MDG) is a United Nations initiative to reduce mortality in children under 5 years of age by two-thirds by the year 2015 (UN, 2006). Although the situation in North America is significantly different than that in the developing world, these numbers draw attention to the importance of the impact of waterborne illnesses worldwide, including Canada, and emphasize the need for new knowledge and ongoing research in the area of the prevention of waterborne diseases.

1.3.1 Waterborne Microorganisms

Currently, there are more than 140 microorganisms recognized as waterborne pathogens (Reynolds *et a*l, 2008). Typically, these waterborne pathogens are enteric microorganisms that are excreted in feces and that can survive to be transmitted through either drinking or recreational water. However, there are a few waterborne parasites, such as *Legionella* or the *Mycobacterium avium*-complex, that are transmitted through contaminated water but do not originate from fecal contamination.

There are three groups of waterborne pathogens (viruses, bacteria and protozoa), all of which behave differently in the environment, in their resistance to drinking water treatment, and in their impact on human health (see Table 2-1).

Viruses are currently amongst the most challenging class of microorganisms that microbiologists study, due to their small size, difficulties in culturing *in vitro*, their low concentrations in environmental samples and current methods of detection. They range in size from 10-300 nm. While viruses typically cause gastroenteritis in humans, they may also cause more severe illness such as meningitis and myocarditis. They are unable to replicate in water, but demonstrate extremely long environmental persistence. Their threat to public health is potentially high because of their low infectious dose (typically 1-10 viral particles) and there is limited capacity to monitor these organisms in water supplies. Furthermore, the small size of viruses may allow for greater ingress into groundwater supplies compared to bacteria and parasites. Enteric viruses are the most common type of waterborne virus, typically excreted from an infected human host. Viruses tend to host-specific and zoonotic transmission is rare, in comparison to bacterial and protozoan agents.

Table 1-1-1: Common waterborne pathogens and their relative environmental survival capability, chlorine resistance and overall health impact. Adapted from: WHO, 2006.

Pathogen	Environmental Survival	Chlorine Resistance	Relative Infectivity in Humans	Health Significance	Zoonotic
VIRUSES					
Adenoviruses	Long	Moderate	High	Moderate	No
Enteroviruses	Long	Moderate	High	High	No
Astroviruses	Long	Moderate	High	Moderate	No
Hepatitis A virus	Long	Moderate	High	High	No
Hepatitis E virus	Long	Moderate	High	High	Potentially
Noroviruses	Long	Moderate	High	High	Potentially
Sapoviruses	Long	Moderate	High	High	Potentially
Rotavirus	Long	Moderate	High	High	No
BACTERIA					
Campylobacter spp.	Moderate	Low	Moderate	High	Yes
Escherichia coli (EHEC, ETEC)	Moderate	Low	Low	Moderate	Yes
Legionella spp.	May multiply	Low	Moderate	High	No
Mycobacterium avium complex	May multiply	High	Low	Low	No
Pseudomonas aeruginosa	May multiply	Moderate	Low	Moderate	No
Salmonella enteritica	Moderate	Low	Low	High	No
Other *Salmonellae*	May multiply	Low	Low	High	Yes
Shigella spp.	Short	Low	High	High	No
Vibrio cholera	Variable survival	Low	Low	High	No
Yersinia enterocolitica	Long	Low	Low	Moderate	Yes
PROTOZOA					
Acanthamoeba spp.	May multiply	Low	High	High	No
Cryptosporidium spp.	Long	High	High	High	Yes
Cyclospora cayetanensis	Long	High	High	High	No
Entamoeba histolytica	Moderate	High	High	High	No
Giardia lamblia	Moderate	High	High	High	Yes
Naegleria fowleri	May multiply	Low	Moderate	High	No
Toxoplasma gondii	Long	High	High	High	Yes

In the United States, between 1971 and 2004, data indicated that viruses were found to be responsible for 8% of waterborne outbreaks of disease, from either recreational or drinking water (Karim *et al*., 2009). This is likely an underestimation, however, of the true burden of waterborne viral gastroenteritis. It has been suggested that in nearly half of waterborne outbreaks in the United States in which the etiological agent was not identified, the etiology was viral in origin (Ferguson *et al*., 2003). The identification of waterborne outbreaks of gastroenteritis is limited by the availability of laboratory tools to study this group of organisms in both water and stool samples. This is particularly true in water; viruses are both difficult to concentrate and identify and many are not culturable *in vitro*. A study by Payment *et al*., (2000) found that in a survey of surface water and treated water, 63% of samples were below the detection limit for viruses. Investigations into a surface water source in Netherlands suspected to be contaminated with sewage, recovered enteric viruses in 100% of samples but at low concentrations (Lodder and de Roda Husman, 2005). Recent development in virus concentration and molecular detection methods will contribute to greater knowledge of virus prevalence and concentrations in water supplies (Hill *et al*., 2009; Polaczyk *et al*., 2007; Hamza *et al*., 2009). Further research into this area is greatly needed.

Bacteria are the most recognized waterborne pathogens. They include both enteric and non-enteric disease-causing organisms. Unlike viruses, some bacteria may multiply in water, and this replication is dependent on the temperature and nutrient content of the water as well as characteristics of the bacteria. They are more susceptible than viruses or protozoa to environmental stresses and thus their environmental persistence is limited. There is evidence that some bacteria, such as *Helicobacter* and *Campylobacter,* are capable of becoming environmentally resistant by entering a metabolically inactive state (viable but not culturable, VBNC) (Byrd *et al*., 1991; Rollins and Colwell, 1986). This ability to adapt and become VBNC affords a survival advantage. Bacteria are particularly susceptible to chlorination and other disinfection methods, although some species such as *Escherichia coli* have been demonstrated to reactivate following UV disinfection through DNA repair enzymes (Zimmer and Slawson, 2002). Non-enteric bacteria such as *Legionella, Aeromonas, Pseudomonas* and *Mycobacterium avium*-complex occur and proliferate naturally in fresh water, but with the exception of *Legionella*, are not frequent sources of waterborne outbreaks of disease.

Infectious doses of bacterial pathogens tend to be higher than viruses and protozoa, although some pathogens such as *Shigella* have infectious doses as low as 10 cells (Kothary and Babus, 2001). Bacterial pathogens are frequently zoonotic and as such, all sources of fecal contamination (sewage,

manure, wildlife, domestic pets etc...) may contain human-infective bacterial pathogens and are therefore a threat to public health.

Overall, bacterial pathogens are easier to study in a variety of matrices and as such, the knowledge related to their occurrence is broader than that of waterborne viruses or protozoa. Furthermore, because of the ease of concentration and growth *in vitro*, enteric species are used as indicator organisms for the routine surveillance of drinking water quality contamination and for determination of drinking water treatment effectiveness (see Section 2.4 below).

Bacterial pathogen concentrations vary by pollution source, geography, seasonal variations and pathogen-specific characteristics. A study in the South Nation Basin in Southern Ontario found *Campylobacter, Salmonella* and *E.coli* (EHEC/VTEC) in 17.8%, 7% and 0.25% of surface water samples, respectively (Wilkes *et al*., 2009). Pathogens were detected at highest concentrations in the fall and were not detected in winter months. While culturing is still the predominant detection and amplification method for bacterial pathogens in water, studies are increasingly incorporating nucleic acid amplification (NAT) assays, with sequencing of isolates for subtyping (genotyping) purposes and real-time polymerase chain reaction (qPCR) for rapid enumeration.

Protozoa, the largest in size of the three main groups of waterborne microorganisms, are a particular challenge to drinking water suppliers. Typically resistant to chlorine, they are only effectively removed from water supplies by filtration and UV irradiation or ozonation. Furthermore, they are extremely resilient to environmental stresses and can survive in cold water for up to 6 months. Compared to bacteria, protozoa in water demonstrate greater resistance to environmental stress, such as temperature, UV and desiccation and therefore their occurrence in water cannot be predicted using classical bacterial indicators (Chauret *et al*., 1998). They do not replicate in the environment, however, but demonstrate low infectious doses, as low as 10 organisms by human challenge experiments, creating a significant health risk should they enter a drinking water supply.

The two most commonly recognized protozoan genera causing of waterborne disease are *Giardia* and *Cryptosporidium*. These organisms each have a two-staged life cycle; a replicative form responsible for the symptoms of disease and an environmentally resistant form, which is transmitted from host-to-host or via an environmental transmission vehicle.

In British Columbia, *Giardia* and *Cryptosporidium* were responsible for 16 out of the 29 documented waterborne outbreaks since 1980 (PHO, 2001). In the United Kingdom, *Cryptosporidium* and *Giardia* were responsible for 83% of waterborne outbreaks between 1992 and 2003, with *Cryptosporidium* being the most common etiological agent (69%) (Smith *et al*., 2006). A survey of raw water in sixty-six drinking water treatment plants in the United States and Canada found *Giardia* and *Cryptosporidium* in 81% and 87% of water samples respectively (LeChevellier *et al*., 1991). Similarly, Hansen and Ongerth estimated that 80-96% of surface waters are contaminated with *Giardia* and/or *Cryptosporidium* (1991).

Other protozoan agents found in water and/or associated with waterborne outbreaks include *Cyclospora cayetanensis, Toxoplasma gondii, Naegleria fowleri, Entamoeba histolytica/dispar* and *Acanthamoeba* species. Of these, *T.gondii* and *C.cayetanensis* are most likely to impact North American drinking water supplies. A large documented outbreak of toxoplasmosis occurred in Victoria, BC in 1995, where a contaminated drinking water reservoir was identified as the most probable source of an estimated 4,000 infections (Bowie *et al*., 1997). *C.cayetanensis* has been responsible for waterborne outbreaks in Nepal and is frequently detected in water sources in Southeast Asia. Its potential impact on North American water sources has not yet been determined (Sterling and Ortega, 1999; Tram *et al*., 2008).

1.3.2 Indicator Organism Testing

Microbial water quality is typically assessed in public health or water purveyor laboratory settings using bacterial surrogates or indicator organisms, such as total coliforms, fecal coliforms and *Escherichia coli (*LeClerc *et al*., 2001). Historically, these indicators were chosen for monitoring use over pathogen-specific testing, due to the ease of their detection and low costs. Their presence in water suggests recent fecal contamination and points to the possibility of (but does not confirm) the presence of human microbial pathogens. In addition to water quality monitoring, indicator organisms may be enumerated for determination of water treatment process efficacy, particularly in evaluating re-growth in the distribution system. Many studies, however, indicate a failure to establish a correlation between pathogen occurrence (such as parasites) and the presence of indicator organisms, making these methods not completely reliable for the protection of public health (Horman *et al.*, 2004; Payment *et al.* 2000). Not surprisingly, waterborne outbreaks have occurred in the absence of bacterial indicator organisms.

The most commonly used indicators in the drinking water industry and in public health are total coliforms, fecal coliforms and *Escherichia coli*. Total coliforms are gram negative, rod-shaped, non-spore forming bacteria. While these organisms are found in the intestines of warm-blooded animals, they can also originate from environmental sources. Presence of these organisms in treated water is suggestive of treatment failure or distribution system re-growth. Fecal coliforms are a subset of total coliforms, originating only from the intestines of warm-blooded animals. They can grow in the presence of bile salts, are oxidase negative and produce lactic acid and gas within 48 hours at 44 ºC ± 0.5ºC. While most members of the fecal coliform group are fecal in origin, it also includes members of the *Klebsiella* genus, which are not necessarily of human origin. In most Canadian provinces and US states, monitoring for fecal coliforms has been replaced with *E.coli. E.coli* is only found in the intestines of humans and other warm-blooded animals and while it occurs in lower numbers in contaminated water sources, it is more representative of recent fecal contamination. In British Columbia, drinking water testing requires a minimum of four *E.coli* and total coliform tests per month, with the number of tests required increasing in proportion to the population size the drinking water system serves (BC Reg 200/2003). In Ontario, a minimum of two *E.coli* and total coliform tests are required per month for small water systems; the number of tests required increases with water system size and treatment equipment used (O. Reg 170/2003).

1.3.3 Drinking Water Treatment

The purpose of drinking water treatment is to render raw water potable, that is, suitable for human consumption. It should remove any microbial or other biological materials, as well as any chemical contaminants. Ideally, the resulting water should be free of taste and odour problems, clear and relatively soft (Gray, 1999). The amount of treatment required is largely dependent on the quality of the source water and typically the type of treatment required is governed by drinking water legislations and regulations.

In the typical water treatment system obtained from surface water sources, there are four treatment processes, as required as part of the current standard: 1) Pre-treatment, 2) Coagulation and flocculation, 3) Clarification and 4) Disinfection and other treatment. In pre-treatment, water is passed through coarse screens to remove larger objects such as plants and smaller screens to remove fine solids and algae. Pre-treatment may also include adjusting the pH for subsequent treatment steps and may also include pre-conditioning and pre-chlorination. This is followed by coagulation and flocculation, in which a chemical coagulant is added to help remove the remaining small particles

(typically smaller than ten microns). The interaction of the particles and the coagulant produce flocs, a process that can be enhanced by mixing. This step is followed by clarification, which promotes sedimentation of the flocs. This step can be further improved by using filtration, which will remove even smaller particles, including protozoa. Slow sand filtration and membrane filtration are the most commonly applied filtration techniques in developed countries.

The final step in drinking water treatment is disinfection, whose purpose is to eliminate remaining microbial pathogens. Chlorine is the most commonly used disinfectant and it is used both as primary disinfection and for residual disinfection in the distribution system. Chlorination can result in the formation of disinfection by-products such as trihalomethanes (THMs), which have been demonstrated to cause bladder cancer in laboratory animals (Villanueva *et al,* 2007). As an alternative to chlorine, ozonation can be used as a disinfectant, which does not produce disinfection by-products and is more effective at disinfecting organisms that are not susceptible to chlorine, such as *Cryptosporidium*. Ultraviolet irradiation is another alternative to chlorination; it is able to kill difficult-to-treat organisms and leaves no disinfection by-products but is easily impacted by turbidity and as such, is typically only used in conjunction with filtration.

1.4 *Giardia*

1.4.1 Biology of *Giardia*

The genus *Giardia* is a group of flagellated parasites that belongs to the Order Diplomonadida and Family Hexamitidae (Adam, 2001). It was first described by in 1681 by Anton van Leeuwenhoek, who examined the organism microscopically from his own stool. *Giardia* has been referred to as the 'missing link' between prokaryotes and eukaryotes as it has demonstrates characteristics of both groups (Kabnick and Peattie, 1991). It is much more simplistic than typical eukaryotes, with respect to its physiological, structural and metabolic structure (Adam, 2001). It lacks mitochondria, peroxisomes and other cellular structures necessary for oxidative phosphorylation (Adam, 2001). It does, however, have a well-developed cytoskeleton and components of the Golgi complex as well as an endoplasmic reticulum. Its genome is compact, lacks introns, with simplified nucleic acid replication, transcription machinery and metabolic pathways (Morrison *et al*., 2007). The primitive nature of the organelles and its metabolism, as well as phylogeny based on the small-subunit rRNA, has led to the proposal that members of the *Giardia* genus are among the most primitive eukaryotes.

This primitive nature is further supported by appearing to only undergo asexual reproduction (Adam, 2001).

However, recent evidence is consistent with the hypotheses that *Giardia* is much more developed than previously thought and may have undergone organelle and genomic reduction, rather than failing to acquire these characteristics. These studies suggest that primitive, inactive, meiotic genes exist in the genome of *Giardia lamblia* (Ramesh *et a*l., 2005; Birky Jr 2005). A population genetics study using single nucleotide polymorphisms (SNPs) also suggests that recombination events have occurred, which is possible only in sexually reproducing organisms (Cooper *et al*., 2007). It has also been suggested that *Giardia* may represent a divergent lineage or organisms which has adapted to a microaerophilic lifestyle, rather than diverging before the endosymbiosis of the mitochondrial ancestor (Monis *et al*., 2009). A phylogeny of related gastrointestinal or waterborne parasites is shown below (Figure 2-1).

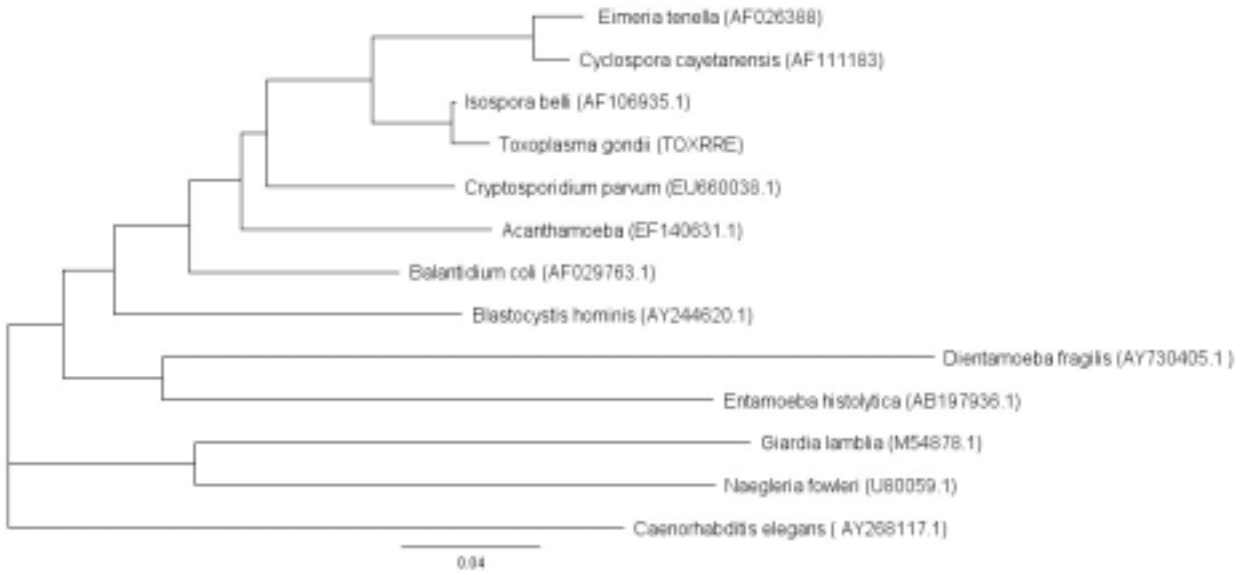


Figure 1-1: Phylogenetic tree demonstrating the genetic relatedness between *G.lamblia* and related gastrointestinal or waterborne protozoa. Tree constructed using Geneious Tree Neighbour Joining Model.

G. lamblia likely has a ploidy of four and has a confirmed genome size of approximately 11.7 Mb divided among five chromosomes (Adam, 2001; Morrison *et al*., 2007). As a diplomonad, it has two identical nuclei that are both transcriptionally active and replicate at the same time (Adam, 2000). Most genes have short 5′ and 3′ untranslated regions and promoter regions that are near the initiation codon.

Trophozoites (the replicative form, see description on next page) exhibit antigenic variation of an extensive repertoire of cysteine-rich variant-specific surface proteins (VSPs) (Nash and Mowatt, 1992). This likely allows the organism to evade the host immune system. VSP expression is allele specific, and changes in expression from one *vsp* gene to another have not been associated with sequence alterations or gene rearrangements and appear to be under the regulation of small interfering RNA (siRNA) (Prucca *et al*., 2008). Since it lacks mitochondria and components of oxidative phosphorylation cascade, like *Trichomonas* spp. and *Entamoeba* spp., it undergoes fermentative metabolism.

Giardia lamblia has a simple life cycle consisting of two stages: a cyst stage and a trophozoite stage (Adam, 2001). The cyst is infectious and is relatively resistant to environmental pressures such as desiccation, UV light and mechanical stress. Cysts are shaped similarly to a rugby ball and measure 5 x 7-10 microns in diameter. Each cyst contains two trophozoites and therefore four nuclei (see Figure 2-2).

Figure 1-2: Typical appearance of environmentally obtained *G.lamblia* cysts, under immunofluorescence microscopy (left), stained with 4'-6-diamidino-2-phenylindole (DAPI) to show nuclei (middle) and under differential interference (DIC) microscopy to show internal morphology (right). The slight irregularity in the shape of the cyst is consistent with environmental stresses, causing deformation of the typical smooth features of the cyst wall. It is quite rare to observe all nuclei in an environmentally obtained cyst.

The trophozoites are the replicative form and are responsible for the symptoms of infection. They are pear-shaped, ventrally flattened, measuring 12-15 microns long and 5-9 microns wide. They contain two nuclei (without nucleoli) that are situated anteriorly, on each side of the long axis. The cytoskeleton comprises a ventral disk (used for attachment), four pairs of flagella and a median body.

1.4.2 *Giardia lamblia* Infection

G.lamblia causes villus atrophy and crypt hyperplasia, although knowledge pertaining to its pathogenesis is limited (Müller and von Allmen, 2005; Farthing 1993; Wright *et al*., 1977). The mechanism of damage to the small intestine microvillus layer is unknown however, recent studies have suggested that this might be associated with by the infiltration of inflammatory cells and host T-cell mediated damage (Scott *et al*., 2004). There is no evidence of toxins or virulence factors and a survey of the sequenced genome has not revealed any putative toxin or virulence genes (Morrison *et al*., 2007). Since microvillous damage may be generalized and remote from any observed or structural damage and the presence of a *G.lamblia* toxin has not been ruled out, there is still a possibility of damage via toxin injury.

1.4.3 *Giardia* Taxonomy

Early species designations were based on the animal from which *Giardia* was isolated, resulting in a multitude of *Giardia* species with little or no morphological differences. Reassessment of the taxonomy, based on light microscopy, electron microscopy and 18s rRNA sequencing, has yielded six recognized *Giardia* species, all of which are adapted to a specific type of host, with the exception of *G.lamblia* (Caccio *et al*., 2005) (See Table 2-2). *G.lamblia* is the only zoonotic *Giardia* species recognized to date and is capable of infecting a wide range of vertebrates including humans, livestock, cats, dogs, beavers and rats. *G.microti* is the species most similar to *G.lamblia*, both morphologically and genetically, but differs with respect to its host range (*G.microti* primarily infects voles and muskrats)

Table 1-1-2: Species of the *Giardia* genus, their hosts, morphology and molecular data. Adapted from Adam, 2001.

Species Name	Hosts	Trophozoite Morphology	Molecular Phylogeny
G. muris	Rodents	Short and rounded; small rounded median body	Distant from *G. lamblia*
G. lamblia	Numerous mammals, including humans	Pear shaped; one or two transverse, claw-shaped median bodies	Clade with multiple genotypes
G. ardeae	Herons	Same as *G. lamblia*	Closer to *G. lamblia* than to *G. muris*
G. psittaci	Psittacine birds	Same as *G. lamblia*	NA
G. microti	Voles and muskrats	Same as *G. lamblia*	Similar to *G. lamblia* genotypes
G. agilis	Amphibians	Long and slender; teardrop-shaped median body	NA

1.4.4 *Giardia lamblia* Taxonomy

The organism now known as *Giardia duodenalis* (syn *lamblia* and *intestinalis*) was first named *Cercomonas intestinalis* by Dr. F. Lambl, but then renamed by 1915 in honour of Professor A. Giard and Dr. F. Lambl. There continues to be debate regarding the species nomenclature; the proper zoological nomenclature is *G.duodenalis,* yet *G.lamblia* continues to be applied in the medical community. For the purposes of this thesis, *G.lamblia* will be used.

The taxonomy of *Giardia lamblia* has undergone considerable changes in recent years and is still widely debated (Thompson *et al*., 2000; Monis *et al*., 2009). Genetic and biotypic analyses of the *G.lamblia* isolates have identified significant differences between isolates, with differences observed in the genome sequence, host specificity and clinical presentation of disease. Additional differences observed amongst isolates include: differences in metabolism and biochemistry, DNA content, in vitro and in vivo growth rates, drug sensitivity, duration of infection, pH preference, infectivity and susceptibility to infection with a double stranded RNA virus (Monis *et al*., 2009). It has been suggested the *G.lamblia* represents a species-complex (hence the usage of the terminology Assemblage for genotype designation). Recently, efforts have commenced to re-evaluate of *Giardia* genus taxonomy (Aurrecoechea *et al*., 2009; Monis *et al*., 2009; Sprong *et al*., 2009).

Genotyping using molecular methods have shown that there is a genetic basis for the phenotypic variations observed. Early molecular assessments of *G.lamblia* subtypes used isoenzyme (zymomeme) analysis (Bertram *et al* 1983; Meloni *et al*., 1988; Cedillo-Rivera *et al*., 1989). This approach evaluates the migration of enzymes which have identical function but different amino acid sequences, through a starch gel in the presence of an electric field. Different *G.lamblia* subtypes demonstrate different isoenzyme patterns, reflecting differences at the protein level which ultimately demonstrate differences at the genetic level. Subsequently, pulse field gel electrophoresis (PFGE) was applied, due to its usefulness in the bacteriology field (Adam *et al.,* 1988; Campbell *et al*., 1990; Isaac-Renton *et al*., 1993). This approach, based on the migration of chromosomes in an electrophoretic field, demonstrated limited usefulness at distinguishing between *G.lamblia* isolates, due to frequent chromosomal rearrangement (Adam, 1992). More recently, subtyping has focused on polymerase chain reaction (PCR) amplification and subsequent typing by DNA sequencing or restriction fragment length polymorphisms (RFLP) (Baruch *et al*., 1996; Homan *et al*., 1998; Caccio *et al*., 2002; Amar *et al*., 2002).

Homan *et al*., identified two groups of human isolates named Belgian and Polish, corresponding to their geographical isolation (1992). Nash *et al* had earlier identified three groups (groups 1, 2 and 3) (1985). Mayrhofer *et al* later classified human isolates into two groupings, Assemblages A and B, which is the current accepted nomenclature by taxonomists and has been applied to animal isolates as well (1995). The correspondence of the various taxonomies is listed below (see Table 2-3).

Table 1-1-3: Comparison of the early taxonomy of *G.lamblia* human isolates.

Nash *et al* (1985)	**Homan (1992)**	**Mayrhofer *et al* (1995)**
1 & 2	Polish	Assemblage A
3	Belgian	Assemblage B

Currently, there are seven acknowledged Assemblages, named Assemblages A through G. Two (Assemblages A and B) are zoonotic and capable of causing infection in humans. The remaining Assemblages (Assemblages C through G) are host-adapted organisms and cannot cause disease in humans (Table 2-4). Assemblage A appears to be capable of infecting a larger range of animal hosts, including human, livestock and rodents such as beavers.

There have been efforts to link the different human Assemblages to differing severity of illness, with anecdotal evidence suggesting that Assemblage A causes mild, intermittent diarrhea while Assemblage B causes severe, acute and persistent diarrhea (Homan and Mank, 2001). However, Read *et al*. found the opposite relationship (that Assemblage A causes severe, acute and persistent diarrhea) (2002). These inconsistencies of the relationships between phenotype and genotype warrant further study.

Table 1-1-4: Assemblages of *Giardia lamblia* and their host specificities.

Assemblage	Host Specificity
A	Humans, other primates, dogs, cats, livestock, rodents, wild mammals
B	Humans, other primates, dogs, some species of wild mammals
C/D	Dogs and other canids
E	Livestock (cattle and other hoofed livestock)
F	Cats
G	Rats

Genetic sequencing has shown that there is greater genetic heterogeneity amongst Assemblage B isolates than amongst Assemblage A isolates. Assemblage A isolates tend to be more homologous genetically (Mayrhofer *et al*., 1995; Meloni *et al*., 1995) (See Figure 2-4). Studies on the phenotypic differences between Assemblages A and B have been complicated by disparate growth abilities of these two groups during axenic culture (Binz *et al*., 1992). It appears that Assemblage A is better adapted to axenic culture and is used as the predominant lab strain (strain WB). However, there is some evidence that Assemblage B has a growth advantage in animal models such as the suckling mouse (Thompson and Lymbery, 1996). It is possible that these differences in ideal growth conditions may contribute to the observed differences in infection rates and clinical manifestations.

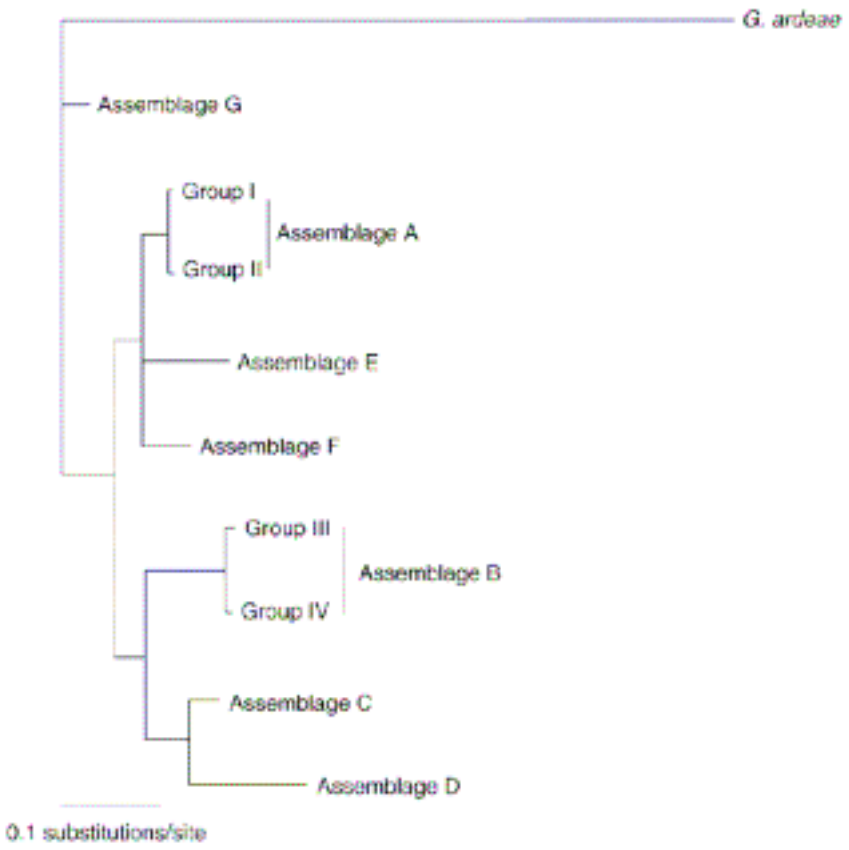


Figure 1-3: Genetic relatedness amongst *Giardia lamblia* Assemblages, based on sequences of the 18s rRNA gene (Monis *et al*., 2009).

The two major Assemblages of *G. lamblia* that infect humans are so different genetically and biologically, that they may warrant separate species or subspecies designations (Monis *et al*., 2009; Aurrecoechea *et al*., 2009) (See Table 2-5). Furthermore, some of the Assemblages are more closely related to *G.muris* than to one another, particularly for Assemblage A and B. Monis, Thompson and Caccio have proposed species designations for all of the Assemblages, based largely on their differences of host specificity (2009). Pursuing this evidence of differences amongst Assemblage A and B phenotypes, a whole genome scan comparing Assemblage A and B reference isolates (isolates WB and H3) has indicated sufficient genetic difference to suggest novel classification of the Assemblages into separate species, according to the study's authors (Frazen *et al*., 2009).

Table 1-1-5: Proposed reclassification *of G.lamblia* Assemblages into species, by Monis *et al* (2009).

Genus	Species	Assemblage
Giardia	*duodenalis*	A
	enterica	B
	canis	C/D
	bovis	E
	cati	F
	simondi	G

1.4.5 *Giardia lamblia* Ecology and Epidemiology

Transmission of *Giardia lamblia* occurs via the fecal-oral route, following direct or indirect contact with *G.lamblia* cysts. Transmission to humans can be through person-to-person transfer, animal-to-person (zoonotic), waterborne or foodborne. Animal-to-animal transmission cycles are extremely important in the dispersion and ecology of this organism. Furthermore, there is evidence of human-to-animal (anthrozoonotic) transmission in free-ranging mountain gorillas of Uganda (Graczyk *et al*, 2002).

Waterborne transmission of *Giardia* can occur via consumption of treated water, consumption of raw water, contact with recreational water (surface water or chlorinated water, including pools, beaches and water parks) or use of contaminated water for food preparation or bathing. Water sources may be contaminated through point-sources (i.e. wastewater effluent) or non-point sources (stormwater and overland flows). Typical sources of *Giardia* contamination in water include wastewater effluent, septic tank leaks, overland flow from manure piles and direct inputs from animal grazing near water sources. It is difficult to determine the percent attribution of each of these sources to *G.lamblia* contamination, particularly for non-point sources.

Even in pristine areas without human impact, pathogen loadings can be significant from wildlife (Hansen and Ongerth, 1991). Pathogen shedding from an infected host can be extremely high during early stages of infection and shedding may occur in non-symptomatic hosts. It is estimated that an

infected calf can shed up to 2.6 x 10^{10} cysts per kilogram of feces (Bradford and Schivjen, 2002). An infected human host can shed up to 1 x 10^{6} cysts per day.

Giardia infections in domestic dogs are common and the potential for transmission to a human host depends on if the animal is carrying a canine-specific isolate (belonging to Assemblages C or D) or a zoonotic isolate (Assemblages A or B). Carriage of zoonotic versus canine-specific *G.lamblia* strains is dependent on the transmission ecology in the area. Early studies, without genotyping, found that 10% of well-cared for dogs and up to 100% of dogs in breeding facilities carried *Giardia* (Hahn *et al*., 1988). A more recent study in Belgium found a similar rate; *Giardia* was the most commonly detected parasitic infection in dogs and was detected in 9.3% of domestic dogs (Claerebout *et al*., 2009).

Similarly, *G.lamblia* infection is common in cattle which may be infected with either a livestock-specific Assemblage (Assemblage E) or a zoonotic Assemblage (Assemblage A). The proportion of Assemblage A and Assemblage E found in cattle herds differs from study to study. Appelbee *et al* (2003) found that *G.lamblia* prevalence ranged from 7- 60% on farms in Alberta, Canada, with Assemblage E being the predominant genotype. Trout *et al*. (2005) found a *Giardia* prevalence of 52% in dairy cows and found Assemblages A and E in 13% and 87%, respectively, of the samples collected from farms in Eastern United States, respectively. In contrast, two studies of dairy calves in New Zealand have detected only Assemblage A (Hunt *et al*, 2000; Winkworth *et al*., 2008).

The range of other organisms infected by *G.lamblia* is wide. *G.lamblia* has been detected in goats and sheep (Castro-Hermida *et al*., 2007), pigs (Maddox-Hyttel *et al*., 2006), deer (Lalle *et al*., 2007; Trout *et al*., 2003), wild moose and reindeer (Robertson *et al*., 2007), muskrat (Bitto and Aldras, 2009), beaver (Wallis *et al*, 1984), mountain gorillas (Nizeyi *et al*. 1999), ferrets (Abe *et al*, 2005) and wild ducks (Kuhn *et al*, 2002), to name a few. Furthermore, it has been shown that flies can serve as a transmission vector for the parasite (Szostakowska *et al*., 2004). An average of 5.9 cysts was found on the exoskeleton of wild-caught synanthropic flies collected from a dairy farm and a municipal landfill; of these cysts, 4.8 were found to be viable (81%).

In the United States, giardiasis occurs more frequently in the summer and early fall, with almost a two-fold increase in the summer months (Yoder and Beach, 2007). This is not surprising, as swimming, camping and other recreational activities are all risk factors for acquiring giardiasis (see Table 2-6). Travel, contact with infected persons and consumption of contaminated recreational or

drinking water are major risk factors in developing giardiasis. Consumption of lettuce (and likely other non-skinned raw fruits and vegetables) has also been identified as a risk factor, suggesting that contaminated irrigation water is a concern (Caccio *et al*,. 2005). Giardiasis typically occurs more frequently in children ages 1-9 and adults ages 30-39, slightly skewed towards the male population (Yoder and Beach, 2007; Caccio *et al*., 2005). In the developed world, giardiasis occurs more frequently in northern geographical areas, including Canada, Scandinavia and the northern United States.

Table 1-1-6: Risk factors for acquiring giardiasis in North America (Yoder and Beach, 2007)

Rank	Risk Factors
1	Travelers to disease-endemic areas
2	Children in childcare
3	Close contact with infected persons
4	Ingestion of contaminated drinking water
5	Ingestion of contaminated recreational water
6	Taking part in outdoor activities such as camping
7	Contact with infected animals
8	Men who have sex with men

1.4.6 *Giardia* Detection in Water

The most widely utilized approach for detecting *Giardia* cysts in water supplies is Method 1623, developed by the US Environmental Protection Agency (USEPA) to simultaneously detect both *Giardia* and *Cryptosporidium* from a single sample using the same reagents (2005)

This method begins with filtration of large volumes of water through an EPA-approved filter (FiltaMax by IDEXX or Envirochek capsule by Pall) (Figure 1-4). The filtration volume is largely determined by the turbidity of the water and the type of water; up to 1000L of treated water and up to 50L of raw (untreated) water can be filtered. The filtrate is eluted from the filter and the resulting eluate is centrifuged to produce a pellet. The pellet is purified by immunomagnetic separation, using antibodies directed against all members of *Giardia* and *Cryptosporidium* genera which are conjugated to magnetic beads. This step allows the specific removal of these organisms from other organisms,

organic material and particulate matter co-concentrated during the filtration step. The organisms are then enumerated using indirect immunofluorescent microscopy, again using antibodies directed against all members of *Giardia* and *Cryptosporidium* genera. The results are confirmed using 4'-6-diamidino-2-phenylindole (DAPI) to confirm nuclei and differential interference contrast (DIC) microscopy to confirm internal structures for identification.

Figure 1-4: Method 1623. A: Filtration using IDEXX's FiltaMax; B: Immunomagnetic Separation (Dynabeads GC Combo, Dynal/Invitrogen); C: Indirect immunofluorescence assay (Easy-Stain, Biotechnology Frontiers). *Giardia* cysts are shown on the right; the larger ovoid structures (smaller structures are *Cryptosporidium* oocysts)

Of particular concern for public health officials and drinking water purveyors alike is that the antibodies used in both the IMS and IFA steps of Method 1623 are directed against all members the *Giardia* and *Cryptosporidium* genera. This is despite the fact that only a subset of *Giardia lamblia* genotypes and *Cryptosporidium* species are known to infect humans (Caccio *et al*., 2005). The remaining genotypes and species are tightly adapted to specific hosts and have not yet crossed the animal-human barrier, thus providing no threat to human health. Furthermore, the antibodies used in the immunofluorescence microscopy step for parasite detection may also cross-react with algal species frequently found in surface water supplies, resulting in possible false positive test results (Rodgers, 1998). Many of these inadequacies may be addressed by applying new molecular methods, which have greater specificity and provide additional, more reliable information for both watershed and public health interventions.

Use of this method is legislated for ongoing water testing in the United States and a similar method is employed by the Drinking Water Inspectorate in the United Kingdom (USEPA 2005; DWI, 1999). Other nations, such as Canada and Australia, have opted against legislating *Giardia* and *Cryptosporidium* testing due to the lack of reliable and sensitive detection methods (Health Canada, 2004). Despite the lack of regulations, some larger municipalities in these countries have adopted routine testing by Method 1623 for these organisms nonetheless. In Western Canada, this includes Metro Vancouver Region and Victoria in British Columbia, and Calgary and Edmonton in Alberta.

Method 1623 is labour intensive, requires highly trained staff and results in unreliable, often low recoveries. Furthermore, the method is cost-prohibitive, with reagent and labour costs approaching $750 (CND) per test. Lastly, it does not provide a full scope of information needed to make public health decisions. Supplementation of Method 1623 with molecular analyses has proven to be useful from the perspective of confirming the presence of a potential human-infective species, as well as for studying *Giardia* and *Cryptosporidium* ecology.

1.4.7 *Giardia* and Drinking Water Treatment

Giardia can be removed most effectively from contaminated water supplies through a combination of flocculation, filtration and disinfection. For safe drinking water, a *Giardia* removal of 99.9% (three log) through drinking water treatment is desired (USEPA, 2005; Health Canada, 2004). In a typical drinking water plant, two log reduction is achieved by conventional treatment (coagulation, sedimentation and filtration) while the final log reduction is achieved through disinfection (chlorination or UV disinfection) (Betancourt and Rose, 2004). The physical removal of *Giardia* from water is complicated by its small size and filtration remains as the best barrier to cyst removal. Effective filtration of *Giardia* from water supplies requires properly functioning filters and adequate coagulation and flocculation conditions.

Giardia and *Cryptosporidium* are typically referred to as chlorine resistant; this is true of *Cryptosporidium* but is misleading with respect to *Giardia*. *Giardia* is moderately sensitive to chlorine disinfection; however, it requires high chlorine concentrations and long contact times for disinfection, which may not be possible in typical water treatment facilities. Furthermore, disinfection success is greatly impacted by water temperature. In a study by Jarroll *et al* (1981), cysts were destroyed at 25°C after exposure to 1.5mg/L chlorine for 10 minutes. However, at 5°C (more typical

of water supplies), 2mg/L chlorine killed cysts after 60 minutes of exposure at pH 6 and 7, but not pH 8. Disinfection with a contact time of 10 minutes was only achieved with chlorine concentrations of 8mg/L at pH 6 and 7 and was not achievable at pH 8. *Giardia* is sensitive to both UV disinfection and ozonation (Belosevic *et al*, 2001; Haas and Kaymak, 2003).

1.4.8 Giardiasis Outbreaks in Water

While waterborne outbreaks of giardiasis are likely to have occurred for centuries, the first documentation of a suspected outbreak of waterborne giardiasis occurred in a Tokyo apartment complex in 1946 (Craun, 1979). Attribution of this outbreak to a water source was through epidemiological analysis. Similarly, the largest suspected waterborne outbreak of giardiasis (50,000 suspected cases) occurred in Portland, Oregon in 1954-1955, but the organism could not be detected in the suspect water source (Hunter, 1997). It is hypothesized that this outbreak was due to contamination of the source water and exacerbated by limited drinking water treatment (disinfection only). The first confirmed case of waterborne giardiasis occurred in Tennessee in 1973, when a 44-year old patient with severe giardiasis reported frequent visits to her parents' farm (Craun 1979; Hunter 1997). According to the publications, analysis of the farm's water supply (underground rain-water collecting cistern) revealed numerous trophozoites, which according to current knowledge appears to be unlikely due to the delicate nature of trophozoites. However, confirmation of infection in five other farm residents consuming the same water source is the best evidence that this is the first documented waterborne outbreak of giardiasis.

Worldwide, there have been 108 waterborne outbreaks of giardiasis documented in peer-reviewed journals, although this number likely does not reflect the true number of waterborne outbreaks. Many outbreaks may not have been documented in peer-reviewed journals, particularly those that occurred prior to 1990s. Furthermore, outbreaks may have been missed by public health officials, particularly those that occurred in earlier years. Of these outbreaks, the majority were attributed to surface water supplies and inadequate treatment, combined with contamination, were the main factors responsible for waterborne outbreaks.

The most recent, large-scale outbreak of waterborne giardiasis attributed to a municipal water supply occurred in Bergen, Norway in 2004 (Nygard *et al*., 2006). More than 1300 laboratory confirmed cases were documented and an estimated 2500 individuals became infected and symptomatic. The

outbreak appears to be the result of a combination of inadequate water treatment and cross-connections of the drinking water distribution systems with sewage pipes.

1.5 Drinking Water and Public Health in Canada

1.5.1 Legislative Framework

In Canada, drinking water quality legislation and regulations are mandated at the provincial level. The Canadian Council of Ministers of the Environment (CCME) suggest drinking water guidelines at the federal level, however, it is at the discretion of each province and territory to interpret and amend these guidelines and include them in provincial/territorial legislation and regulations. As such, water quality legislation and regulations differ significantly across Canada.

British Columbia is one of few jurisdictions worldwide that consider drinking water to be a public health issue, rather than an environmental or sanitation issue only. Until 1992, the Sanitation Regulation in the Health Act regulated drinking water quality in the province. The *Safe Drinking Water Act* was implemented in 1992 to regulate waterworks systems and to ensure that water suppliers were providing water that was of adequate quality for human consumption (potable). The legislation was further changed in 2003, when the *Drinking Water Protection Act* and the *Drinking Water Protection Regulation* came into effect. These new regulations continue to address drinking water quality as a public health issue, but also emphasize source water assessments, source protection, and the multi-barrier approach as crucial aspects of the legislation (BC Ombudsman, 2008).

Under the most current legislation, any surface water system containing more than two connections must adhere to the *Drinking Water Protection Regulations*. The regulations require, at a minimum, disinfection (chlorine or other approved disinfection), along with regular water quality surveillance for bacterial indictors. Currently, under the *Drinking Water Protection Act* and *Regulation*, groundwater disinfection is discretionary. This, however, may change with the expected creation of BC groundwater legislation

In British Columbia, the majority of residents are served by public surface water supplies (76%), while 24% are served by private or public groundwater sources (PHO, 2001) (Figure 1-5). An inventory conducted by the BC Office of the Public Health Officer in 2000 found that there were 3,016 known water systems in the province and this number is likely to increase as more water

systems are identified and reported (PHO, 2001). Two large suppliers (Metro Vancouver Water District and Capital Regional District in Victoria) supply more than 50% of BC residents with drinking water. However, more than two-thirds of water systems in BC are small water systems (with two to fourteen connections) and it is often these water supplies that are under-monitored and at greatest risk of causing waterborne illness.

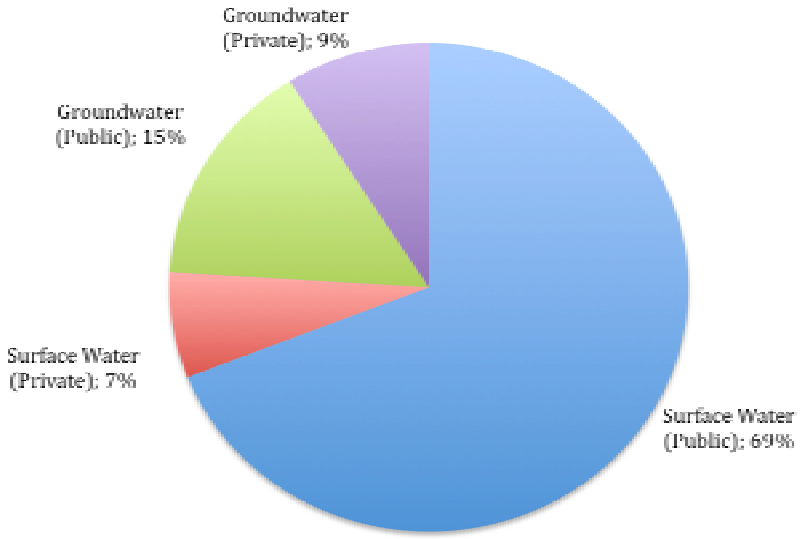


Figure 1-5: Distribution of drinking water sources in British Columbia. Source: Provincial Health Officer, 2001.

1.5.2 Waterborne Outbreaks of Disease in British Columbia

In British Columbia, there is a perception that water supplies are abundant, and pristine. Despite this perception, there is evidence of significant health risks associated with BC water supplies, as demonstrated by 29 documented outbreaks of waterborne diseases since 1980 (PHO, 2001) (see Table 2). These outbreaks occurred over a large geographical range, associated with both large and small drinking water systems, with varying types of treatment. In all but one outbreak (Princeton, BC), the water originated from either a surface water supply or a mixture of surface water and groundwater. In British Columbia, groundwater utilization is lower in comparison to other provinces; 24% of residents rely on groundwater sources for drinking water, compared to 30.3% in the rest of Canada (PHO,

2001). Because of the nature of groundwater, having natural filtration and serving smaller groups of residents, outbreaks of gastrointestinal illness due to groundwater are less likely to occur. In most of the outbreaks, there was evidence of animal contamination, such as the outbreak in Creston BC where a beaver was found residing in the drinking water intake culvert. This emphasizes the role of animal-to-human (zoonotic) transmission, rather than human-to-human (anthropogenic) transmission. Lastly, while conclusive evidence of treatment deficiencies is not available for all outbreaks, there is a common theme of insufficient drinking water treatment given the etiological agent of the outbreak or no drinking water treatment at all. This is in contrast to evidence of outbreaks in North America and Europe, where treatment breakdown, rather than insufficient treatment, is the most commonly associated with waterborne outbreaks (Hrudey and Hrudey, 2007).

The majority of the documented outbreaks in BC were attributed to protozoa (17 outbreaks or 59% of all the outbreaks), with *Giardia* being the most common etiological agent (13 outbreaks), followed by *Campylobacter* (6 outbreaks) and *Cryptosporidium* (3 outbreaks) (Table 1-1-7). This pattern is consistent with patterns observed in North America and Europe and reflects the ubiquity of protozoa in the environment and their resistance to typical treatment approaches (Craun *et al.,* 2006; Hrudey and Hrudey, 2007). These documented outbreaks resulted in 1,734 laboratory confirmed cases of disease, which like have had significant economic and societal impacts on the residents of BC (Corso *et al*., 2003; Baker *et al*., 1979). This is likely an underestimation of the true number of infections, as only a small percentage of infected patients seek medical attention for gastroenteritis. A study by Majowicz *et al*. (2006), suggested that for every diagnosed case of gastroenteritis in Ontario, there are one hundred undiagnosed cases in the community. It is estimated, at current costs that a single case of gastroenteritis in British Columbia has a mean annual cost of CAN$1,342.57 in direct costs, indicating a significant financial burden of infectious diseases (Henson *et al*, 2008). It should be noted, however, that the majority of cases of gastrointestinal illness are foodborne. Furthermore, it is difficult to determine the impact of waterborne pathogens on endemic illness (non-outbreak related) and outbreaks only represent acute incidents of waterborne gastroenteritis (Colford *et al*., 2006).

Table 1-1-7: Documented outbreaks of waterborne illness in British Columbia, since 1980

Year	Location	Etiological Agent	Lab Confirmed (Epid Estimate)	Suspected Source	Water Source	Notes
1980	Nakusp	*Campylobacter*	12 (800)	Wildlife	Surface	
1981	100 Mile House	*Giardia*	69	Beaver	Surface	Negative coliform (total and fecal) tests
1982	Kimberly	*Giardia*	not available	Wildlife	Surface	Preventable: better source protection and treatment
1984	Chilliwack	*Salmonella*	82	Human sewage	Surface/groundwater	
1985	Creston	*Giardia*	72	Beaver	Surface	Preventable: better source protection and treatment
1986	Penticton	*Giardia*	362	Beaver	Surface/groundwater	Insufficient treatment
1987	Penticton	*Giardia*	109 (3,125)	Beaver	Surface/groundwater	Insufficient treatment
1987	Black Mountain	*Giardia*	60	Wildlife/cattle	Surface	Insufficient treatment
1988	Kamloops	*Campylobacter*	not available	Wildlife	Surface	
1990	Near Lytton	*Salmonella*	not available	Wildlife	Surface (spring fed)	Caused by human error
1990	Kitimat	*Giardia*	28	Beaver	Surface	Preventable: better source protection and treatment
1990	Creston	*Giardia*	130	Wildlife	Surface	
1990	Fernie	*Giardia*	50	Wildlife	Surface (spring fed)	Preventable: better source protection and treatment
1990	West Trail/Rossland	*Giardia*	>40	Wildlife	Surface	
1990	Matsqui	*Not ID*	organisms not ID	Unknown	Surface	
1991	Barriere	*Giardia*	25		Surface	
1991	Granisle	Not ID	organisms not ID	Unknown	Surface	No chlorination, likely preventable with treatment
1991	Fort Fraser	Not ID	organisms not ID	Unknown	Surface	
1992	Kaslo	*Campylobacter*	10	Wildlife	Surface	
1993	Fernie	*Campylobacter*	35	Cattle	Surface	
1995	Victoria	*Toxoplasma*	110 (3,000)	Cats/cougar	Surface	Poor reservoir/intake design (now decommissioned)
1995	Revelstoke	*Giardia*	62	Beaver/wildlife	Surface	Chlorination, problems with source.
1995	Revelstoke	*Campylobacter*	71	Beaver/wildlife	Surface	
1996	Cranbrook	*Cryptosporidium*	29 (2,097)	Cattle	Surface	Likely cattle contamination, although source never identified
1996	Kelowna	*Cryptosporidium*	177 (10,000)	Presumed human	Surface	
1996	Valemount	*Giardia*	10	Wildlife	Surface	
1997	Princeton	Unidentified	146	Human sewage	Groundwater	Likely Norovius
1998	Chilliwack	*Cryptosporidium*	19	Cattle	Surface/groundwater	
1998	Camp Malibu	*Campylobacter*	26	Wildlife	Surface	Water not chlorinated; campers were not informed of the risk.

1.5.3 Boil Water Advisories in British Columbia

In addition to documentation of waterborne outbreaks of disease, boil water advisories (BWA) may be used as an indicator of poor water quality and infrastructure deficiencies. When water quality exceeds the acceptable level of indicator organisms, contains excess organic material or, for any other reason such as known treatment inadequacy, is considered risk to consume, a BWA is issued (DWPR, 2009). A recent analysis of boil water advisories across Canada found 1,766 boil water advisories across Canada; the majority of these advisories were located in Ontario and British Columbia (Edgerton, 2008). As of February 2009, there were 275 active boil water advisories in British Columbia, the majority of which were on drinking water systems located within the Interior Health Authority (see Table 1-1-8).

Table 1-1-8: Distribution and length of boil water advisories (BWA) in British Columbia, February 2009.

Health Authority	No. BWA (%)	Length of BWA		
		<1 year	>5 years	>10 years
Interior Health	124 (45.1%)	7 (14.9%)	96 (52.2%)	45 (70.3%)
Fraser Health	16 (5.8%)	4 (8.5%)	10 (5.4%)	4 (6.3%)
Coastal Health	32 (11.6%)	15 (31.9%)	9 (11.6%)	5 (7.8%)
Vancouver Island	57 (20.7%)	14 (29.8%)	15 (8.2%)	5 (7.8%)
Northern Health	46 (16.7%)	7 (14.9%)	18 (9.8%)	5 (7.8%)
Totals	**275**	**47**	**184**	**64**

A significant number of BWAs in BC have been in place for over five years (66.9%), while a number of boil water advisories have been in place for over ten years (23.3%). The longest standing BWA in the province is located in the community of Edmont, located on the Sechelt Inlet on the southern mainland coast of British Columbia. This BWA has been in place for over nineteen years. BWAs are intended to bring a short-term resolution to a water quality issues. Longer-term advisories are, therefore, suggestive of inadequate infrastructure for the protection of public health. Overall, BWAs are a broad indicator of regulatory compliance and suggestive of overall inadequacies of some of BC's water supplies in the provision of water deemed potable by public health officials.

1.5.4 Giardiasis in British Columbia

Giardiasis is the most common parasitic infection worldwide. In British Columbia, it is the third leading cause of gastroenteritis, third only to Norovirus and *Campylobacter* infections (BCCDC, 2009). In 2008, there were 17.33 cases of giardiasis for every 100,000 people and while this is a declining trend, it is still elevated compared to the national average. Giardiasis rates are not evenly distributed throughout the province; rates were highest in Vancouver, a highly urban area (26.4 cases per 100,000) and Kootenay Boundary, a much more rural community (24.5 cases per 100,000) (Figure 1-6). Giardiasis rates in BC do not show the same seasonal variability observed in other geographical areas, peaking in mid-September rather than in summer months.

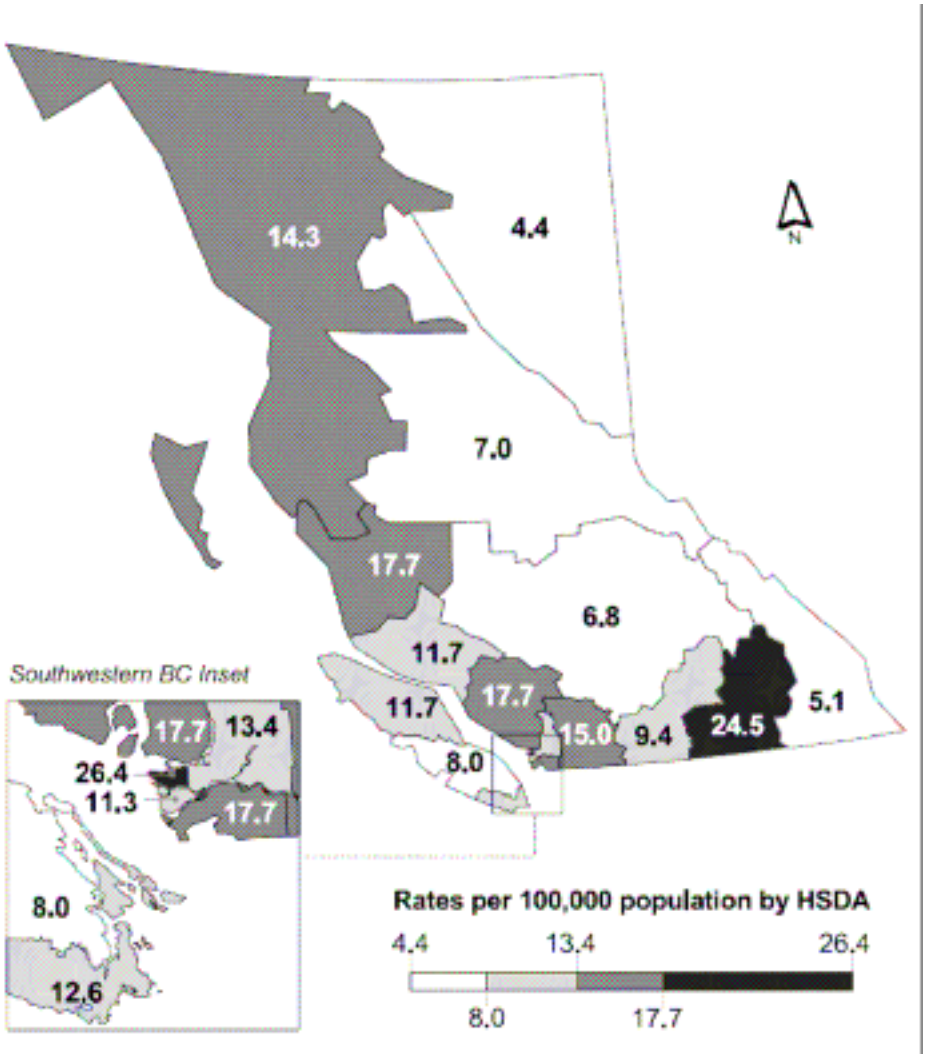


Figure 1-6: Rates of giardiasis in British Columbia in 2008, according to health service delivery areas (HSDA). Source: BCCDC, 2009

As with all infectious diseases, incidence data for giardiasis is expected to be a vast underestimation of the true community incidence. Reported cases of communicable disease are suggested to be the 'tip of the iceberg' in comparison to actual numbers of cases occurring in a community (Straif-Bourgeois and Ratard, 2007). This is partly due in giardiasis to the fact that not all infected persons become symptomatic and many symptomatic individuals do not seek medical attention. Furthermore, even if an infected individual seeks medical attention, laboratory tests may not be ordered to confirm infectious agent. Lastly, there is a chance that reported cases may not be recorded and forwarded to public health officials.

It still has not been elucidated why British Columbians experience a greater incidence of giardiasis than the rest of Canada. During early disease investigation, it appeared that many cases were attributed to waterborne outbreaks of giardiasis. However, there have not been any documented outbreaks in BC of waterborne giardiasis since 1996. It has been speculated that some of the observed decrease in giardiasis incidence could be attributed increased compliance with drinking water treatment regulations. Investigations into the prevalence and environmental sources of *G.lamblia* may provide information regarding transmission and ecology of the organism in BC and could elucidate reasons to the continued difficulties with giardiasis in BC.

1.6 References

Abe N, Read C, Thompson RC and Iseki M. 2005. Zoonotic genotype of *Giardia intestinalis* detected in a ferret. Journal of Parasitology. 91(1): 179-182.

Adam, RD. 2001. Biology of *Giardia lamblia.* Clinical Microbiology Reviews. 14(3): 447-475.

Adam RD. 2000. The *Giardia lamblia* genome. International Journal for Parasitology. 30(4): 475-484.

Adam RD. 1992. Chromosome-size variation in Giardia lamblia: the role of rDNA repeats. Nucleic Acids Research. 20(12): 3057-3061.

Adam RD, Nash TE and Wellems TE. 1998. The *Giardia lamblia* trophozoite contains sets of closely related chromosomes. Nucleic Acids Res. 16(10): 4555-4567.

Amar CF, Dear PH, Pedraza-Díaz S, Looker N, Linnane E and McLauchlin J. 2002. Sensitive PCR-restriction fragment length polymorphism assay for detection and genotyping of *Giardia duodenalis* in human feces. Journal of Clinical Microbiology. 40(2):446-452.

Appelbee AJ, Frederick LM, Heitman TL and Olson ME. 2009. Prevalence and genotyping of *Giardia duodenalis* from beef calves in Alberta, Canada. Veterinary Parasitology. 112(4): 289-294.

Aurrecoechea C, Brestelli J, Brunk BP, Carlton JM, Dommer J, Fischer S, Gajria B, Gao X, Gingle A, Grant G, Harb OS, Heiges M, Innamorato F, Iodice J, Kissinger JC, Kraemer E, Li W, Miller JA, Morrison HG, Nayak V, Pennington C, Pinney DF, Roos DS, Ross C, Stoeckert CJ Jr, Sullivan S, Treatman C and Wang H. 2009. GiardiaDB and TrichDB: integrated genomic resources for the eukaryotic protist pathogens *Giardia lamblia* and *Trichomonas vaginalis*. Nucleic Acids Research. 37(database issue): D526-D530.

Baker EL Jr, Peterson W, Von Allmen S and Fleming J. 1979. Economic impact of a community-wide waterborne outbreak of gastrointestinal illness. American Journal of Public Health. 69(5): 501-502.

Baruch AC, Isaac-Renton J and Adam RD. 1996. The molecular epidemiology of *Giardia lamblia*: a sequence-based approach. Journal of Infectious Disease. 74(1): 233-236.

BC Ombudsman. 2008. Fit to Drink: Challenges in Providing Safe Drinking Water in British Columbia. British Columbia Office of the Ombudsman.

BC Reg 200/2003. Drinking water protection regulation. Province of British Columbia.

Belosevic M, Craik SA, Stafford JL, Neumann NF, Kruithof J and Smith DW. 2001. Studies on the resistance/reactivation of *Giardia muris* cysts and *Cryptosporidium parvum* oocysts exposed to medium-pressure ultraviolet radiation. FEMS Microbiology Letters. 204(1): 197-203.

Betancourt WQ and Rose JB. 2004. Drinking water treatment processes for removal of *Cryptosporidium* and *Giardia*. Veterinary Parasitology. 126(1-2): 219-234.

Binz N, Thompson RC, Lymbery AJ, Hobbs RP. 1992. Comparative studies on the growth dynamics of two genetically distinct isolates of *Giardia duodenalis* in vitro. International Journal for Parasitology. 22(2): 195-202.

Birky Jr, CW. 2005. Sex: is *Giardia* doing it in the dark? Current Biology. 15(2): R56-R58

Bitto A and Aldras A. 2009. Prevalence of *Giardia* and *Cryptosporidium* in muskrats in northeastern Pennsylvania and New Jersey. Journal of Environmental Health. 71(8): 20-26.

Bowie WR, King AS, Werker DH, Isaac-Renton JL, Bell A, Eng SB, Marion SA and the BC Toxoplasma Investigation Team. 1997. Outbreak of toxoplasmosis associated with municipal drinking water. Lancet. 350(9072): 173-177.

Bradford SA and Schijven J. 2002. Release of *Cryptosporidium* and *Giardia* from dairy calf manure: impact of solution salinity. Environmental Science and Technology. 36(18): 3916-3923.

British Columbia Centre for Disease Control (BCCDC). 2009. British Columbia Annual Summary of Reportable Disease. Provincial Health Services Authority. Vancouver, British Columbia.

Byrd J, Xu H and Colwell R. 1991. Viable but nonculturable bacteria in drinking water. Applied and Environmental Microbiology. 57(3): 875-878.

Cacciò SM, Thompson RC, McLauchlin J and Smith HV. 2005. Unraveling *Cryptosporidium* and *Giardia* epidemiology. Trends in Parasitology. 21(9): 430-437.

Cacciò SM, De Giacomo M and Pozio E. 2002. Sequence analysis of the beta-giardin gene and development of a polymerase chain reaction-restriction fragment length polymorphism assay to genotype Giardia duodenalis cysts from human faecal samples. International Journal of Parasitology. 32(8):1023-30.

Castro-Hermida JA, Almeida A, González-Warleta M, Correia da Costa JM, Rumbo-Lorenzo C and Mezo M. 2007. Occurrence of *Cryptosporidium parvum* and *Giardia duodenalis* in healthy adult domestic ruminants. Parasitology Research. 101(5): 1443-1448.

Campbell SR, van Keulen H, Erlandsen SL, Senturia JB and Jarroll EL. 1990. *Giardia* sp.: comparison of electrophoretic karyotypes. Experimental Parasitology. 71(4): 470-82.

Chauret C, Nolan K, Chen P, Springthorpe S and Sattar S. 1998. Aging of *Cryptosporidium parvum* oocysts in river water and their susceptibility to disinfection by chlorine and monochloramine. Canadian Journal of Microbiology. 44(12): 1154-1160.

Claerebout E, Casaert S, Dalemans AC, De Wilde N, Levecke B, Vercruysse J and Geurden T. 2009. *Giardia* and other intestinal parasites in different dog populations in Northern Belgium. Veterinary Parasitology. 161(1-2): 41-46.

Colford, JM, Roys, S Beach, MJ, Hightower A., Shaw SE and Wade TJ. 2006. A review of household drinking water trials and an approach to the estimation of endemic waterborne gastroenteritis in the United States. Journal of Water Health. 4(Suppl 2): 71-88.

Cooper M, Adam R, Worobey M and Sterling C. 2007. Population genetics provides evidence for recombination in *Giardia*. Current Biology. 17(22): 1984-1988.

Corso PS, Kramer M, Blair K, Addiss, D., Davis, J. and Haddix A. 2003. Cost of illness in the 1993 waterborne *Cryptosporidium* outbreak, Milwaukee, Wisconsin. Emerging Infectious Diseases. 9(4): 426-431.

Craun, MF, Craun FG, Calderon RL and Beach MJ. 2006. Waterborne outbreaks in the United States. Journal of Water and Health. 4(Suppl 2): 19-30.

Craun GF. 1979. Waterborne giardiasis in the United States: a review. American Journal of Public Health. 69(8): 817-819.

Drinking Water Inspectorate (DWI). 1999. Guidance on assessing risk from *Cryptosporidium* oocysts in treated water supplies to satisfy the Water Supply (Water Quality) (Amendment) Regulations. 1999. SI 1524. Department of the Environment, Transport and the Regions, London.

Farthing MJ. 1993. Pathogenesis of giardiasis. Transactions of the Royal Society of Tropical Medicine and Hygiene. 87(Supp 3): 17-21.

Ferguson CM, Altavilla N, Ashbolt NJ and Deere DA. 2003. Prioritizing watershed pathogen research. Journal of the American Waterworks Association. 95(2): 92–102.

Franzén O, Jerlström-Hultqvist J, Castro E, Sherwood E, Ankarklev J, Reiner DS, Palm D, Andersson JO, Andersson B and Svärd SG. 2009. Draft genome sequencing of *Giardia intestinalis* Assemblage B isolate GS: is human giardiasis caused by two different species? PLoS Pathogens. 5(8): e1000560.

Garcia, LS. 2001. Diagnostic Medical Parasitology. ASM Press, Washington, DC.

Graczyk TK, Bosco-Nizeyi J, Ssebide B, Thompson RC, Read C and Cranfield MR. 2002. Anthropozoonotic *Giardia duodenalis* genotype (Assemblage) A infections in habitats of free-ranging human-habituated gorillas, Uganda. Journal of Parasitology. 88(5): 905-909.

Gray, F. 1999. Water technology: an introduction for scientists and engineers. Wiley, New York City, New York.

Haas CN and Kaymak B. 2003. Effect of initial microbial density on inactivation of *Giardia muris* by ozone. Water Research. 37(12): 2980-2988.

Hahn NE, Glaser CA, Hird DW and Hirsh DC. 1988. Prevalence of *Giardia* in the feces of pups. Journal of the American Veterinary Medical Association. 192(10): 1428-1429.

Hamza I, Jurzik L, Stang A, Sure K, Uberla K and Wilhelm M. 2009. Detection of human viruses in rivers of a densely-populated area in Germany using a virus adsorption elution method optimized for PCR analyses. Water Research. 43(10): 2657-2668.

Hansen J and Ongerth J. 1991. Effects of time and watershed characteristics on the concentration of *Cryptosporidium* oocysts in river water. Applied and Environmental Microbiology. 57(10): 2790-2795.

Health Canada. 2004. Canadian Guidelines for Drinking Water Quality: Supporting Documentation. Protozoa: *Giardia* and *Cryptosporidium*. Water Quality and Health Bureau, Health Canada. Ottawa, Canada.

Henson SJ, Majowicz SE, Masakure O, Sockett PN, MacDougall L, Edge VL, Thomas MK, Fyfe M, Kovacs SJ and Jones AQ. 2008. Estimation of the costs of acute gastrointestinal illness in British Columbia, Canada. International Journal of Food Microbiology. 127(1-2): 43-52.

Hill V, Polaczyk A, Kahler A, Cromeans T, Hahn D and Amburgey J. 2009. Comparison of hollow-fiber ultrafiltration to the USEPA VIRADEL technique and USEPA Method 1623. Journal of Environmental Quality. 38(2): 822-825.

Homan WL and Mank TG. 2001. Human giardiasis: genotype linked differences in clinical symptomatology. International Journal for Parasitology. 31(8): 822-826.

Homan WL, Gilsing M, Bentala H, Limper L and van Knapen F. 1998. Characterization of *Giardia duodenalis* by polymerase-chain-reaction fingerprinting. Parasitology Research. 84(9):707-714.

Homan WL, van Enckevort FH, Limper L, van Eys GJ, Schoone GJ, Kasprzak W, Majewska AC and van Knapen F. 1992. Comparison of *Giardia* isolates from different laboratories by isoenzyme analysis and recombinant DNA probes. Parasitology Research. 78(4): 316-323.

Hörman A, Rimhanen-Finne R, Maunuzla L, von Bonsdorff C, Torvela N, Heikinheimo A and Hänninen M. 2004. *Campylobacter* spp., *Giardia* spp., *Cryptosporidium* spp., noroviruses, and indicator organisms in surface water in southwestern Finland, 2000-2001. Applied and Environmental Microbiology. 70(1): 87-95.

Hrudey SE and Hrudey EJ. 2007. Published case studies of waterborne disease outbreaks- evidence of recurrent threat. Water Environment Research. 79(3): 234-245.

Hunt CL, Ionas G and Brown TJ. 2000. Prevalence and strain differentiation of *Giardia intestinalis* in calves in the Manawatu and Waikato regions of North Island, New Zealand. Veterinary Parasitology. 91(1-2): 7-13.

Hunter PR. 1997. Waterborne Disease: Epidemiology and Ecology. Wiley, Chichester, United Kingdom.

Isaac-Renton JL, Cordeiro C, Sarafis K and Shahriari H. 1993. Characterization of *Giardia duodenalis* isolates from a waterborne outbreak. J Infect Dis. 167(2): 431-440.

Jarroll EL, Bingham AK and Meyer EA. 1981. Effect of chlorine on *Giardia lamblia* cyst viability. Applied and Environmental Microbiology. 41(2): 483-487.

Kabnick, KS and Peattie DA. 1991. *Giardia*: A missing link between prokaryotes and eukaryotes**.** American Scientist. 79(1): 34-43.

Karim MR, Rhodes ER, Brinkman N, Wymer and Fout GS. 2009. New electropositive filter for concentrating enteroviruses and noroviruses from large volumes of water. Applied and Environmental Microbiology. 75(8): 2393-2399.

Kothary M and Babu U. 2001. Infective dose of foodborne pathogens in volunteers: a review. Journal of Food Safety. 21(1): 49-73.

Krewski D, Balbus J, Butler-Jones D, Haas C, Isaac-Renton J, Roberts KJ and Sinclair M. 2002. Managing health risks from drinking water--a report to the Walkerton inquiry. Journal of Toxicology Environmental Health A. 65(21): 1635-1823.

Kuhn RC, Rock CM and Oshima KH. 2002. Occurrence of *Cryptosporidium* and *Giardia* in wild ducks along the Rio Grande River valley in southern New Mexico. Applied Environmental Microbiology. 68(1): 161-165.

Lalle M, Frangipane di Regalbono A, Poppi L, Nobili G, Tonanzi D, Pozio E and Cacciò SM. 2007. A novel *Giardia duodenalis* assemblage A subtype in fallow deer. Journal of Parasitology. 93(2): 426-428.

LeChevallier MW, Norton WD and Lee RG. 1991. Occurrence of *Giardia* and *Cryptosporidium* spp. in surface water supplies. Applied and Environmental Microbiology. 57(9): 2610-2616.

Leclerc H, Mossel DA, Edberg SC and Struijk CB. 2001. Advances in the bacteriology of the coliform group: their suitability as markers of microbial water safety. Annual Reviews in Microbiology. 55: 201-234.

Lodder WJ and de Roda Husman AM. 2005. Presence of noroviruses and other enteric viruses in sewage and surface waters in The Netherlands. Applied and Environmental Microbiology. 71(3): 1453-1461.

MacKenzie WR, Hoxie NJ, Proctor ME, Gradus MS, Blair KA, Peterson DE, Kazmierczak JJ, Addiss DG, Fox KR, Rose JB, *et al*. 1993. A massive outbreak in Milwaukee of cryptosporidium infection transmitted through the public water supply. New England Journal of Medicine. 331(3):161-167.

Maddox-Hyttel C, Langkjaer RB, Enemark HL and Vigre H. 2006. *Cryptosporidium* and *Giardia* in different age groups of Danish cattle and pigs--occurrence and management associated risk factors. Veterinary Parasitology. 141(1-2): 48-59.

Majowicz SE, McNab WB, Sockett P, Henson TS, Doré K, Edge VL, Buffett MC, Fazil A, Read S, McEwen S, Stacey D and Wilson JB. 2006. Burden and cost of gastroenteritis in a Canadian community. Journal of Food Protection. 69(3): 651-659.

Mayrhofer, G., Andrews, R.H., Ey, P.L. and Chilton, N.B. 1995. Division of *Giardia* isolates from humans into two genetically distinct assemblages by electrophoretic analysis of enzymes encoded at 27 loci and comparison with *Giardia muris*. Parasitology. 111(1): 11–17.

Meloni BP, Lymbery AJ and Thompson RC. 1995. Genetic characterization of isolates of *Giardia duodenalis* by enzyme electrophoresis: implications for reproductive biology, population structure, taxonomy, and epidemiology. Journal of Parasitology. 81(3): 368-383.

Monis PT, Caccio SM and Thompson RC. 2009. Variation in *Giardia*: towards a taxonomic revision of the genus. Trends in Parasitology. 25(2): 93-100.

Morrison H, McArthur A, Gillin F, Aley S, Adam R, Olsen G, Best A, Cande W, Chen F, Cipriano M, Davids B, Dawson S, Elmendorf H, Hehl A, Holder M, Huse S, Kim U, Lasek-Nesselquist E, Manning G, Nigam A, Nixon J, Palm D, Passamaneck N, Prabhu A, Reich C, Reiner D, Samuelson J, Svard S and Sogin M. 2007. Genomic minimalism in the early diverging intestinal parasite *Giardia lamblia.* Science. 317(5846): 1921-1926

Müller N and von Allmen N. 2005. Recent insights into the mucosal reactions associated with *Giardia lamblia* infections. International Journal for Parasitology. 35(13):1339-1347.

Nash TE and Mowatt MR. 1992. Characterization of a *Giardia lamblia* variant-specific surface protein (VSP) gene from isolate GS/M and estimation of the VSP gene repertoire size. Molecular and Biochemical Parasitology. 51(2): 219–227.

Nash TE, McCutchan T, Keister D, Dame JB, Conrad JD and Gillin FD. 1985 Restriction-endonuclease analysis of DNA from 15 *Giardia* isolates obtained from humans and animals. Journal of Infectious Diseases. 52(1): 64-73.

Nizeyi JB, Mwebe R, Nanteza A, Cranfield MR, Kalema GR and Graczyk TK. 1999. *Cryptosporidium* sp. and *Giardia* sp. infections in mountain gorillas (*Gorilla beringei*) of the Bwindi Impenetrable National Park, Uganda. Journal of Parasitology. 85(6): 1084-1088.

Nygård K, Schimmer B, Søbstad Ø, Walde A, Tveit I, Langeland N, Hausken T and Aavitsland P. 2006. A large community outbreak of waterborne giardiasis-delayed detection in a non-endemic urban area. BMC Public Health. 6: 141-151.

O.Reg 170/03. Safe Drinking Water Act 2002, Ontario Regulation 170/03 Drinking Water Systems.

Payment P and Hunter PR. 2001. Endemic and epidemic infectious intestinal disease and its relationship to water. In: Fewtrell L and Bartram J (eds). Water Quality: Standards, Guidelines and Health. International Water Association Publishing. Cornwall, UK.

Payment P, Berte A, Prévost M, Ménard B and Barbeau B. 2000. Occurrence of pathogenic microorganisms in the Saint Lawrence River (Canada) and comparison of health risks for populations using it as their source of drinking water. Canadian Journal of Microbiology. 46(6): 565-576.

Polaczyk AL, Narayanan J, Cromeans TL, Hahn D, Roberts JM, Amburgey JE and Hill VR. 2008. Ultrafiltration-based techniques for rapid and simultaneous concentration of multiple microbe classes from 100-L tap water samples. Journal of Microbiological Methods. 73(2): 92-99.

Prucca CG, Slavin I, Quiroga R, Elías EV, Rivero FD, Saura A, Carranza PG and Luján HD. 2008 Antigenic variation in *Giardia lamblia* is regulated by RNA interference. Nature. 456(7223): 750-754.

Public Health Officer (PHO). 2001. Drinking water quality in British Columbia: the public health perspective. Officer of the Public Health Officer of British Columbia, Victoria, Canada.

Ramesh M, Malik S and Logsdon JM Jr. 2005. A phylogenomic inventory of meiotic genes; evidence for sex in *Giardia* and an early eukaryotic origin of meiosis. Current Biology. 15(2): 185-191.

Read C, Walters J, Robertson ID and Thompson RC. 2002. Correlation between genotype of *Giardia duodenalis* and diarrhoea. International Journal for Parasitology. 32(2): 229-231.

Reynolds KA, Mena KD and Gerba CP. 2008. Risk of waterborne illness via drinking water in the United States. Reviews in Environmental Contamination and Toxicology. 192: 117-158.

Robertson LJ, Forberg T, Hermansen L, Hamnes IS and Gjerde B. 2007. *Giardia duodenalis* cysts isolated from wild moose and reindeer in Norway: genetic characterization by PCR-rflp and sequence analysis at two genes. Journal of Wildlife Diseases. 43(4): 576-585.

Rodgers MR, Flanigan DJ, and W Jakubowski. 1995. Identification of algae which interfere with the detection of *Giardia* cysts and *Cryptosporidium* oocysts and a method for alleviating this interference. Applied and Environmental Microbiology. 61(10): 3759-3763.

Rollins D and Colwell R. 1986. Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in natural, aquatic environment. Applied and Environmental Microbiology. 52(3): 531–538.

Scott KG, Yu LC and Buret AG. 2004. Role of CD8+ and CD4+ T lymphocytes in jejunal mucosal injury during murine giardiasis. Infection and Immunity. 72(6): 3536-3542.

Smith A, Reacher M, Smerdon W, Adak GK, Nichols G and Chalmers RM. 2006. Outbreaks of waterborne infectious intestinal disease in England and Wales, 1992-2003. Epidemiology and Infection. 134(6): 1141-1149.

Sprong H, Caccio SM, van der Glessen JWB and ZOOPET network and partners. 2009. Identification of zoonotic genotypes of *Giardia duodenalis*. PLoS Neglected Tropical Diseases. 3(12): e558.

Statistics Canada. 2003. Human Activity and the Environment: Annual Statistics 2003.

Sterling, CR and Ortega YR. 1999. *Cyclospora*: An enigma worth unraveling. Emerging Infectious Diseases. 5(1): 48-53.

Straif-Bourgeois S, Ratard R. Infectious Diseases. In: Ahrens W, Pigeot I (Eds) Handbook of Epidemiology. Springer, Germany.

Szostakowska B, Kruminis-Lozowska W, Racewicz M, Knight R, Tamang L, Myjak P and Graczyk TK. 2004. *Cryptosporidium parvum* and *Giardia lamblia* recovered from flies on a cattle farm and in a landfill. Applied Environmental Microbiology. 70(6): 3742-3744.

Tram NT, Hoang LM, Cam PD, Chung PT, Fyfe MW, Isaac-Renton JL and Ong CS. 2008. *Cyclospora* spp. in herbs and water samples collected from markets and farms in Hanoi, Vietnam. Tropical Medicine and International Health. 13(11): 1415-1420.

Thompson RC, Hopkins RM and Homan WL. 2000. Nomenclature and genetic groupings of *Giardia* infecting mammals. 16(5): 210-213.

Thompson RC and Lymbery AJ. 1996. Genetic variability in parasites and host-parasite interactions. Parasitology. 112(Suppl): S7-22.

Trout JM, Santín M, Greiner E and Fayer R. 2005. Prevalence and genotypes of *Giardia duodenalis* in post-weaned dairy calves. Veterinary Parasitology. 130(3-4): 177-183.

Trout JM, Santin M and Fayer R. 2003. Identification of assemblage A *Giardia* in white-tailed deer. Journal of Parasitology. 89(6): 1254-1255.

United Nations (UN). 2006. The Millennium Development Goals Report: 2006. United Nations, New York.

United States Environmental Protection Agency (USEPA). 2005. Method 1623: *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA. Office of Water, U.S. Environmental Protection Agency, Washington, DC.

Villanueva CM, Cantor KP, Grimalt JO, Malats N, Silverman D, Tardon A, Garcia-Closas R, Serra C, Carrato A, Castaño-Vinyals G, Marcos R, Rothman N, Real FX, Dosemeci M AND Kogevinas M. 2007. Bladder cancer and exposure to water disinfection by-products through ingestion, bathing, showering, and swimming in pools. American Journal of Epidemiology. 165(2): 148-156.

Wallis PM, Buchanan-Mappin JM, Faubert GM and Belosevic M. 1983. Reservoirs of *Giardia* spp. in southwestern Alberta. Journal of Wildlife Diseases. 20(4): 279-283.

Wilkes G, Edge T, Gannon V, Jokinen C, Lyautey E, Medeiros D, Neumann N, Ruecker N, Topp E and Lapen DR. 2009 Seasonal relationships among indicator bacteria, pathogenic bacteria, *Cryptosporidium* oocysts, *Giardia* cysts, and hydrological indices for surface waters within an agricultural landscape. Water Research. 43(8): 2209-2223.

Winkworth CL, Learmonth JJ, Matthaei CD and Townsend CR. 2008. Molecular characterization of *Giardia* isolates from calves and humans in a region in which dairy farming has recently intensified. Applied Environmental Microbiology. 74(16): 5100-5105.

World Health Organization (WHO). 1999. Report on infectious diseases – removing obstacles to healthy development. World Health Organization, Geneva.

Wright SG, Tomkins AM and Ridley DS. 1977. Giardiasis: clinical and therapeutic aspects. Gut. 18: 343-350.

Yoder JS, Beach MJ and Centers for Disease Control and Prevention (CDC). 2007. Giardiasis surveillance-United States, 2003-2005. MMWR Surveillance Summary. 56(7): 11-18.

Zimmer JL and Slawson RM. 2002. Potential repair of *Escherichia coli* DNA following exposure to UV radiation from both medium- and low-pressure UV sources used in drinking water treatment. Applied and Environmental Microbiology. 68(7): 3293-3299.

Chapter 2

Genetic Analysis of *Giardia* Isolates Specimens Collected from Humans, Drinking Water and Animals[1]

2.1 Introduction

First discovered by Van Leeuwenhoek in 1681, *Giardia lamblia* was not recognized as a human pathogen until the late 1970s (Borehan *et al*., 1990). It is now recognized as the most common cause of diarrhea worldwide. The disease, giardiasis, causes diarrhea and malabsorption but infection is typically self-limiting in most hosts. *G.lamblia* is also the most frequently identified parasite causing with waterborne outbreaks. Transmission through water supplies occurs globally, in both developing and developed nations alike (Thompson, 2000; Marshall *et al*., 1997; Kappus *et al*., 1994).

G.lamblia most commonly infects mammals, but is also found in reptiles and birds. Transmission is via the fecal-oral route, with waterborne and person-to-person transmission being the most frequent routes of spread. It is ubiquitous in water supplies; a survey of North American water supplies found *Giardia* cysts in 81% of raw water supplies (LeChevallier *et al*., 1991). Due to its prevalence in water and its resistance to traditional drinking water disinfection, it has been included in national drinking water monitoring guidelines in the United States and the United Kingdom (USEPA, 2005; DWI, 1999).

G.lamblia in water supplies has been a particular concern in British Columbia (BC), Canada, for the past 25 years. *G.lamblia* was the etiological agent in 13 of the 29 documented waterborne outbreaks since 1980 (PHO, 2001). Despite a considerable decrease in the incidence in giardiasis rates in BC since 1990 (68 cases per 100,000), giardiasis continues to be more prevalent compared to the rest of Canada (14.2 cases per 100,000 in 2008) (BCCDC, 2009). It is likely that a combination of risk factors and geographic characteristics contribute to the problem in BC. Varied terrain (mountainous, coastal and agricultural), climate (marine to continental) and participation in activities known to be risk factors for giardiasis, such as travel and camping, may all contribute to these elevated rates. A study in 1992 revealed that consumption of untreated surface water resulted in a 12-fold increased

[1] A version of this chapter will be submitted for publication. Prystajecky N, Huck PM and Isaac-Renton, JL. Genetic analysis of *Giardia* isolates specimens collected from humans, drinking water and animals

risk of acquiring giardiasis in British Columbia (Isaac-Renton and Philion, 1992). Reduction in giardiasis rates province-wide may reflect improvements to water resource management, including increased watershed management and upgrades to drinking water treatment facilities. However, this relationship between water resource management and the rates of giardiasis have never conclusively linked by scientific studies.

Water was identified as an important transmission vector for *G.lamblia* in the late 1970s in North America and subsequently methods were developed for the detection of the pathogen in water supplies. Early methodologies included filtration of large volumes of water through string-wound filters, followed by purification by density centrifugation and microscopic identification, as described in the Standard Methods for the Examination of Water and Wastewater (ASTM, 1991). Testing for *G.lamblia* and the related parasite *Cryptosporidium* were later incorporated into the United States Environmental Protection Agency's drinking water microbiology program. The current approach, Method 1623 for the detection of *Giardia* and *Cryptosporidium* in water, is capable only of detection to the genus level (USEPA, 2005). Further characterization to species or subspecies level is only achievable by genotypic methods.

Genotyping is an essential molecular epidemiological tool in understanding *G.lamblia* biology, taxonomy and transmission dynamics. Under the current taxonomic regime, *G.lamblia* is divided into seven Assemblages (aka subtypes) named Assemblages A through G, based on genetic sequence and host specificity (Mayrhofer *et al*., 1995). Of these, only two Assemblages, Assemblages A and B, are infectious to humans. Assemblages A and B are also zoonotic, infecting humans and animal species. Assemblages A and B differ in their biology and host specificity; it appears that Assemblage A infects a wide range of hosts including livestock and many mammals, while Assemblage B tends to be limited to humans, beavers and domestic animals (Monis *et al*., 2009). The remaining subtypes, Assemblages C through G, are tightly adapted to specific animal hosts and have yet to be shown to be infectious to humans. This disparity between detection capabilities and host specificity of *Giardia* species and *G.lamblia* subtypes impacts current water quality monitoring protocols and their applicability to risk assessment since non-human infectious isolates may be detected. As such, genotyping is crucial in determining the potential risk of *Giardia* isolates captured from water.

In addition to the taxonomic data provided by conducting molecular epidemiologic studies, genotyping of patient and environmental samples may provide insight into regional transmission. As well, as laboratory genotyping methods continue to improve, archived isolates need to be further

characterized to provide new knowledge as well as to be useful in ongoing evaluation of new detection methods.

In this paper, isolates collected from a variety of British Columbian sources (humans, animals and water) were further characterized further using new genotyping, classification and analysis approaches.

2.2 Methods

2.2.1 Sample Purification

Identification of *Giardia lamblia* infection in both human and animal specimens was determined by microscopy, using standard ova and parasite examination (O&P) (Garcia and Bruckner, 1997). Specimens found to be positive and relevant to future studies were further processed. Cysts were purified from fecal materials using sucrose density centrifugation (Roberts-Thompson *et al*., 1976). Water samples were collected according to Standard Methods for the Examination of Water and Wastewater (American Society for Testing and Materials, 1991). Large volumes of water were filtered through a string-wound filter, processed using a stomacher and the resulting pellet was purified by Percoll-sucrose density centrifugation. Purified cysts were enumerated by immunofluorescence microscopy, using commercially available antibody kits (Hydrofluor™ Combo, Oxoid, Nepean, Ontario). *Giardia* was recovered from trapped beavers by intestinal lavage and sucrose density centrifugation (Isaac-Renton *et al*., 1988).

2.2.2 Isolates Studied

Isolates were collected from a variety of sources, including *Giardia* infected patients and animals, as well as parasite contaminated water samples. A total of 78 isolates were analyzed (Table 2-1). Stool specimens were collected from both sporadic and outbreak-associated human cases and tested for the presence of *Giardia* cysts as described above. Animal isolates were collected from unpreserved animal scat (sheep and dog) or from the intestinal tracts (trapped beavers). Water samples were collected by filtration and *Giardia* cysts were detected as described above. Additional purified and cultured specimens were donated from researchers in Canada, the United States, China and Australia

Table 2-1: Distribution of archived sample types used in the study.

Sample Type	Location	Number
Human	BC	27
Human	International	6
Water	BC & CAN	24
Beaver	BC & AB	13
Other Animals	BC & AB	4
Total		**78**

British Columbia isolates were compared to additional isolates from different geographic locales. A map showing the geographic location of the human BC isolates studied is indicated below (Figure 2-1).

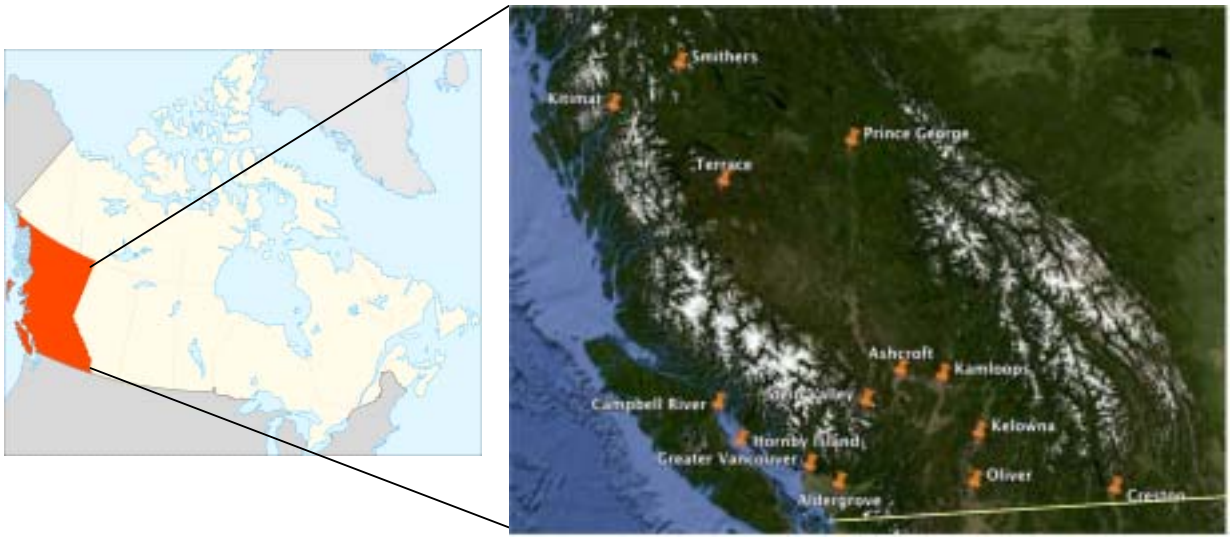


Figure 2-1: Geographic location of BC human isolates under investigation. Yellow line denotes the border with the United States.

2.2.3 Isolates

Purified *Giardia* cysts were excysted and cultured axenically in Keister's modified TYI-33 media (supplemented with bile) for the production of trophozoites (Keister, 1983). A subset of samples were split and cultured in a Mongolian male gerbil (*Meriones unguiculatas*) (Faubert and Belosevic, 1990; McIntyre *et al*., 2000). In brief, animals were given metronidazole (500mg/kg/day for three days) to eliminate previous parasitic infection, then immunosuppressed by administering dexamethasone through drinking water. The gerbils were fed purified cysts and fecal samples were examined for the presence of cysts. Fecal samples from infected gerbils were collected and cysts were purified by methods described above. Trophozoites were also isolated from the intestines of sacrificed gerbils. Isolates were kept at 4ºC for immediate use or stored at -20ºC (short-term) and long-term storage (archiving) at -80 ºC.

2.2.4 Genomic DNA Extraction

Historical DNA extraction from cysts was performed using physical and chemical lysis, followed by phenol/chloroform/isoamyl purification (Weiss *et al*. 1992). In brief, pellets were first washed in TEN buffer (10mM Tris-Cl, 10nM EDTA, 150 mM NaCl, pH 8), followed by two freeze-thaw cycles. Samples were further digested using 0.5mg lysozyme, sodium dodecyl sulfate (SDS) in TEN buffer and proteinase K. Digestion was followed by another freeze-thaw cycle. Samples were purified by repeat phenol/chloroform/isoamyl alcohol (25:24:1) extraction. The upper aqueous layer was then precipitated by cold acetate/ethanol precipitation overnight. Resulting pellets were washed with 70% ethanol and resuspended in TE buffer. Samples not used immediately were stored at -20ºC and long-term storage (archiving) at -80ºC. DNA extraction from trophozoites differed in the lysis step, using DNA Now Reagent (Biogentex, Inc., Seabrook, Texas). Resulting trophozoite lysates were prepared in the same manner as described for cysts.

2.2.5 Pulse Field Gel Eletrophoresis and Isoenzyme Analysis

Giardia positive samples were previously characterized by isoenzyme (IE) analysis and pulse field gel electrophoresis (PFGE). Detailed methods are described in McIntyre *et al*., 2000 and Isaac-Renton *et al*., 1993. Late log-phase trophozoites were harvested from TYI-33 culture and isolates were prepared as described by Isaac-Renton *et al*., 1993. Isoenzyme analysis was performed by electrophoresing samples for two hours at 175V and gels were photographed and compared.

PFGE analysis was performed by harvesting late log-phase trophozoites from TYI-33 cultures and preparing isolates as described by McIntyre *et al*., 2000. The run conditions were as follows: 330 second pulse for 24 hours, 750 second pulse for 24 hours and 1800 second pulse for 48 hours. Gels were stained with ethidium bromide and photographed for comparison.

2.2.6 Polymerase Chain Reaction and DNA Sequencing

As the purpose of this study was both method validation and isolate characterization, efforts were made to bring the quality of archived DNA preparations up to current standards for proper comparisons. DNA preparations from previous analyses were re-purified to remove residual phenol or other contaminants from previous extraction using DNA Micro Kit (Qiagen), resuspended in 50µl of molecular grade water and stored at -20ºC.

PCR was performed on all isolates at the 18s rRNA gene, as described by Appelbee *et al*., 2003 and Hopkins *et al*., 1997. PCR was performed using the forward primer Gia2029 (5'-AAGTGTGGTGCAGACGGACTC-3') and reverse primer Gia2150c (5'-CTGCTGCCGTCCT-TGGATGT-3') to produce a 497bp product (Table 2-2). PCRs were performed using 1.0-2.5 µl of DNA, 2.0 mM $MgCl_2$, 10 pmol each primer, 200mM each dNTP, 5% DMSO, 10mg/ml BSA, 1x Qiagen PCR buffer and 0.5 Qiagen HotStarTaq polymerase. PCR was conducted using a 15-minute hot-start at 95ºC, followed by 35 cycles of PCR (96ºC for 45s, 55ºC for 30s, and 72ºC for 45s) and a four minute extension at 72ºC. PCRs were carried out on either a Perkin-Elmer GeneAmp 9600 or an Eppendorf Mastercycle thermocycler. PCR products were visualized using ethidium bromide and UV illumination.

Table 2-2: Summary of primers used during the study.

Name	Sequence	Target (Size)	Reference
Gia2029	5'-AAGTGTGGTGCAGACGGACTC-3	18s rRNA (497bp)	Appelbee *et al*., 2003
Gia2150c	5'-CTGCTGCCGTCCTTGGATGT-3'		
BGF1	5'-AAGCCCGACGACCTCACCCGCAGTGC3'	*β-giardin* (750bp)	Lalle *et al*., 2005
BGR1	5'-GAGGCCGCCCTGGATCTTCGAGACGAC-3'		
AL3543	5´-AAATIATGCCTGCTCGTCG-3'	*Tpi* (605 bp)	Sulaiman *et al*., 2003
AL3546	5´-CAAACCTTITCCGCAAACC-3´		

PCR was also performed, using similar conditions, on the *tpi* and *β-giardin* genes. For *β-giardin,* PCR was performed using forward primer BGF1 (5'-AAGCCCGACGACCTCAC-CCGCAGTGC-3') and reverse primer BGR1 (5'-GAGGCCGCCCTGGATCTTCGAGACGAC-3') to amplify a product of 750bp (Lalle *et al*., 2005). The PCR conditions were similar to those described above, except DMSO was omitted and 1.5 mM $MgCl_2$ and 25 pmoles of each primer were used. The PCR reaction was performed for 35 cycles (94°C for 45s, 65°C for 45s and 72°C for 60s), preceded by a hot-start step (94°C for 15 minutes) and a final extension step (72°C for 10 minutes) using a thermocycler (Mastercycler, Eppendorf).

Tpi PCR was performed using primers with degenerate bases, as described by Sulaiman *et al*., 2003. Forward primer AL3543 (5´- AAATIATGCCTGCTCGTCG-3´) and reverse primer AL3546 (5´-CAAACCTTITCCGCAAACC-3´) were used to amplify a 605 bp product. The PCR conditions were performed similar to above, with an increased $MgCl_2$ concentration (3.0 mM) and increased *Taq* polymerase content (2.5U) using Qiagen HotStarTaq Plus polymerase. PCR conditions were as follows: 5 minute hot-start at 94°C, followed by 35 cycles of 94°C for 45s, 50°C for 45s, and 72°C for 60s and a final extension at 72°C for 10 minutes.

PCR products were purified using QiaQuick Purification Kit according to manufacturer's instructions (Qiagen) and quantified using a spectrophotometer (Ultraspec 3100 Pro, BioChrom). Samples were sequenced using Applied Biosystems (ABI) platform, using the BigDye Terminator Version 3.1 Cycle Sequencing Kit on an ABI 3100 automated multicapillary sequencer. Sequencing conditions included 2µl of BigDye Terminator sequencing reagents, 6µl of BigDye Terminator sequencing

Buffer, 3.2 pmoles primer and 10-20nmol PCR product. The cycle sequencing was performed for 25 cycles (96°C for 10 s, 50°C for 5 s, and 60°C for 4 min) on a 9600 Perkin-Elmer GeneAmp thermocycler. Sequence products were ethanol-acetate precipitated, cleaned with 75% ethanol and dried, followed by reconstitution in Hi-Di formamide and loaded into the ABI 3100 sequencer. Each sample was sequenced in both the forward and reverse directions.

2.2.7 Genetic Data Analysis

All statistical analyses were performed using Microsoft Excel (2003). Sequence analysis was performed using DNAStar SeqMan, MegAlign Programs and Geneious DNA Software. Bootstrap analyses were performed using Geneious DNA software.

2.3 Results

A total of 78 of isolates were selected for genetic characterization. All 78 isolates had been previously characterized using IE and PFGE. All isolates were successfully characterized at the *tpi* (78) and 18s rRNA loci (78); six isolates were not typable at the *β-giardin* locus due to poor sequencing results.

2.3.1 Comparison of PFGE, IE and PCR/Sequencing Results

Comparability of sequencing results between sequencing methods and compared to the historical results was high for all isolates. Listed below (Table 2-3) is a subset of comparisons, including some isolates that were not consistent amongst current genotyping methods. Only three isolates demonstrated inconsistencies in Assemblage designation using the three PCR/sequencing approaches surveyed in this study (VANC/92/UBC/107, VANC/92/UBC/99 and VANC/90/UBC/62). These three samples represent two environmental specimens and a travel-related human specimen. It is possible that the inconsistent classification may be a result of mixed genotypes present in a single specimen. Further evidence of mixed genotype in the human specimen (VANC/92/UBC/107) was observed by isoenzyme analysis and an unusual PFGE pattern, but was not observed for the two environmental samples.

The sub-Assemblage groupings differed between the historical nomenclature and current nomenclature; this likely reflects differences in grouping knowledge after exhaustive sequencing efforts in the 2000s.

Table 2-3: Comparisons of a subset of isolates, using five genotyping approaches (PFGE, IE, sequencing at tpi, β-giardin and 18s rRNA loci). PFGE and IE designations are given in historical genotyping nomenclature. X indicates unsuccessful sequencing.

Isolate	Description	PFGE	IE	*Tpi*	*18s*	*B-giardin*
VANC/90/UBC/50	BC Outbreak	A	7	B	B	B
VANC/92/UBC/107	BC Travel	A	7	B	B	B
VANC/89/UBC/48	BC Endemic	A	1	B	B	B
VANC/91/UBC/69	BC Water	B	1	B	B	B
VANC/91/UBC/68	BC Water	C	1	B	B	X
VANC/94/UBC/122	BC Water	B	1	B	B	B
VANC/94/UBC/123	BC Water	B	1	B	B	B
VANC/92/UBC/107	Int'l Travel	D+	5 & 2	B	A	A
SI/16	AB Sheep	D	2	B	A	A
VANC/87/UBC/23	BC Endemic	D	3	A	A	A
VANC/90/UBC/62	BC Water	C	3	A	B	A
VANC/92/UBC/99	BC Water	A	4	B	A	A
VANC/85/UBC/2	BC Endemic	D	5	A	A	A
VANC/87/UBC/29	BC Beaver	D	5	A	A	A
VANC/85/UBC/7	BC Beaver	D	5	A	A	A
BE-2/14	AB Beaver	D	5	A	A	A
BE-1/15	AB Beaver	D	5	A	A	A
CH3/20	US Endemic	D	5	A	A	A
D3/18	BC Dog	D	5	A	A	A
VANC/85/UBC/5	BC Endemic	D	5	A	A	A
VANC/87/UBC/24	Int'l Travel	D	5	A	A	A
VANC/89/UBC/33	BC Travel	D	5	A	A	A

2.3.2 Sequence Analysis – Triose Phosphate Isomerase Gene

The triose-phosphate isomerase (*tpi*) gene was selected for detailed analysis, for the purposes of this publication as it provided a greater amount of sequence variation than the 18s rRNA gene and greater sequence quality than the β-giardin gene. This is a reflection of the intermediate stability of the gene, in comparison to compared to 18s rRNA gene (extremely stable) and β-giardin gene (encodes an antigenic protein, therefore more variable). (Sulaiman *et al*., 2003) .All isolates examined, including isolates retrieved from water, dog and sheep, belonged to Assemblages A and B. This may reflects that the observation that axenic culture is best suited for growth of isolates belonging to zoonotic Assemblage (Assemblages A and B).

Amongst human isolates, there was 90.9% sequence homology between isolates and 67% of bases were identical. Assemblage A accounted for 69.7% of human isolates and 30.3% of human isolates belonged to Assemblage B (Figure 2-2). Sequence variability between Assemblage B isolates was much greater than sequence variability between Assemblage A isolates, consistent with current literature. International isolates did not consistently group with one another and neither did samples isolated from endemic cases. Sequence analysis revealed no consistent nucleotide changes that were consistent amongst human isolates or specific outbreaks, however, a single nucleotide change could be used to distinguish some international isolates from BC isolates (Figure 3-3).

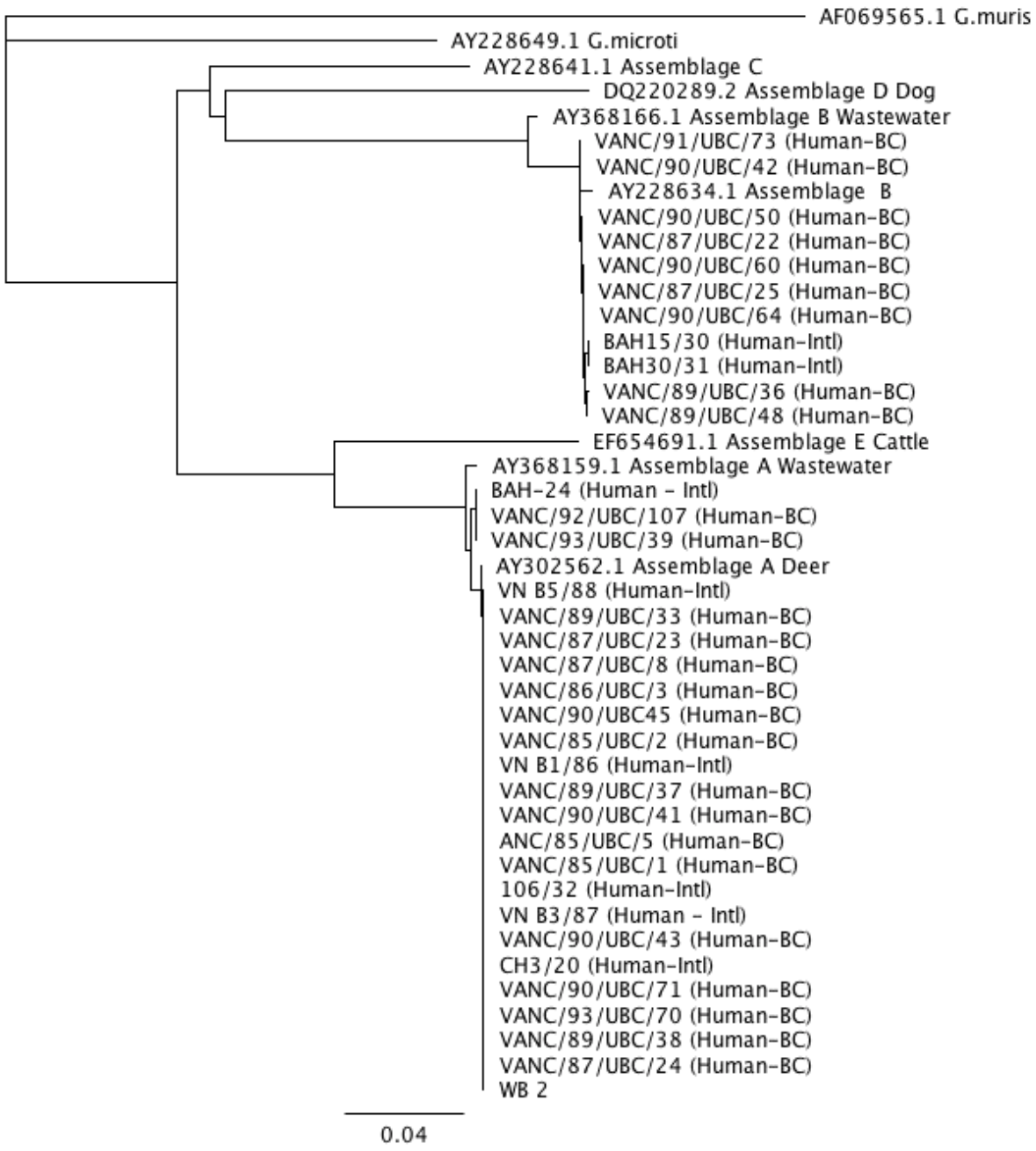


Figure 2-2: Phylogenetic alignment of human *tpi* sequences, using neighbor-joining tree, using Jukes-Cantor genetic distance model and *G.muris* as root.

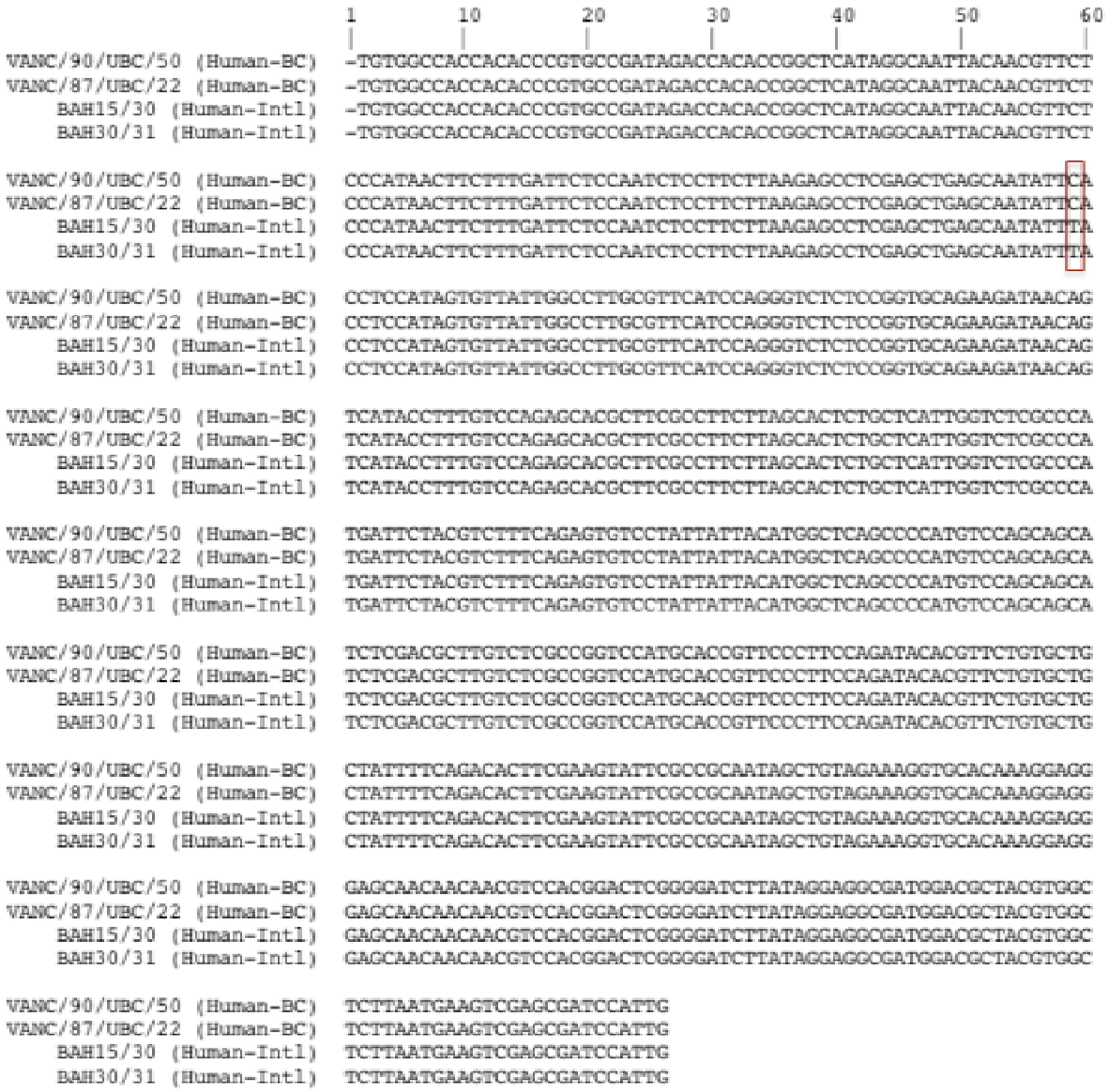


Figure 2-3: Sequence alignment of two human BC outbreak isolates and two human international cases, demonstrating identical sequences with the exception of a single nucleotide polymorphism at bp 119 (T→C)

Amongst isolates retrieved from beavers, there was 89.1% sequence homology between isolates, with 74% of bases were identical. The majority of beaver isolates analyzed at the *tpi* locus belonged to Assemblage A (60%), while the remaining isolates (40%) belonged to Assemblage B (Figure 2-3). There was no consistent pattern in the geographic location of isolate collection and Assemblage determination. Similar sequence distances were obtained for isolates obtained from British Columbia water samples; sequences demonstrated 89.4% homology and 69.9% of isolates being identical. It should be noted that animal model should theoretically allow for only one predominant genotype due to competition, despite multiple genotypes expected in highly contaminated water samples.

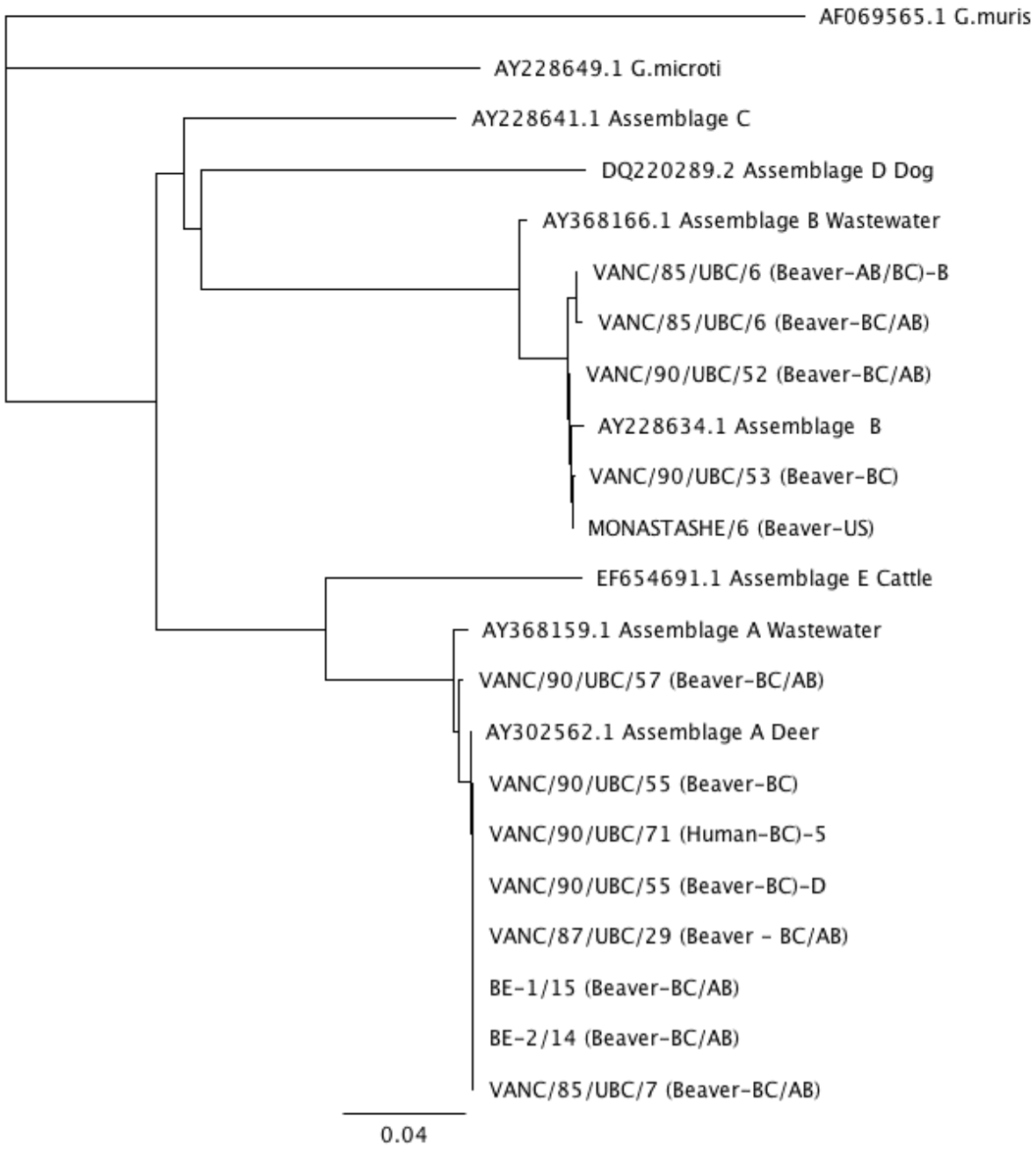


Figure 2-3: Phylogenetic alignment of beaver *tpi* sequences, neighbor-joining tree, using Jukes-Cantor genetic distance model and *G.muris* as root.

2.3.3 Suitability of Validated Genotyping Methods For Environmental Study

One of the objectives of this research was to develop an in-house library of *G.lamblia* sequences, for use in subsequent prospective environmental molecular epidemiological studies. The main objective, however, was to determine the most suitable methods for environmental studies. The criteria used to evaluate the suitability of the 18s rRNA, *tpi* and *β-giardin* genes were PCR quality, raw sequence quality and information provided by sequencing data (i.e. genotype resolution).

PCR quality was determined by visual inspection of band strength after gel electrophoresis, presence of non-specific bands and the need for repeating failed PCRs and/or nested primary PCR products to yield high quality PCR products for sequencing. Only 18s rRNA PCR was achievable for all sequences without nesting, likely because it is a multi-copy gene whereas the other targets are single copy genes (see Appendix). Furthermore, for some specimens, *tpi* PCR without nesting yielded non-specific bands, likely due to the presence of degenerate bases in the primers. This may have further impacts on direct environmental specimens, even with nested PCR, because of the presence of interfering genetic material and because it is a semi-nested PCR approach. The *β-giardin* gene did require nested for some, but not all, specimens.

Raw sequence quality was determined by examining the average signal strength for each locus and analysis of the eletropherogram for features such as signal dampening, dye globs, inconsistent base calling and cleanliness of sequences. Only the *β-giardin* gene had several sequences where the sequence quality was too poor to be used (inconsistent base calling and signal dampening), even upon repeat PCR and sequencing. 18s rRNA and *tpi* sequencing both demonstrated high sequence quality based on the parameters above.

Ultimately it was determined that the ability to sequence 18s rRNA samples without nesting and the high quality of sequence electropherograms that 18s rRNA should be selected for use in molecular epidemiological analyses for environmental specimens.

2.4 Discussion

This study successfully evaluated three new genotyping methods and further characterized a library of *Giardia* isolates obtained from ongoing work in British Columbia (BC). The three genotyping methods were optimized for a variety of specimen types (humans, animal and water). By further characterizing isolates from various sources (human, animal, water), new knowledge was provided

about this ubiquitous enteric parasite in BC as well as new information on important genotyping methods that could be used in ongoing environmental epidemiological and surveillance programs.

Isolates analyzed represented a wide range of sources. Isolates demonstrated a large range in sequence distances, which reflects the substantial sequence distances between Assemblage A and B at all genetic loci. Even when considering only British Columbia isolates, sequence distances were large between Assemblage A and Assemblage B isolates. Furthermore, isolates belonging to Assemblage B demonstrate greater intra-Assemblage sequence variance, compared to Assemblage B, consistent with other studies in humans, animals and environmental specimens (Sulaiman *et al*., 2003). This large difference in genetic sequence between Assemblage A and Assemblage B isolates is consistent with attempts to revisit *Giardia* taxonomy to reflect these large differences (Monis *et al*., 2009).

The lack of non-zoonotic isolates in this study of archived parasites (Assemblages C through G) may not be a true absence of host-adapted isolates in British Columbia but rather may reflect previous methods used to obtain isolates. TYI-33 media was developed for culturing human pathogens and has been shown to be inadequate for successful culture of non-human isolates (Meloni and Thompson, 1986). Furthermore, it has been demonstrated that TYI-33 media is best adapted for Assemblage A isolates, with Assemblage B isolates taking longer to double (Karanis and Ey, 1998). This is reflected in other studies where axenic culture has resulted in predominant detection of Assemblage A over Assemblage B (Nash and Mowatt 1992; Monis *et al*., 1996). It was expected that some of the animal isolates, specifically from dogs and sheep, would be classified as members of the host-adapted Assemblages based on previous publications (Santin *et al*., 2007; Palmer *et al*., 2008; Souza *et al*., 2007). However, there is evidence of zoonotic infection in these animal species as well and appears to be geographically specific (Leonhard *et al*., 2007; Giangaspero *et al*., 2005). Likewise, it was expected that some environmental specimens may also contain non-zoonotic Assemblages but all samples contained either Assemblages A or B.

The predominance of Assemblage A in all sample types (human, animal and water) could reflect selection of the culturing method for Assemblage A isolates, as described above (Nash and Mowatt 1992; Monis *et al*., 1996). However, it is also possible that Assemblage A may be the most commonly represented *Giardia lamblia* Assemblage in British Columbia. The predominance of Assemblage A in British Columbia would have interesting impacts on pathogen ecology and transmission dynamics; Assemblage A has the widest host range (thus the greatest ecological impact) but has been reported to cause less severe diseases and therefore less shedding (thus less transmission

from a single infected host) (Homan and Mank, 2001; Monis *et al*., 2009). This hypothesis certainly warrants further study using methods that do not require culturing in TYI-33 media (i.e. detection by molecular analyses).

While giardiasis was commonly referred to as beaver fever, there is substantial evidence that many mammalian species, not just *Castor canadensis*, contribute to *G.lamblia* contamination of water supplies. Early outbreaks in British Columbia were linked genotypically and epidemiologically with infected beavers at the drinking water intakes (McIntyre *et al*., 2000; Isaac-Renton *et al*., 1993). However, zoonotic isolates of *G.lamblia* has been detected in a wide range of animals including cattle (O'Handley *et al*., 2000; Ey *et al*., 1997), sheep (Giangaspero *et al*., 2005), dogs (Lalle *et al*., 2005), deer (Trout *et al*., 2003) and moose (Robertson *et al*., 2007).

There were three instance of discordance of Assemblage designation by current genotyping methods. Current knowledge of *Giardia* biology still suggests reproduction occurs asexually, meaning that recombination that could result in discordant genotype designations is unlikely. There is some evidence that *Giardia* may have cryptic sexual reproduction (Birky Jr, 2005), however, a more likely explanation is the presence of mixed genotypes or contaminating sequences due to substantial manipulation of isolates amongst many culturing and genotyping projects. However, there was no discordance between historical genotyping methods and current genotyping methods. Sequencing did provide greater resolution between isolates, providing fingerprinting at a subtype level. This is particularly important in working with *G.lamblia,* because the current taxonomic classification does not provide much information regarding isolate source or characteristics. Further subtyping is necessary for *Giardia* source-tracking activities, used in source attribution studies in food and water to determine sources of contamination (Yang *et al*., 2008; Smith *et al*., 2006). It is also beneficial in outbreak investigation, to link cases to each other and to potential sources of contamination (Eisenstein *et al*., 2008; Robertson *et al*., 2006). Source attribution studies have also been performed in cases of non-outbreak cases of giardiasis, for the development of understanding of endemic transmission cycles (Lalle *et al*., 2005; Eligio-García *et al*., 2005).

In the drinking water industry, there is an increasing expectation of laboratories to characterize parasite isolates at the genetic level (fingerprinting) to define their potential impact on human health, to answer the question of isolate related and to predict sources of contamination. There is growing focus of interest in using sequencing of specific pathogens as a 'source-tracing' tool, to identify

possible sources of parasite contamination for land-management purposes (Simpson *et al*., 2002; Ruecker *et al*., 2005; Goss and Richards 2008).

This pathogen-specific approach for water quality management is distinct from the traditional approach of using bacterial indicators, which only indicates recent fecal contamination (LeClerc *et al*., 2001). There is substantial evidence to indicate that bacterial indicators cannot predict parasitic contamination of water supplies (Wilkes *et al*., 2009). Although more expensive, pathogen-specific typing could eventually be scaled-up into high-throughput molecular approaches that clinical laboratories have incorporated into routine testing without substantial financial costs (Mahoney *et al*., 2007; Wong *et al*., 2003), This would be a substantial step forward for drinking water monitoring for the purpose of public health. While sequence data for *Giardia* isolates has yet to be incorporated into risk assessment models, the use of molecular data to predict health risk may be a next step. This can only be successful with high quality sequence data that is representative of a large geographic range and incorporates all potential source of *Giardia* (both infectious and non-infectious). This study confirms the usefulness of genotyping as an additional tool in the amelioration of monitoring drinking water, which will have significant impacts on the protection of public health.

2.5 References

American Society for Testing and Materials (ASTM). 1991. Proposed test method for the detection of *Giardia* cysts and *Cryptosporidium* oocysts in low turbidity water by a fluorescent antibody procedure. In: Annual Book of ASTM Standards 11.01. American Society for Testing and Materials.

Appelbee AJ, Frederick LM, Heitman TL and Olson ME. 2003. Prevalence and genotyping of *Giardia duodenalis* from beef calves in Alberta, Canada. Veterinary Parasitology. 112(4): 289-294.

Bertram MA, Meyer EA, Lile JD and Morse SA. 1983. A comparison of isozymes of five axenic *Giardia* isolates. Journal of Parasitology. 69(5):793-801.

Birky CW Jr. 2005. Sex: is *Giardia* doing it in the dark? Current Biology. 5(2): R56-R58.

Borehan PF, Upcroft JA and Upcroft P. 1990. Changing approaches to the study of *Giardia* epidemiology: 1681–2000. International Journal for Parasitology. 20(4): 479-487.

British Columbia Centre for Disease Control (BCCDC). 2009. British Columbia Annual Summary of Reportable Disease. Provincial Health Services Authority. Vancouver, British Columbia.

Cedillo-Rivera R, Enciso-Moreno JA, Martínez-Palomo A and Ortega-Pierres G. 1989. *Giardia lamblia*: isoenzyme analysis of 19 axenic strains isolated from symptomatic and asymptomatic patients in Mexico. Transactions of the Royal Society of Tropical Medicine and Hygiene. 83(5):644-646.

Drinking Water Inspectorate (DWI). 1999. Guidance on assessing risk from *Cryptosporidium* oocysts in treated water supplies to satisfy the Water Supply (Water Quality) (Amendment) Regulations. 1999. SI 1524. Department of the Environment, Transport and the Regions, London.

Eisenstein L, Bodager D and Ginzl D. 2006. Outbreak of giardiasis and cryptosporidiosis associated with a neighborhood interactive water fountain--Florida, 2006. Journal of Environmental Health. 71(3): 18-22.

Eligio-García L, Cortes-Campos A, Jiménez-Cardoso E. 2005. Genotype of *Giardia intestinalis* isolates from children and dogs and its relationship to host origin. Parasitology Research. 97(1): 1-6.

Ey PL, Mansouri M, Kulda J, Nohýnková E, Monis PT, Andrews RH and Mayrhofer G. 1997. Genetic analysis of *Giardia* from hoofed farm animals reveals artiodactyl-specific and potentially zoonotic genotypes. Journal of Eukaryotic Microbiology. 44(6): 626-635.

Faubert, G and Belosevic M. 1990. Animal models for *Giardia duodenalis* type organisms. In Giardiasis, E. A. Meyer (ed.). Elsevier Science Publishers, Amsterdam, The Netherlands.

Garcia L and Bruckerner D. 1997. Diagnostic Medical Parasitology. ASM Press, Washington, DC.

Giangaspero A, Paoletti B, Iorio R and Traversa D. 2005. Prevalence and molecular characterization of *Giardia duodenalis* from sheep in central Italy. Parasitology Research. 96(1): 32-37.

Goss M and Richards C. 2008. Development of a risk-based index for source water protection planning, which supports the reduction of pathogens from agricultural activity entering water resources. Journal of Environmental Management. 87(4):623-632.

Homan WL and Mank TG. 2001. Human giardiasis: genotype linked differences in clinical symptomatology. International Journal for Parasitology. 31(8): 822-826.

Isaac-Renton JL, Cordeiro C, Sarafis K and Shahriari H. 1993. Characterization of *Giardia duodenalis* isolates from a waterborne outbreak. Journal of Infectious Diseases. 167(2): 431-440.

Isaac-Renton JL and Philion JJ. 1992. Factors associated with acquiring giardiasis in British Columbia residents. Canadian Journal of Public Health. 83(2): 155-158.

Isaac-Renton JL, Byrne SK and Prameya R. 1988. Isoelectric focusing of ten strains of *Giardia duodenalis*. Journal of Parasitology. 74(6): 1054-1056.

Kappus KD, Lundgren RG, Juranek DD, Roberts JM and Spencer HC. 1994. Intestinal parasitism in the United States: update on a continuing problem. American Journal of Tropical Medicine and Hygiene. 50(6): 705-713.

Karanis P and Ey PL. 1998. Characterization of axenic isolates of *Giardia intestinalis* established from humans and animals in Germany. Parasitology Research. 84(6): 442-449.

Lalle M, Jimenez-Cardosa E, Caccio SM and Pozio E. 2005. Genotyping of *Giardia duodenalis* from humans and dogs from Mexico using a beta-giardin nested polymerase chain reaction assay. Journal of Parasitology. 91(1): 203-205.

LeChevallier MW, Norton WD and Lee RG. 1991. Occurrence of *Giardia* and *Cryptosporidium* spp. in surface water supplies. Applied and Environmental Microbiology. 57(9): 2610-2616.

Leonhard S, Pfister K, Beelitz P, Wielinga C and Thompson RC. 2007. The molecular characterization of *Giardia* from dogs in southern Germany. Veterinary Parasitology. 150(1-2):33-38.

Mahony J, Chong S, Merante F, Yaghoubian S, Sinha T, Lisle C, Janeczko R. 2007. Development of a respiratory virus panel test for detection of twenty human respiratory viruses by use of multiplex PCR and a fluid microbead-based assay. Journal of Clinical Microbiology. 45(9): 2965-2970.

Marshall MC, Naumovitz D, Ortega Y and Sterling CR. 1997. Waterborne protozoan pathogens. Clinical Microbiology Reviews. 10(1): 67-85.

Mayrhofer, G., Andrews, R.H., Ey, P.L. and Chilton, N.B. 1995. Division of *Giardia* isolates from humans into two genetically distinct assemblages by electrophoretic analysis of enzymes encoded at 27 loci and comparison with *Giardia muris*. Parasitology. 111(1): 11–17.\

McIntyre L, Hoang L, Ong CS, Lee P, Isaac-Renton JL. 2000. Evaluation of molecular techniques to biotype *Giardia duodenalis* collected during an outbreak. Journal of Parasitology. 86(1): 172-177.

Meloni BP, Lymbery AJ and Thompson RC. 1988. Isoenzyme electrophoresis of 30 isolates of *Giardia* from humans and felines. American Journal of Tropical Medicine and Hygiene. 38(1):65-73.

Meloni BP and Thompson RC. 1986. Comparative studies on the axenic *in vitro* cultivation of *Giardia* of human and canine origin: evidence for intraspecific variation. Transactions of the Royal Society of Tropical Medicine and Hygiene. 81(4): 637-640.

Monis PT, Caccio SM and Thompson RC. 2009. Variation in *Giardia*: towards a taxonomic revision of the genus. Trends in Parasitology. 25(2): 93-100.

Monis PT, Mayrhofer G, Andrews R, Homan W, Limper L and Ey PL. 1996. Molecular genetic analysis of *Giardia intestinalis* isolates at the glutamate dehydrogenase locus. Parasitology. 112(Pt 1): 1-12.

Nash TE and Mowatt MR. 1992. Identification and characterization of a *Giardia lamblia* group-specific gene. Experimental Parasitology. 75(4): 369-378.

O'Handley RM, Olson ME, Fraser D, Adams P and Thompson RC. 2000. Prevalence and genotypic characterisation of *Giardia* in dairy calves from Western Australia and Western Canada. Veterinary Parasitology. 90(3):193-200.

Palmer CS, Traub RJ, Robertson ID, Devlin G, Rees R and Thompson RC. 2008. Determining the zoonotic significance of *Giardia* and *Cryptosporidium* in Australian dogs and cats. Veterinary Parasitology. 154(1-2): 142-147.

Public Health Officer. 2001. Drinking water quality in British Columbia: the public health perspective. Officer of the Public Health Officer of British Columbia, Victoria, Canada.

Ruecker NJ, Bounsombath N, Wallis P, Ong CS, Isaac-Renton JL and Neumann NF. 2005. Molecular forensic profiling of *Cryptosporidium* species and genotypes in raw water. Applied and Environmental Microbiology. 71(12): 8991-8994.

Roberts-Thomson IC, Stevens DP, Mahmoud AA and Warren KS. 1976. Acquired resistance to infection in an animal model of giardiasis. Journal of Immunology. 117(5-2): 2036-2037.

Robertson LJ, Forberg T, Hermansen L, Hamnes IS and Gjerde B. 2007. *Giardia duodenalis* cysts isolated from wild moose and reindeer in Norway: genetic characterization by PCR-rflp and sequence analysis at two genes. Journal of Wildlife Diseases. 43(4): 576-585.

Robertson LJ, Hermansen L, Gjerde BK, Strand E, Alvsvåg JO and Langeland N. 2006. Application of genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn and winter 2004. Applied and Environmental Microbiology. 72(3): 2212-2217.

Santín M, Trout JM and Fayer R. 2007. Prevalence and molecular characterization of *Cryptosporidium* and *Giardia* species and genotypes in sheep in Maryland. Veterinary Parasitology. 146(1-2): 17-24.

Simpson JM, Santo Domingo JW, Reasoner DJ. 2002. Microbial source tracking: state of the science. Environmental Science and Technology. 36(24):5279-5288.

Smith HV, Cacciò SM, Tait A, McLauchlin J and Thompson RC. 2006. Tools for investigating the environmental transmission of *Cryptosporidium* and *Giardia* infections in humans. Trends in Parasitology. 22(4): 160-167.

Souza SL, Gennari SM, Richtzenhain LJ, Pena HF, Funada MR, Cortez A, Gregori F and Soares RM. 2007. Molecular identification of *Giardia duodenalis* isolates from humans, dogs, cats and cattle from the state of São Paulo, Brazil, by sequence analysis of fragments of glutamate dehydrogenase (gdh) coding gene. Veterinary Parasitology. 149(3-4):258-264.

Sulaiman IM, Fayer R, Bern C, Gilman RH, Trout JM, Schantz PM, Das P, Lal AA and Xiao L. 2003. Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis.* Emerging Infectious Diseases. 9(11): 1444-1452.

Thompson, RC. 2000. Giardiasis as a re-emerging infectious disease and its zoonotic potential. International Journal for Parasitology. 30(12-13): 1259-1267.

Trout JM, Santin M and Fayer R. 2003. Identification of assemblage A *Giardia* in white-tailed deer. Journal of Parasitology. 89(6): 1254-1255.

Yang W, Chen P, Villegas EN, Landy RB, Kanetsky C, Cama V, Dearen T, Schultz CL, Orndorff KG, Prelewicz GJ, Brown MH, Young KR and Xiao L. 2008. *Cryptosporidium* source tracking in the Potomac River watershed. Applied and Environmental Microbiology. 74(21): 6495-6504.

Weiss JB, Van Keulen H and Nash TE. 1992. Classification of subgroups of *Giardia lamblia* based upon ribosomal RNA gene sequence using the polymerase chain reaction. Molecular and Biochemical Parasitology 54(1): 73-86.

Wilkes G, Edge T, Gannon V, Jokinen C, Lyautey E, Medeiros D, Neumann N, Ruecker N, Topp E and Lapen DR. 2009. Seasonal relationships among indicator bacteria, pathogenic bacteria, *Cryptosporidium* oocysts, *Giardia* cysts, and hydrological indices for surface waters within an agricultural landscape. Water Resources. 43(8): 2209-2223.

Wong DA, Yip PC, Tse DL, Tung VW, Cheung DT, Kam KM. 2003. Routine use of a simple low-cost genotypic assay for the identification of mycobacteria in a high throughput laboratory. Diagnostic Microbiology and Infectious Disease. 47(2): 421-426.

Chapter 3

Occurrence of *Giardia lamblia* and *Cryptosporidium* species in a mixed urban-rural watershed[2]

3.1 Introduction

Giardia lamblia and members of the *Cryptosporidium* genus are amongst the most common human enteric parasites. Affecting human and animal populations alike, these organisms have a wide host range (Hunter and Thompson, 2005). They infect most mammalian species as well as avian and reptilian species. Zoonotic and anthropogenic subtypes of these organisms exist, thus transmission cycles can be complex and difficult to investigate.

Human infections of *Giardia* and *Cryptosporidium* typically occur either as a result of person-to-person transmission or through secondary transmission through contaminated food or water (Smith *et al*, 2007; Caccio *et al*., 2005). Contaminated water is the most common transmission vehicle reported (Karanis *et al*., 2007). These two pathogens represent a significant challenge to drinking water purveyors due to their survival in water, their resistance to some drinking water treatment approaches and their low infectious dose, should they enter drinking water distribution systems (Black *et al*, 1996; Betancourt and Rose, 2004).

Genotyping of *Giardia lamblia* and *Cryptosporidium* spp. isolates from human, animal and environmental sources is contributing to the knowledge of pathogen host range, transmission ecology and the impact on disease outcomes (Xiao and Fayer, 2008). Genotyping has identified six *G.lamblia* Assemblages (syn. subtypes), of which only two subtypes (Assemblages A and B) are infective to humans (Caccio *et al*., 2005). Both these Assemblages are zoonotic; the remaining Assemblages are host-adapted and are non-infectious to humans. There are 13 recognized *Cryptosporidium* species and it is hypothesized that over twenty species may exist (Xiao *et al*., 2004). Of the 13 species, only two (*C.parvum* and *C.hominis*), routinely infect humans. *C.parvum* is able to infect humans and other mammalian species, while the host specificity of *C.hominis* is limited to only humans (Morgan-Ryan *et al*., 2002).

[2] A version of this chapter will be submitted for publication. Prystajecky N, Huck PM, Schreier H and Isaac-Renton JL. Occurrence of *Giardia lamblia* and *Cryptosporidium* species in a mixed urban-rural watershed in southern British Columbia.

The gold-standard method for the detection of *Giardia* and *Cryptosporidium* in water is Method 1623, (USEPA, 2005). This method uses commercially available immunofluorescence assays and immunomagnetic separation kits, which use antibodies that directed against the all members of the *Giardia* and *Cryptosporidium* genera. Thus, detection may identify species and subtypes of these organisms that are not a threat to human health. As such, for a thorough understanding of the human health impact of these organisms in water supplies, supplementation of the gold-standard detection method with genotyping is needed.

In British Columbia (BC), due to the geographic characteristics of this province in Western Canada, there is a general perception that water supplies are abundant and pristine. However, public health surveillance has shown that British Columbians suffer from a greater incidence of gastrointestinal illness and have experienced a greater number of waterborne outbreaks of disease than residents in other parts of Canada (PHO, 2001). There have been 29 documented waterborne outbreaks of disease since 1980, the majority of which were caused by *Giardia* (13 outbreaks) or *Cryptosporidium* (three outbreaks). Lack of adequate treatment of drinking water was the main reason for the majority of outbreaks. While the incidence of giardiasis and cryptosporidiosis in BC has declined significantly in the past decade, likely due to increased regulatory compliance to drinking water treatment standards, the incidence of these diseases still exceeds rates in the rest of Canada (PHO, 2001). In British Columbia in 2008, rates of cryptosporidiosis were 2.6 cases per 100,000 and rates of giardiasis were 14.2 cases per 100,000 (BCCDC, 2009).

This study was conducted as a substudy in a larger source-to-tap investigation in a BC mixed urban-rural community. Genotyping of *Giardia* and *Cryptosporidium* isolates were determined to identify genotype prevalence and variability. The results were compared to traditional chemical and bacteriological water quality indices. This report provides follow-up to previous *Giardia* and *Cryptosporidium* studies conducted in BC and how these organisms continue to impact BC's water supplies and the public health.

3.2 Materials and Methods

3.2.1 Site Description

Water samples were collected in the Township of Langley, an urban-rural community located 40 km east of Vancouver, in the Fraser Valley of BC, Canada (Figure 3-1). The Township's inhabitants use a variety of drinking water sources, including untreated groundwater from private wells, treated groundwater from municipal wells and a mixture of municipal treated groundwater and treated surface water supplied by the Greater Vancouver Water District. Treated municipal groundwater samples were collected from the Hopington Aquifer at one site (Arcadia Municipal Well) and surface water samples were collected from Coghlan Creek and the Salmon River sites (Figure 3-2).

Figure 3-1: Location of the Township of Langley within British Columbia.

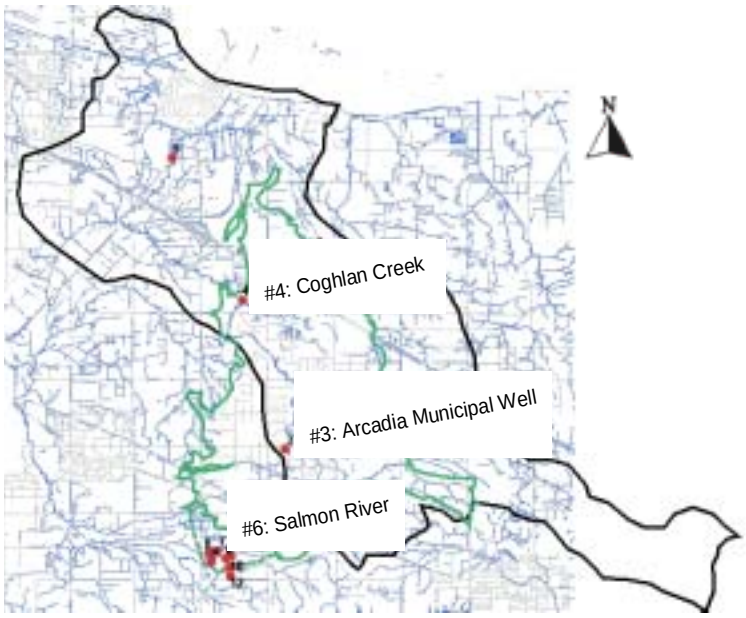


Figure 3-2: Sampling sites within the Salmon River watershed. Sites numbered 3, 4 and 6 were used in this study for genotyping of *Giardia* and *Cryptosporidium* isolates. The Hopington Aquifer is outlined in black.

Coghlan Creek is a tributary of the Salmon River and flows through the Hopington Aquifer. It is largely influenced by groundwater in the summer months (Wernick *et al*., 1998). The Hopington Aquifer provides drinking water for 3,000 families, yet is described as the most vulnerable aquifer in the Lower Fraser Valley. Persistent high nitrate values suggest that both the surface water and groundwater may have significant pollution impacts (Schreier *et al*., 1999; Wernick *et al*., 1998).

3.2.2 *Giardia* and *Cryptosporidium* Sampling

Samples were collected bi-weekly over a two-year study period, from September 2004 to November 2006. Method 1623 protocols were followed for sample collection (USEPA, 2005). Treated groundwater and untreated surface water samples were collected by filtration using an open-disc foam filter (IDEXX Filta-Max, Westbrook ME, USA) using a pump at a flow rate of 0.2-4.5L/minute. Filtered water volumes varied with the water source; up to 550L of treated water was filtered and up to 50L of raw water was filtered, depending on the turbidity of the source water. Samples were transported in coolers on ice to the British Columbia Centre for Disease Control (BCCDC), stored at

4°C and processed within 96 hours. Sampling of the Arcadia municipal groundwater well was discontinued after a year due to lack of parasite detection during the first year of study.

3.2.3 Sample Processing

All samples were processed using automatic wash station (IDEXX, Westbrook, ME, USA), which allows both filter elution and sample concentration to be completed within the same unit. The resulting sediment was eluted by repeated expansions and compressions of the foam disc using a wash buffer and concentrated using a concentrator tube fitted with a 3µm membrane to remove non-target debris and to reduce sample volume, following the manufacturer's instructions.

3.2.4 Immunomagnetic Separation

The resulting concentrates were further processed by immunomagnetic separation (IMS) (Dynabeads-GC Combo Kit, Dynal Biotech, Brown Deer, WI, USA) to target *Giardia* and *Cryptosporidium* specifically and remove potential PCR inhibitors. IMS purification followed the manufacturer's procedure with the exception of the dissociation step; the suggested acid dissociation was replaced with heat dissociation (Ware *et al*., 2003). The resulting purified sample was then used for an immunofluorescence assay (IFA) for the enumeration of *Giardia* cysts and *Cryptosporidium* oocysts. If the pellet size following elution exceeded 0.5mL, two IMS tubes were set up.

3.2.5 Immunofluorescence Assay and Microscopic Examination

The purified sample was applied to a Dynal Spot-On slide and dried overnight in a refrigerator at 4°C. The slides were then methanol-fixed and stained with a fluorescein isothiocyanate (FITC) conjugated antibody (Easy-Stain, Biotechnology Frontiers, North Ryde, Australia) and 4'6'-diamidino-2-phenylindole (DAPI). Presumptive positive cysts or oocysts identified by IFA were confirmed with DAPI staining and differential interference contrast (DIC) microscopy.

3.2.6 Slide Scraping Procedure

The overall study approach is indicated in Figure 3-3. Molecular analyses were added after IFA step, commencing with slide-scrapping to recover cysts and oocysts from the IFA slides.

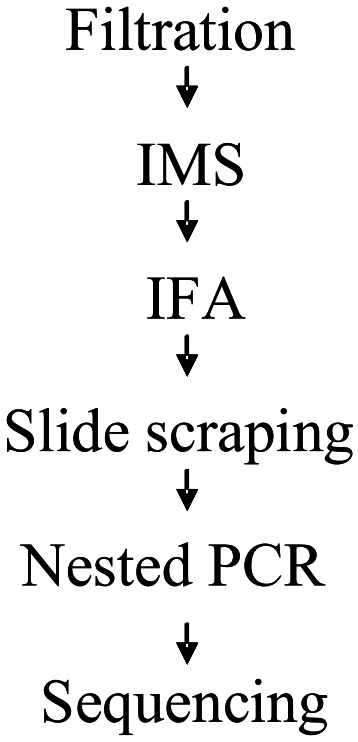


Figure 3-3: Incorporation of molecular analyses into Method 1623.

The microscope slides were then temporarily stored at 4°C until further processing. For long-term storage of the slides, the cover-slips were removed and the slides were gently washed with warmed PBS solution to remove the DIBCO-glycerol. Slides were dried at room temperature and then stored at -20°C. To remove the methanol-fixed cysts and oocysts from the slides for further analysis, slides were removed from the freezer and processed immediately as described by Ruecker *et al*. (10). A total volume of 180µl of tissue lysis buffer (Buffer ATL) was added to the well (Qiagen, Mississauga, ON) in 45µl aliquots, alternating between rinsing the slide and scraping the slide using a pipette tip. Between each step the buffer was transferred to a sterilized microcentrifuge tube and kept on ice. Samples were stored at -20°C until further processing. The detection limit for the recovery of cysts and oocysts from the Spot-On slides was determined using cysts and oocysts purchased from Waterborne Inc (New Orleans, LA, USA) in a series of spiking experiments, spiking slides with 0-30 cysts and oocysts per slide and evaluating recoveries using PCR.

3.2.7 Genomic DNA Extraction

Genomic DNA was extracted by freeze-thaw with proteinase K digestion. Six freeze-thaw cycles were used, alternating between a 2 minute freeze in liquid nitrogen and a five minute thaw at 65°C, followed by the addition of 400µg of proteinase K (Qiagen) and an overnight digestion in a thermomixer at 56°C to further degrade the cyst and oocyst walls and to inactivate DNase activity. The resulting lysates were then purified using a QiaAMP DNA Micro Kit (Qiagen), following the manufacturer's protocol for tissue lysis for samples with low numbers of target DNA. Carrier RNA was added to Qiagen's Buffer AL (lysis buffer) at a concentration of 10µg/L (both supplied in the QiaAMP DNA Micro Kit) before processing each batch of lysates. After the samples were applied to the MinElute columns and two wash steps were performed, DNA was eluted from the column using 50µl of distilled molecular grade water. For samples in which two acid dissociation steps were used, each slide resulting from each acid dissociation step was treated as a separate sample. Samples were stored at -20°C for genotyping. A spiked positive control and a negative control were also included in the genomic extraction step.

3.2.8 Nested PCR

Nested polymerase chain reaction (PCR) protocols were used for the amplification of *Giardia lamblia* DNA and *Cryptosporidium* spp. DNA to provide adequate target sequences for genotyping purposes. For genotyping of *G.lamblia*, the 18s rRNA gene was amplified in a nested PCR reaction as previously described (Appelbee *et al*., 2005 and Hopkins *et al*., 1997).

For primary PCR, primers Gia2029 (5'-AAGTGTGGTGCAGACGGACTC-3') and Gia2150c (5'-CTGCTGCCGTCCTTGGATGT-3') were used to produce a 497bp product. This product was used in the secondary PCR using secondary primers RH11 (5'-CATCCGGTCGATCCTGCC-3') and RH4 (5'-AGTCGAACCCTGATTCTCCGCCAGG-3') to yield a 292bp product. The PCR reaction mix was comprised of 25µl HotStarTaq MasterMix (Qiagen, Mississauga, ON) containing 1X PCR Buffer, 1.5mM $MgCl_2$, 2.5 U of modified *Taq* polymerase, 200µM each deoxynucleoside triphosphate (dNTP), 25pmoles of each primer, 5% DMSO, 10mg/ml BSA and 2.5 - 5µl of purified genomic lysate in a 50µl reaction volume. The PCR reaction was performed using a 15 minute hot-start at 95ºC, followed by 35 cycles of PCR (96ºC for 45s, 55ºC for 30s, and 72ºC for 45s) on a thermocycler (Mastercycler Gradient, Eppendorf, Westbury, NY, USA). The secondary PCR cycle conditions were identical to the primary PCR except the annealing temperature was increased from

55°C to 59°C for the annealing period. Negative controls and positive controls using genomic DNA extracted from purchased cysts and oocysts (Waterborne Inc, New Orleans, LA, USA).

To genotype *Cryptosporidium*-positive samples, a nested PCR targeting a ~825bp segment of the 18s rDNA gene as previously described was used (Xiao *et al*., 1999). For primary PCR, primers CXF1 (5'-TTCTAGAGCTAATACATGCG-3') and CXR1 (5'-CCCTAATCCTTCGAAAGAG-GA-3') were used to amplify the entire 18s rDNA gene. The reaction mix comprised of 1x PCR buffer (Invitrogen), 6mM MgCl2, 1.25U Platinum Taq polymerase (Invitrogen), 200µM each dNTP, 10pmoles of each primer and 5µl of purified genomic lysate in a 100µl reaction volume. The PCR reaction was performed for 35 cycles (94°C for 45s, 55°C for 45s and 72°C for 60s), preceded by a hot-start step (94°C for 3 minutes) and a final extension step (72°C for 7 minutes) using a thermocycler (Mastercycler Gradient, Eppendorf, Westbury, NY, USA). For the secondary PCR, a target of 826bp to 864bp was amplified with primers CXF2 (5'-GGAAGGGTTGTATTTATTAGATAAAG-3') and CXR2 (5'-AAGGAGTAAGGAACAACC-TCCA-3') using the same PCR reaction mix as the primary PCR, with 2µl of primary PCR product replacing the genomic DNA and the $MgCl_2$ concentration reduced to 3µM. Negative controls and positive controls using genomic DNA extracted from purchased cysts and oocysts (Waterborne Inc, New Orleans, LA, USA).

3.2.9 Sequencing

Secondary PCR products were visualized using agarose gel electrophoresis and ethidium bromide staining under UV light. PCR-positive samples were purified using QiaQuick PCR Purification Kit according to the manufacturer's instructions (Qiagen) and quantified using a spectrophotometer (Ultrospec 3100 Pro, BioChrom, Ltd., Cambridge, United Kingdom) and diluted to a final volume of 10ng/µl. The samples were sequenced using the BigDye Terminator Version 3.1 Cycle Sequencing Kit (Perkin-Elmer, Boston, MA, USA) on an ABI 3100 automated sequencer (Perkin-Elmer, Boston, MA, USA) in-house at the BCCDC. Both forward and reverse sequencing of the secondary product were performed for increased sequence accuracy. Sequencing reactions comprised of 2µl of BigDye Terminator sequencing reagents, 6µl of BigDye Terminator sequencing Buffer, 1, 1.6 µl of 2 µM concentrations of primers. Sequencing reactions consisted of 2 µl of BigDye terminator cycle sequencing reagents, 6 µl of BigDye terminator cycle sequencing buffer,3.2 pmoles of primer,1-2µl purified PCR product and molecular grade distilled water to make a 20µl reaction volume. The cycle sequencing was performed for 25 cycles (96°C for 10 s, 50°C for 5 s, and 60°C for 4 min) on a 9600

GeneAmp Thermocycler (Perkin-Elmer, Boston, MA, USA). Sequence products were ethanol-acetate precipitated and dried, followed by reconstitution in Hi-Di formamide and loaded into the ABI 3100 sequencer.

3.2.10 Data Analysis

Sequence data were assembled and analyzed using SeqMan and EditSeq software packages (DNAStar Inc, Madison, WI, USA). Sequences were identified using the nucleotide-nucleotide BLAST program on the NCBI website (blastn algorithm). Published sequences for the 18s rDNA gene for *G.lamblia* and *Cryptosporidium* spp. were obtained from GenBank and used for phylogenetic analyses. Multiple alignments between known sequences and isolates identified in this study were performed using the MegAlign program (DNAStar Inc, Madison, WI, USA), using CLUSTAL V and W alignments for phylogenetic analyses. Microsoft Excel Statistical Package was used for all statistical analyses.

3.2.11 Water Quality Analyses, Climate Data and Human Health Data

Water chemistry data were collected using gas chromatography-mass spectrometry *(*GC-MS*)* for the following parameters: nitrates ($N\text{-}NO_3$), ammonia ($N\text{-}NH_3$), phosphate and chloride. Turbidity and conductivity were determined using a dual function probe, calibrated before each use. Total coliform and fecal coliform testing were conducted at the BCCDC, by membrane filtration. Climate data was obtained from the Environment Canada's National Climate Data and Information Archive, using data from the nearest weather station (Abbotsford A Station, Climate ID 1100030). Giardiasis reportable communicable disease (RCD) data was captured for the first year (2004-2005) of the study period, for cases within the study area.

3.3 Results

3.3.1 Chemical and Biological Indicators

A summary of all study parameters, including ranges, are indicated below (Table 3-1 and Table 3-2). There were no statistically significant differences in chemical parameters between the two study sites. The Coghlan Creek site demonstrated slightly elevated nitrate levels compared to Salmon River site, while the Salmon River site demonstrated elevated chloride levels in comparison to the Coghlan Creek site.

Total coliform and fecal coliforms were detected in 100% of samples collected from the Coghlan Creek site. All but one sample (98%) from the Salmon River sites were positive for total and fecal coliforms. Total coliform and fecal coliform concentrations were higher in the Salmon River site, suggesting that Coghlan Creek is not the predominant source of fecal contamination of the Salmon River. Parasite detection did not follow patterns observed for bacteriological indicators; more detailed analyses of parasite detection are described below.

Table 3-1: Summary of chemical and microbiological indicators collected in Coghlan Creek, Township of Langley, British Columbia

Parameter	Cl^-	PO_4^{3-}	NH_4^-N	NO_3^-N	Conductivity	Turbidity	Total Coliforms	Fecal Coliforms	*Crypto-sporidium*	*Giardia*
	mg/L	mg/L	mg/L	mg/L	MHO	NTU	CFU/100mL	CFU/100mL	#/100L	#/100L
Median Value	11.83	0.02	0.08	3.85	149.62	16.46	3831	327	333	107
Standard Deviation	3.32	0.02	0.12	1.23	33.50	43.34	4236	636	2554	469
Minimum	2.61	0.00	0.00	0.42	69.00	0.60	90	1	0	0
Maximum	31.45	0.08	0.53	5.96	208.30	231.00	23000	3600	20600	3800
% Samples Above Detection Limit	n/a	n/a	n/a	n/a	n/a	n/a	100%	100%	56%	91%

Table 3-2: Summary of chemical and microbiological indicators collected in Salmon River, Township of Langley, British Columbia

Parameter	Cl^-	PO_4^{3-}	NH_4^-N	NO_3^-N	Conductivity	Turbidity	Total Coliforms	Fecal Coliforms	*Crypto-sporidium*	*Giardia*
	mg/L	mg/L	mg/L	mg/L	MHO	NTU	CFU/100mL	CFU/100mL	#/100L	#/100L
Median Value	16.04	0.02	0.09	2.59	162.17	14.95	6295	737	11	32
Standard Deviation	3.72	0.03	0.12	0.64	41.15	21.85	12496	1806	20	104
Minimum	6.15	0.00	0.00	0.86	60.00	2.40	0	0	0	0
Maximum	25.00	0.10	0.56	4.14	238.70	86.00	85000	9600	126	730
% Samples Above Detection Limit	n/a	n/a	n/a	n/a	n/a	n/a	100%	100%	74%	77%

Table 3-3: Summary of chemical and microbiological indicators collected in Arcadia Municipal drinking water well (groundwater), Township of Langley, British Columbia

Parameter	Cl^-	PO_4^{3-}	NH_4^-N	NO_3^-N	Conductivity	Turbidity	Total Coliforms	Fecal Coliforms	*Crypto-sporidium*	*Giardia*
	mg/L	mg/L	mg/L	mg/L	MHO	NTU	CFU/100mL	CFU/100mL	#/100L	#/100L
Median Value	13.31	0.01	0.06	10.71	213.27	0.40	14	11	0	0
Standard Deviation	1.24	0.00	0.08	1.22	18.23	1.05	118	94	0	0
Minimum	9.23	0.00	0.00	5.07	129.20	0.10	0	0	0	0
Maximum	18.03	0.02	0.34	12.16	262.00	6.00	990	790	0	0
% Samples Above Detection Limit	n/a	n/a	n/a	n/a	n/a	n/a	8.20%	1.40%	0%	0%

3.3.2 *Giardia* and *Cryptosporidium* Prevalence

Using the gold-standard method (Method 1623), *Cryptosporidium* spp. were detected in 63% of surface water samples, at concentrations ranging from 0 to 20,600 oocysts per 100L of water (Figure 3-4). *Cryptosporidium* spp. oocysts were detected more frequently in the Salmon River site (73%, n=49) than in the Coghlan Creek site (55%, n=65). Omitting the outlying sample (20,600 oocysts/100L, October 14, 2004, Coghlan Creek), the average *Cryptosporidium* concentration was 14.6 oocysts/100L.

Using the gold-standard method (Method 1623), *Cryptosporidium* spp. were detected in 63% of surface water samples, at concentrations ranging from 0 to 20,600 oocysts per 100L of water (Figure 3-4). *Cryptosporidium* spp. oocysts were detected more frequently in the Salmon River site (73%, n=49) than in the Coghlan Creek site (55%, n=65). Omitting the outlying sample (20,600 oocysts/100L, October 14, 2004, Coghlan Creek), the average *Cryptosporidium* concentration was 14.6 oocysts/100L.

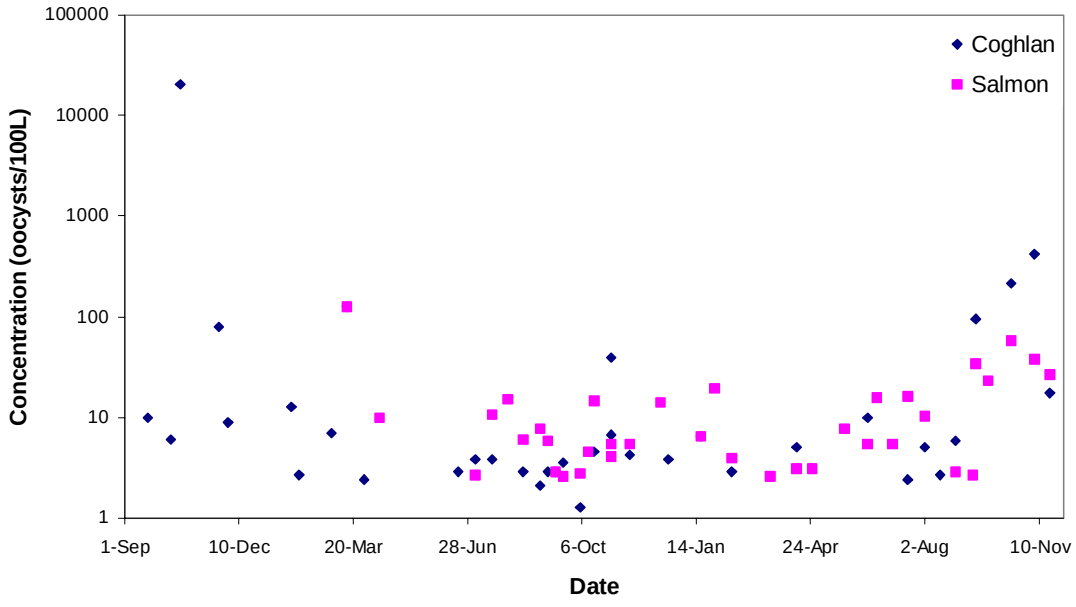


Figure 3-4: Prevalence of *Cryptosporidium* at two sampling sites (Coghlan Creek and Salmon River) in the Township of Langley, British Columbia using Method 1623.

Giardia was detected more frequently than *Cryptosporidium*, and at higher concentrations. *Giardia* cysts were detected in 86.3% of surface water samples, at concentrations ranging from 0 to 3, 800 cysts per 100L of water (Figure 3-5). Omitting the outlying sample (3,800 cysts/100L, March 15, 2005, Coghlan Creek), the average *Giardia* concentration was 41.6 oocysts/100L. *Giardia* cysts were detected more frequently in the Coghlan Creek site (92%, n=65) than in the Salmon River site (78%, n=49).

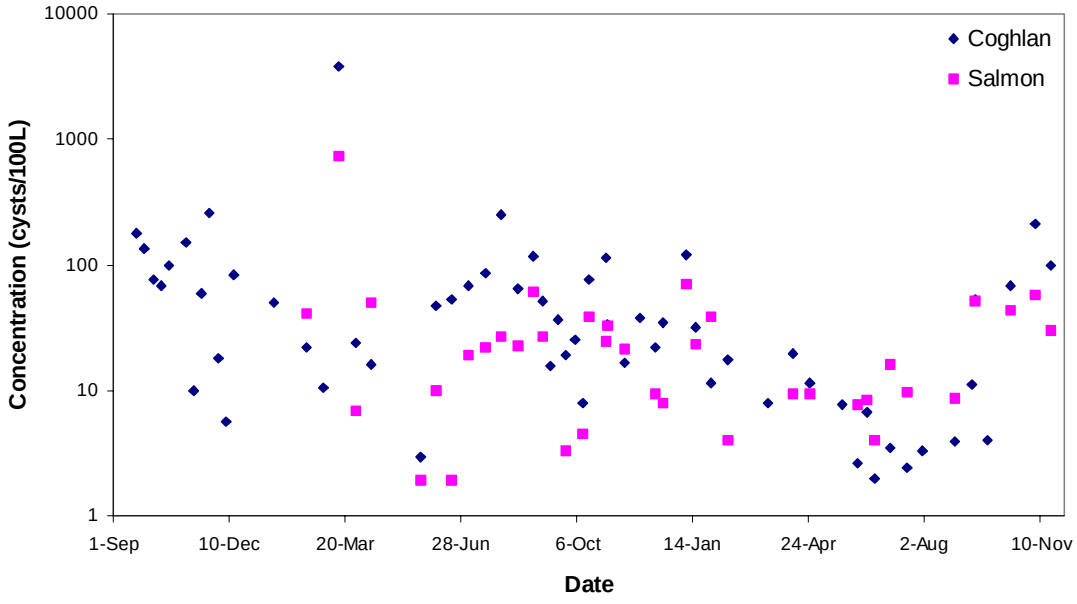


Figure 3-5: Prevalence of *Giardia* at two sampling sites (Coghlan Creek and Salmon River) in the Township of Langley, British Columbia using Method 1623.

At the Coghlan Creek site, two separate incidents of highly elevated *Giardia* and *Cryptosporidium* concentrations were observed. One day (Fall, 2004) *Cryptosporidium* concentrations were 20,600 oocysts per 100L of water and on another (Spring, 2005), *Giardia* concentrations were 3,800 cysts per 100L of water. Standard laboratory methods do not require microscopic confirmation of all detected (oo)cysts; rather, only a subset of organism are examined by DAPI and DIC microscopy. All organisms examined were confirmed to be *Cryptosporidium* (Fall, 2004) or *Giardia* (Spring, 2005). PCR amplification and sequencing confirmed that these samples contained the appropriate organisms.

However, it could not be proven that all cyst and oocyst structures in these two incidents contained only *Giardia* and *Cryptosporidium.*

On one occasion (Spring, 2005), significant cross-reactivity of the IMS and FA antibodies was observed; organisms that were a similar shape to *Giardia* but of a larger size, fluoresced a strong apple-green by IFA. However, the internal structures as observed by DIC microscopy were not consistent with *Giardia* internal morphology and numerous nuclei were observed by DAPI staining.

Neither *Giardia* nor *Cryptosporidium* were detected in the Arcadia Municipal Well, thus sampling was discontinued after one year.

3.3.3 *Giardia* and *Cryptosporidium* Molecular Analyses

Detection limits of the slide-scraping method and subsequent molecular analyses were conducted by spiking parasite preparations, purchased from Waterborne Inc, onto slides. Results showed that nested PCR could detect *Giardia* and *Cryptosporidium* from slides spiked with as low as two cysts and one oocyst, respectively, although slides with less than five organisms demonstrated variable results (results not shown). Since each PCR reaction used 2-5µl of the 50µl nucleic acid preparation, the detection limit per PCR reaction is 0.12-0.2 cysts and 0.04-0.1 oocysts, in slides spiked with parasites in laboratory-grade water. Detection limits in environmental specimens was greater however, due to the presence of inhibitory substances in water samples, degradation of nucleic acid due to environmental exposures and the age of the (oo)cyst(s).

By nested PCR, 77% of *Giardia* samples that were positive by microscopy were also positive by at least one nested PCR (PCR were conducted in duplicate), indicating that some positive samples would have been negative if only PCR was conducted. Performing the PCR in triplicate on a subset of samples did increase PCR sensitivity from 77% to 80%; because of the limited improvement and increased cost, performing PCR in triplicate for *Giardia* was discontinued. Retrospective analysis comparing the microscopy record (fluorescence intensity, DAPI staining of nuclei and DIC characteristics) and PCR did not demonstrate any consistent relationship between a sample that was positive by microscopy but negative by PCR. However, it was noted that the majority of cysts lacked all four nuclei by DAPI staining. This could be a function of the nuclei not being visible (due to organism position on the fixed microscope slide) or it may suggest loss of genetic material which would impact the detection by PCR.

Sequence analyses were performed to determine the Assemblage designation of the *Giardia* cysts detected. The majority of samples were from zoonotic Assemblages (Assemblages A and B) (Figure 3-6). Assemblage A was more predominant (55.5% of sequenced samples) than Assemblage B (34.5%). Despite significant livestock operations in the area, Assemblage E was detected infrequently (1.5%).

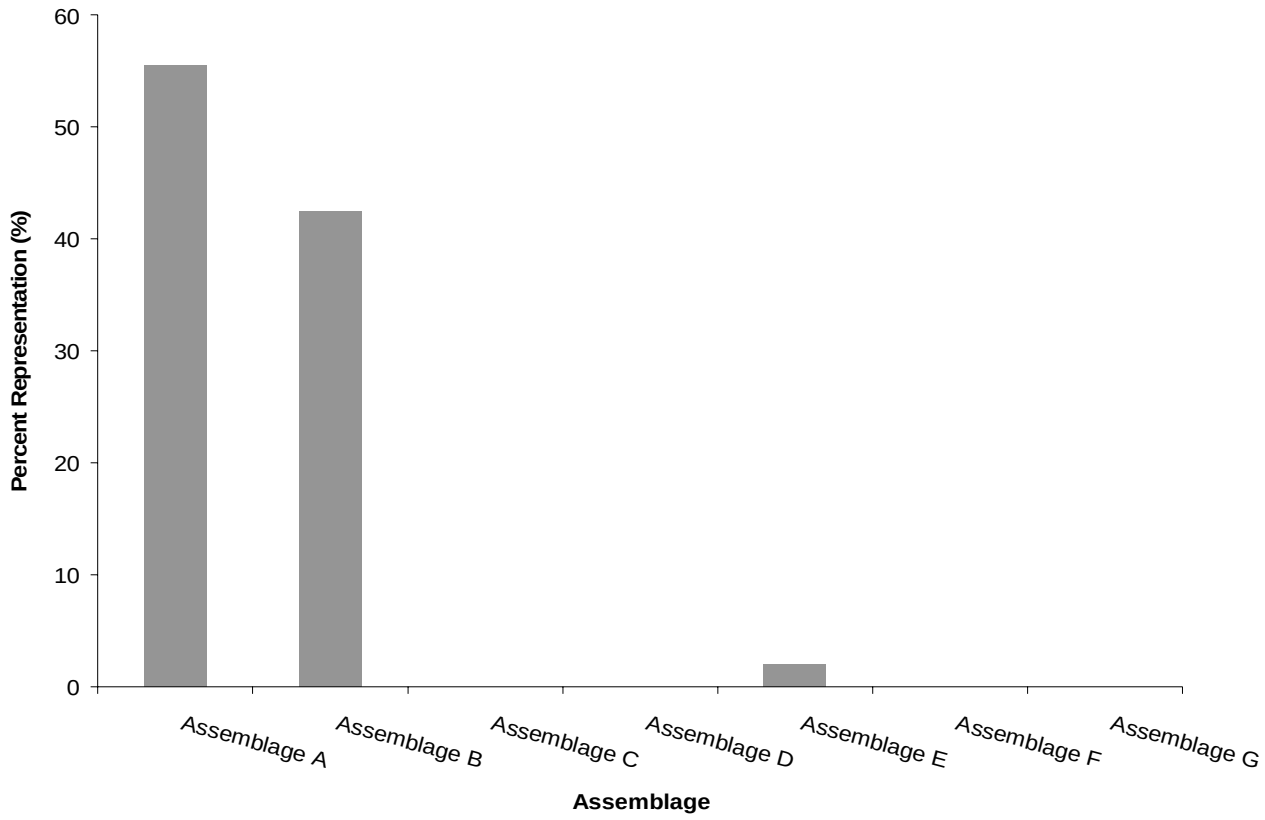


Figure 3-6: Proportion of *Giardia lamblia* Assemblages detected in water sources in the Township of Langley, as determined by nested PCR and sequence analysis, indicating the majority of isolates represent a potential public health risk.

Giardia sequence data was compared, phylogenetically, to published sequenced (Genbank) and to sequences from a library of *G.lamblia* isolates collected primarily in British Columbia. These library sequences were collected from historical clinical, environmental and animal samples collected during previous research studies or giardiasis outbreaks. The phylogenetic analysis indicates that isolates collected in the study have greater sequence homology to one another, than to historical or published sequences (Figure 3-7). Assemblage A isolates clustered most closely with an isolate collected from a white-tailed deer (AY302561), while isolates from Assemblage B clustered in separate outgroups, compared to reference strain H3 (DQ116605). Interestingly, numerous isolates had 100% sequence homology to water samples collected in previous BC studies but not collected in other geographical locations to date.

This may be indicative of the limited geographical range of isolates represented in publications, or may represent genetic variants which are specific to British Columbia. A small number of isolates did not group with published sequences or sequences from the study period; these may represent unique genetic isolates or sequence data may be erroneous due to the presence of mixed genotypes or other interfering genetic material.

For *Cryptosporidium* isolates, 60% of samples were positive by microscopy were also positive by at least one nested PCR (PCR conducted in triplicate). This lower detection rate likely reflects lower concentrations of oocysts on the IFA slides; 48% of slides contained only a single oocyst. Therefore any losses during samples processing, along with non-homogenous distribution of nucleic acids in genomic extracts, could greatly impact PCR success.

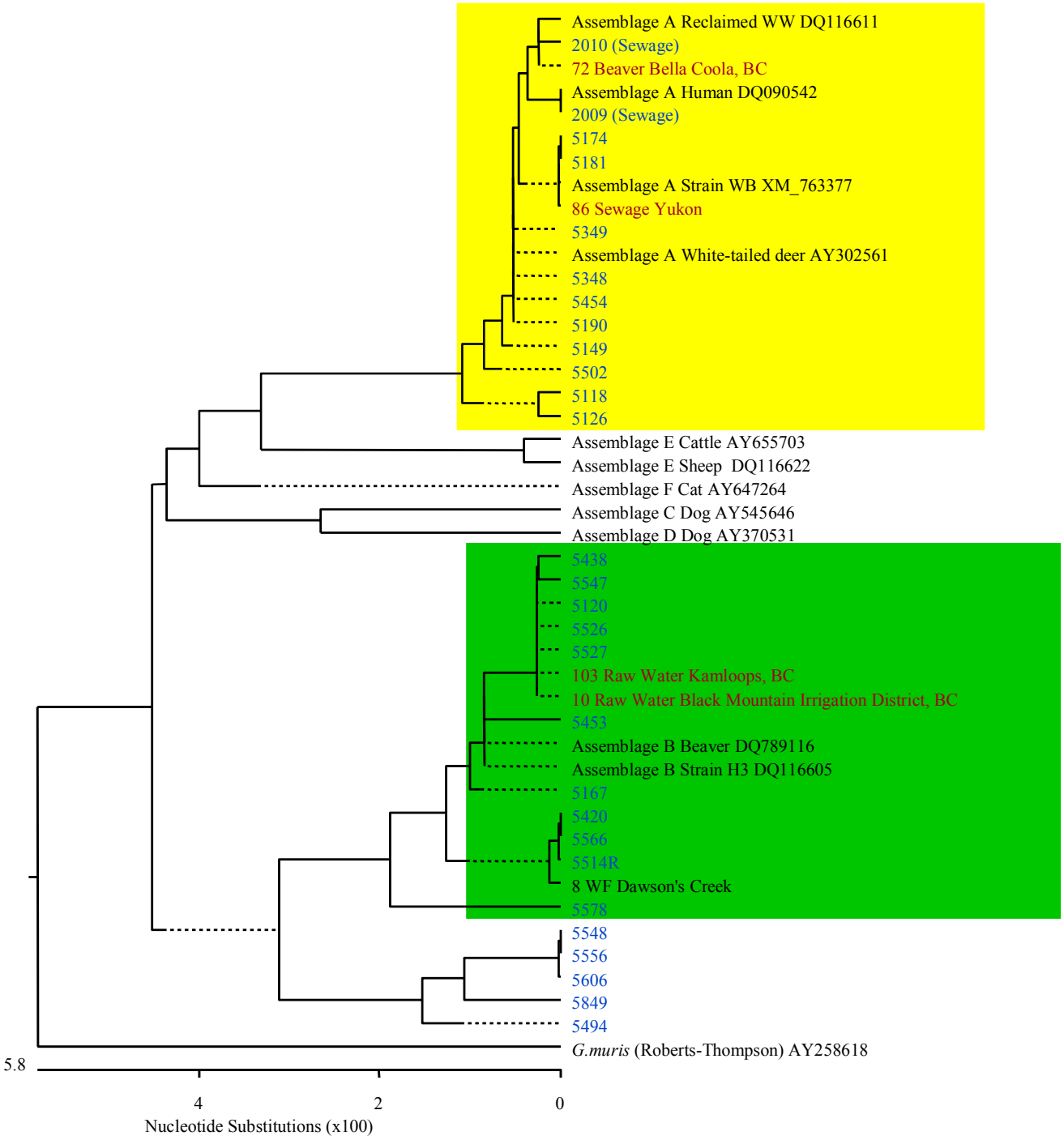


Figure 3-7: Phylogenetic analysis of a subset of *Giardia lamblia* samples (Clustal W alignment, DNAStar). Township of Langley are indicated in blue, red indicates in-house *Giardia* library isolates and isolates in black from GenBank.

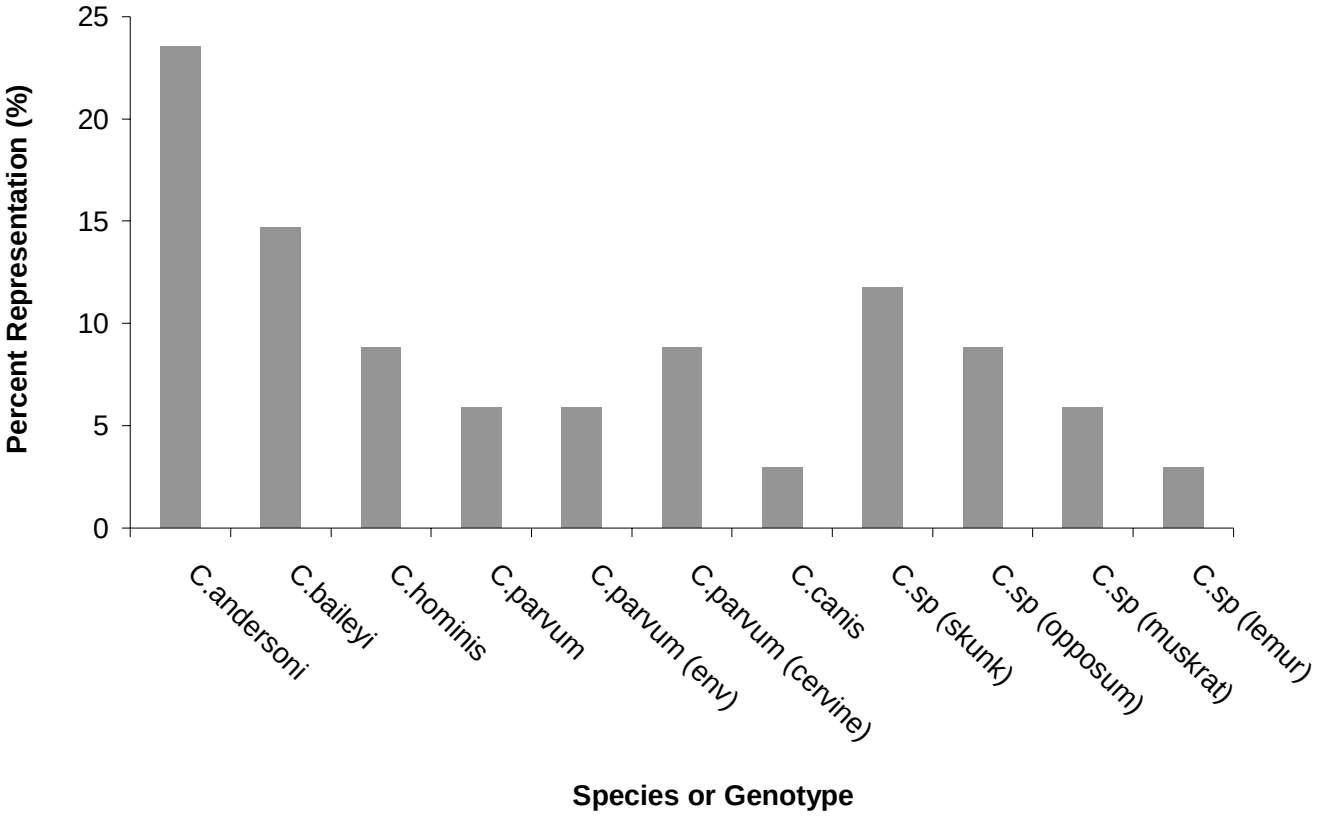


Figure 3-8: Proportion of *Cryptosporidium* spp. detected in water sources in the Township of Langley, as determined by nested PCR and sequence analysis.

Sequencing of the 18s rRNA gene for *Cryptosporidium* demonstrated that *C.andersoni* was detected most frequently (24% of sequences), followed by *C.baileyi* (15%) and *Cryptosporidium* skunk genotype (12%) (Figure 3-8 and

Figure 3-9). These species/genotypes likely pose little threat to human health; both the skunk genotype or *C.baileyi* have never been detected in humans and there is only one report of *C.andersoni* being found in humans. However, a variety of *C.parvum* genotypes (21%) were detected, along with *C.hominis* (9%), which represents a potential threat to human health. While *C.parvum* can occur in both humans and animals, *C.hominis* can only originate only from human sources and is indicative of

human contamination in the watershed. A BC specific library for *Cryptosporidium* isolates is not available and therefore isolates were only compared to published sequences available in Genbank.

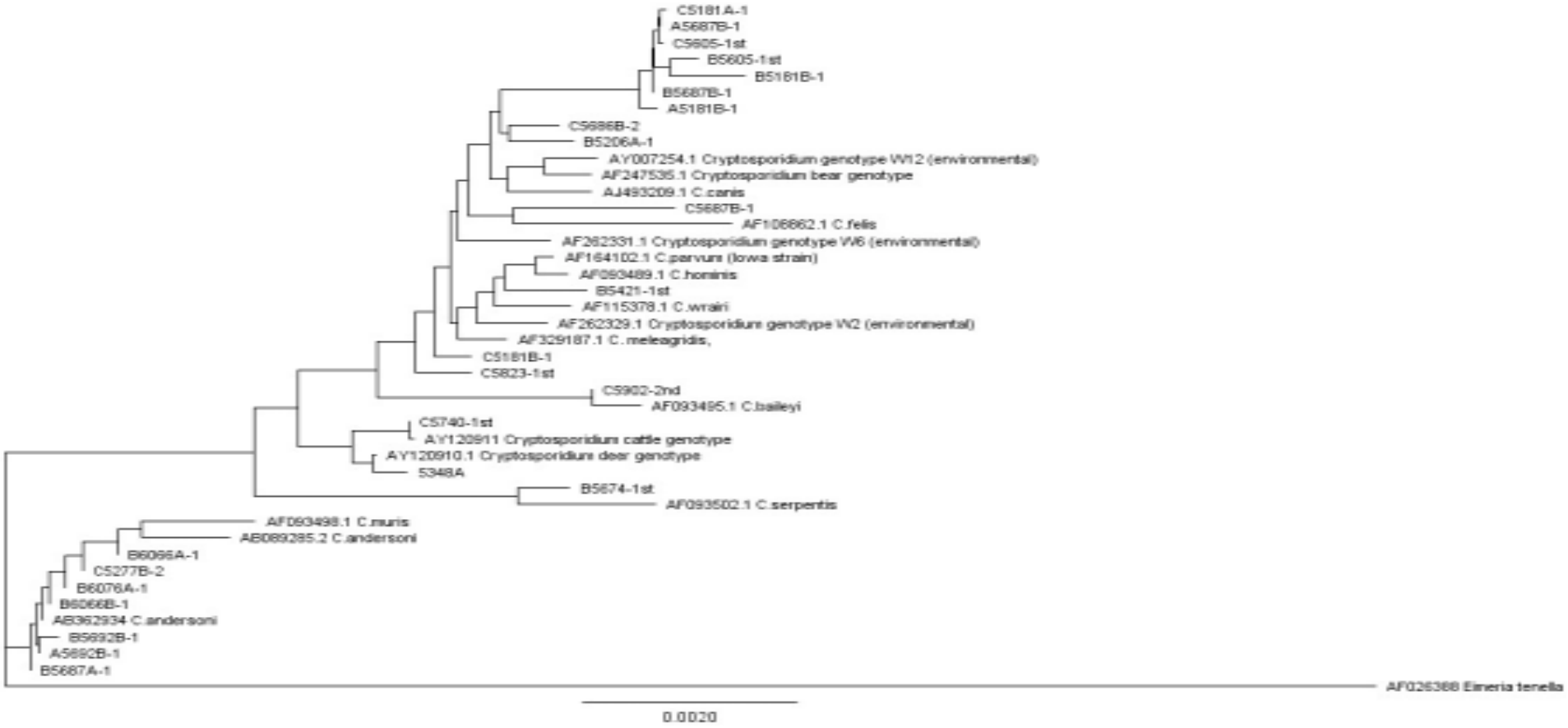


Figure 3-9: Phylogenetic analysis of a subset of *Cryptosporidium* samples collected in the Township of Langley, using *Eimeria tenella* as an outgroup (Geneious Tree Builder, Geneious Pro 4.54)

3.3.4 Statistical Analyses

Correlational analyses were conducted to compare indicator parameters to *Cryptosporidium* and *Giardia* concentrations. The chemical parameters and microbial indicators did not show any correlational relationship to *Cryptosporidium* and *Giardia* concentrations (correlation coefficient r below 0.4) (Table 3-4).

Table 3-4: Pearson correlation comparing chemical and bacteriological indicator parameters to *Cryptosporidium* and *Giardia* concentrations at two study sites in the Township of Langley. R values between 0.4 and 0.7 were considered moderate relationships, while values greater than 0.7 were considered to be strong relationships. The * denotes a statistically significant relationship, as determined by linear regression ($p<0.05$).

	Coghlan Creek		Salmon River	
Parameter	***Cryptosporidium***	***Giardia***	***Cryptosporidium***	***Giardia***
	r	**r**	**r**	**r**
Total Coliforms	-0.09	0.26*	0.02	-0.04
Fecal Coliforms	0.02	-0.04	0.13	-0.01
P	0.13	-0.02	0.16	0.07
Cl-	-0.05	0.00	-0.04	-0.04
NH4-N	-0.05	0.02	0.00	0.05
NO3-N	-0.10	0.00	0.07	-0.08
Conductivity	-0.03	-0.03	0.09	-0.04
Turbidity	0.76*	0.81*	0.56*	0.72*
Cryptosporidium	-	0.14	0.86*	-
Giardia	0.14	-	-	0.86*

Turbidity data was not collected the entire study period; however, for the period in which the data were collected, there was a strong, statistically significant, relationship between parasite occurrence and turbidity. Turbidity was a better predictor of *Cryptosporidium* concentration in Coghlan Creek, than the Salmon River while turbidity was a good predictor of *Giardia* occurrence at both sites. In the Salmon River site, *Giardia* and *Cryptosporidium* concentrations were strongly correlated ($r = 0.86$, $p<0.05$) suggesting common or similar sources of the organisms. In the Coghlan Creek site, *Cryptosporidium* and *Giardia* concentrations were not correlated, suggesting a difference in the

loading patterns (and potentially the sources) of *Giardia* and *Cryptosporidium* between the tributary and main river.

Detailed rainfall analyses were conducted to determine if rainfall occurrence could predict parasite prevalence or concentration (Table 3-5). Cumulative 24-hr, 48-hr, 72-hr and 96-hr rainfall were analyzed against *Giardia* and *Cryptosporidium* concentrations. There was no relationship between parasite occurrence and preceding cumulative rainfall. Of interest, parasite loading continued to occur in the summer months, in the absence of rainfall. Analysis of significant rainfall events, defined as a rainfall event in the 92nd percentile (Thomas *et al.*, 2005) also did not show statistical significance, unless for seasonal norms were accounted for (data not shown).

Table 3-5: Correlational analysis relating cumulative rainfall (24-, 48-, 72- and 96-hr) to *Cryptosporidium* and *Giardia* concentrations at two study sites in the Township of Langley. R values between 0.4 and 0.7 were considered moderate relationships, while values greater than 0.7 were considered to be strong relationships.

Coghlan Creek

Parameter	24-hr r	48-hr r	72-hr r	96-hr r
Giardia	0.02	0.06	0.08	0.08
Cryptosporidium	0.05	0.09	0.09	0.05

Salmon River

Parameter	24-hr r	48-hr r	72-hr r	96-hr r
Giardia	0.03	0.05	0.07	0.08
Cryptosporidium	0.04	0.04	0.06	0.06

3.3.5 Human Health Data

Giardiasis and cryptosporidiosis are reportable communicable diseases (RCD) in British Columbia. Within the study area, there were 10 laboratory confirmed cases of giardiasis and seven cases of cryptosporidiosis. However, public health experts agree that this is an under-estimation of real

incidence of these diseases, due to under-reporting and limited ordering of laboratory tests by physicians (Straif-Bourgeois and Ratard, 2007).

For giardiasis, RCD data were compared to bi-weekly *Giardia* water concentrations (Figure 3-10). RCD cases were determined to be part of the study area using the first three characters in the postal code. Due to the limited number of cases, analyses were accomplished by visual inspection. It should be noted that the average incubation period for giardiasis is a week to 10 days and the average reporting delay for RCD is one to fours weeks. Based on this information, it is expected that a reported case will be reported as a RCD approximately three weeks after exposure to *Giardia* cysts. Giardiasis RCD cases were clustered in the fall and spring seasons, but demonstrated no consistent relationship to (seasons based on solstices and equinoxes, not climate characteristics). Historical RCD data (1996-2005) indicated that giardiasis occurs year-round, with increased incidence in the early fall weeks (data not shown). There was no observable relationship between giardiasis RCD incidence and increased concentrations in surface water during the study period (Figure 4-10).

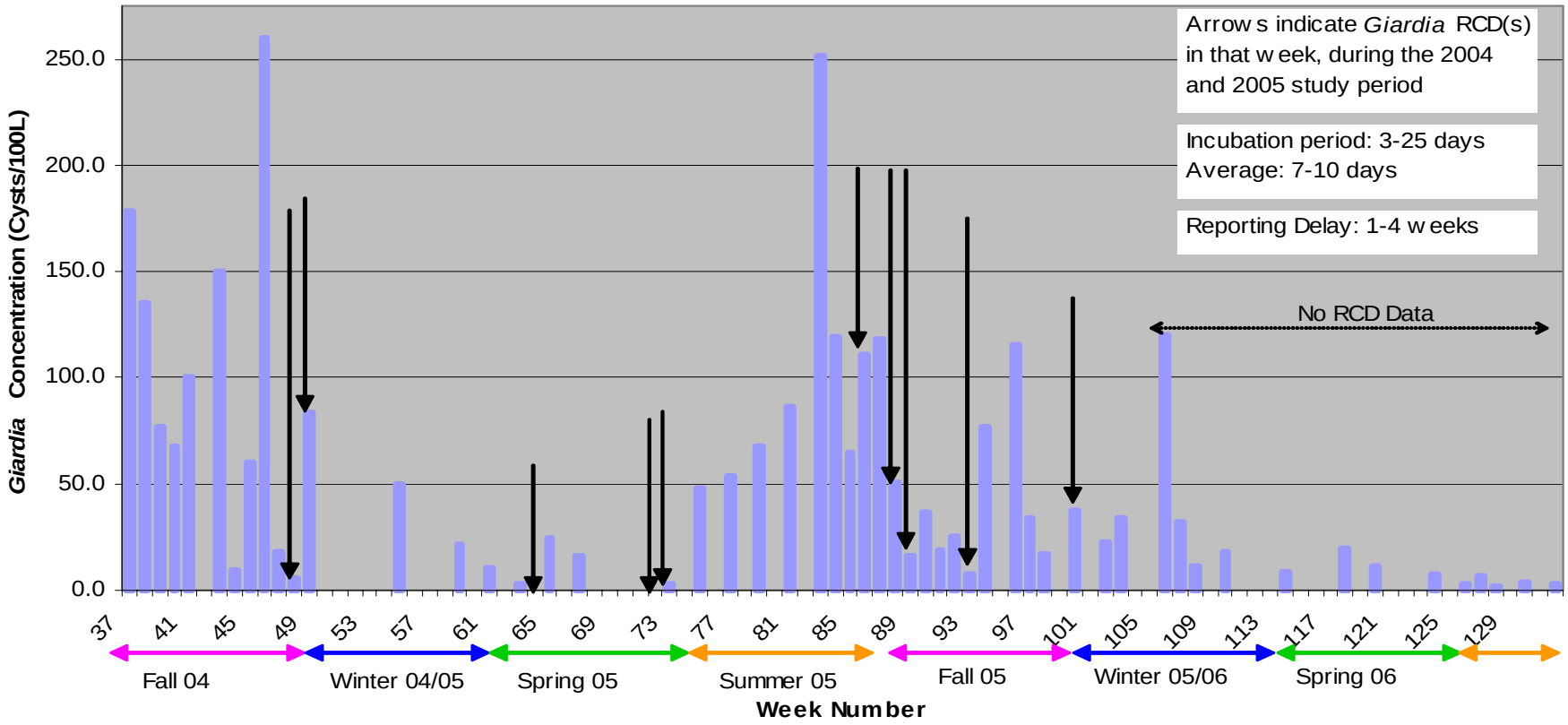


Figure 3-10: Comparison of giardiasis cases in Salmon River watershed in the Township of Langley, compared to *Giardia* water concentrations (cysts/100L) during the 2004-2005 study period

3.4 Discussion

The Township of Langley is a mixed-urban rural community in south-western British Columbia that is representative of many communities across Canada. With a mix of medium-scale farming, hobby farming and forested land, it was hypothesized there would be a large diversity of pathogen sources from livestock, domestic animals and wildlife, reflected in a diversity of parasite sequences.

The Township was selected as a study site as there is increasing concern by the residents of the Township regarding the quality of both the municipal and private drinking groundwater supplies. This has largely arisen because some groundwater wells within the Township have exceeded the maximum allowable concentration of nitrate of 10mg/L, (Guidelines for Canadian Drinking Water Quality). This exceedence emphasizes the vulnerability of the aquifers located within the confines of the Township of Langley; these aquifers are frequently referred to as some of the most vulnerable aquifers in British Columbia. The water supplies within the township appear to be particularly susceptible to contamination, due to a combination of land-use practices and five of the major aquifers within the Township are unconfined and have rapid recharge rate. The aquifer-stream interactions of both the Coghlan Creek and Salmon River show that both streams contributed to aquifer levels in the summer month (losing rivers), therefore contamination of surface water directly impacts groundwater during these months. Because of known agricultural contamination of water resources in the Township, there is a significant chance that these supplies may also be impacted by agricultural pathogens, such as *Giardia* and *Cryptosporidium*.

The results of this study confirm that parasite loadings in this mixed urban-rural were elevated compared to comparable watersheds and occur year-round. In a study of a mixed agricultural watershed in Southern Ontario, Canada, *Cryptosporidium* and *Giardia* were detected in 44% and 31% of samples tested, respectively (Wilkes *et al*. 2009). In contrast, *Cryptosporidium* and *Giardia* were detected in 63% and 86% of samples in the current study. A study in a mixed-use watershed in Portugal found *Cryptosporidium* and *Giardia* concentrations as high as 1,050 oocysts/100L and 1,280 cysts/100L respectively (Castro-Hermida *et al*, 2008). In comparison, the maximum concentrations in the current study were as high as 20,600 oocysts/100L and 3,800 cysts/100L for each *Cryptosporidium* and *Giardia* respectively.

The same study in Portugal found *Cryptosporidium* species *parvum, hominis* and *andersoni*, while *Giardia lamblia* Assemblage A and E were both detected, but the frequency of detection of each

genotype was not given. In the current study, the majority of *Cryptosporidium* (50%) and *Giardia* (98%) isolates belong to species (*Cryptosporidium*) and Assemblages (*G.lamblia*) known to be infectious to humans. Thus, these water sources may be a source of human illness, either through recreational contact or through groundwater intrusion. Despite the predominance of livestock in the area, in both medium-sized farms and hobby farms, cattle specific *G.lamblia* isolates were infrequently detected (Assemblage E). This suggests that in the Township, livestock carry the zoonotic *G.lamblia* Assemblages, rather than the livestock-adapted strain, during the study period. Infection of cattle with only zoonotic Assemblage has been shown in some geographical areas. In New Zealand, host-adapted *G.lamblia* Assemblages (Assemblage E) have never been detected in cattle herds while both Assemblage A and B have been detected in herds (Winkworth *et al*., 2008). In a study in Italy, Assemblages A and B were detected frequently in cattle, while Assemblage E was detected infrequently (Lalle *et al*., 2005). In contrast, more than 90% of isolates collected from cattle on farms in Western Australia and Western Canada were Assemblage E (O'Handley *et al*., 2000). Alternatively, it is possible that Assemblage E is present in the livestock in the watershed, but that loadings are limited due to proper manure management.

The predominance of *Giardia* Assemblage A in this watershed is consistent with the findings of the library of historical isolates (Chapter 3), providing further confirmation that Assemblage A may dominate ecological transmission in British Columbia. Assemblage A does demonstrate the greatest host range, capable of infecting most mammalian species (Thompson, 2000). It is possible that this increased host range, permitting transmission to a large number of hosts, combined with high environmental concentrations may contribute to the increased rates of giardiasis in British Columbia compared to other parts of Canada.

The differences in detection sensitivity by microscopy and PCR for both organisms emphasize the usefulness of molecular epidemiological tools as a complimentary to provide additional data regarding *Giardia* and *Cryptosporidium*, rather than a tool intended to replace microscopic detection. The inability to amplify all (oo)cyst(s) detected by microscopy could arise from the presence of materials inhibitory to PCR, such as humic acids and sediments, which can co-concentrate during the filtration of large volumes of water (Wilson 1997; Miller *et al*., 1999; Rajal *et al*., 2007). Furthermore, detection can be impacted by the quality of nucleic acid, which can be a reflection of the stresses the organism endures in the environment. Furthermore, as indicated on the April 26, 2004 sampling event, strong cross-reactivity with non-*Giardia* and non-*Cryptosporidium* objects is possible and has been confirmed in the literature, which could lead to an over-estimation of the IFA

results. It has been demonstrated previously that antibodies used in IFA microscopy can cross-react with algal species and that careful confirmation of morphology is necessary to identify cross-reacting species (Rodgers *et al*., 1995).

The results confirm that surrogate organisms are poor indicators of *Giardia* and *Cryptosporidium* occurrence. While these organisms are of fecal origin, their survival in water differs significantly from fecal coliforms, due to the structural integrity of the (oo)cyst wall and low metabolic state, allowing prolonged survival in cold waters (Fayer *et al*., 1998; Olsen *et al*., 1999). The best predictor of *Giardia* and *Cryptosporidium* occurrence was turbidity, yet the strength of the relationship differed for the two water sources studied. Not surprisingly, there was a stronger relationship between turbidity and pathogen occurrence in Coghlan Creek, which has lower stream discharge compared to Salmon River. There is evidence of a link between phone-calls to a health information telephone line and drinking water turbidity (Gilbert *et al*., 2006). Furthermore, during the Milwaukee cryptosporidiosis outbreak, there was a strong relationship between turbidity and emergency room visits and hospitalizations (Naumova *et al*., 2003. These findings, along with the simplicity of measuring turbidity, have resulted in efforts to link pathogen occurrence with turbidity. However, many studies have failed to show consistent strong relationships between turbidity and parasite occurrence in raw water supplies (Dorner *et al*., 2007; Goldstein *et al*., 1996). In addition, examination of relationship between *Giardia* and *Cryptosporidium* concentration, there was a stronger correlation between these organisms in Salmon River compared to in Coghlan Creek, suggesting differences in pathogen loadings patterns in the different streams.

Pathogen occurrence was also compared to antecedent rainfall, due to the evidence that significant rainfall preceding many of the waterborne outbreaks of disease that have occurred in Canada (Thomas *et al*., 2006; Auld *et al*., 2004). However, prospective studies of pathogen occurrence with respect to rainfall occurrence in watersheds have not revealed similar relationships (Wilkes *et al*., 2009). In this study, parasites demonstrated no correlation to antecedent rainfall,. The high parasite counts in the summer, in the absence of rainfall, is not consistent with other studies (Caccio *et al*., 2003; Kistemann *et al*., 2002) .The high parasite loading in summer months is suggestive that loadings may originate from grazing animals, in-stream organisms such as muskrat or contributions from various fowl species.

The lack of relationship between parasite reportable communicable disease (RCD) data and parasite occurrence in water is not surprising. While RCD data is the most valid and currently available data

set representing human health parameters, it is an under-representation of actual health data (Mor *et al*., 2009; Brabazon *et al*., 2008). RCD data have been described as the 'tip of the iceberg' and represents only those patients who became symptomatic, who visited a physician, whose physician ordered a laboratory test, where the patient actually submitted a sample for analysis and a positive result was reported to public health authorities (Straif-Bourgeois and Ratard, 2007). It is estimated that laboratory confirmed cases represent less than ten percent of actual cases. Therefore the ten cases of giardiasis in the 2004-2005 study period may actually be closer to 100 cases. Furthermore, in addition to *Giardia* exposure from water (drinking water or recreational water), giardiasis can be spread by other transmission modes including person-to-person, travel-acquired and animal-to-person transmission (Hunter and Thompson, 2005). Hence, direct relationships between giardiasis in humans and parasite concentrations in water, can be difficult to establish.

This study highlights the benefits of integrating gold-standard laboratory methods with alternative detection approaches. This is expected to be particularly useful in the future, as novel technologies for concentration, detection and speciation of *Giardia* and *Cryptosporidium*, may emerge. Frameworks for integrating new technologies to complement gold-standard methods will be crucial for the improvement of microbial water quality surveillance, which will ultimately allow for better protection of public health through purveyance of pathogen-free water.

3.5 References

Adam, RD. 2001. Biology of *Giardia lamblia.* Clinical Microbiology Reviews. 14(3): 447-475.

Appelbee AJ, Frederick LM, Heitman TL and Olson ME. 2003. Prevalence and genotyping of *Giardia duodenalis* from beef calves in Alberta, Canada. Veterinary Parasitology. 112(4): 289-294.

Auld H, MacIver D and Klaassen J. 2004. Heavy rainfall and waterborne disease outbreaks: the Walkerton example. Journal of Toxicology and Environmental Health A. 67(20-22): 1879-1887.

Betancourt WQ and Rose JB. 2004. Drinking water treatment processes for removal of *Cryptosporidium* and *Giardia*. Veterinary Parasitology. 126(1-2): 219-234.

Black EK, Finch GR, Taghi-Kilani R and Belosevic M. 1996. Comparison of assays for *Cryptosporidium* parvum oocysts viability after chemical disinfection. FEMS Microbiology Letters. 135(2-3): 187-189.

Brabazon ED, O'Farrell A, Murray CA, Carton MW, Finnegan P. 2008. Under-reporting of notifiable infectious disease hospitalizations in a health board region in Ireland: room for improvement? Epidemiology and Infection. 136(2): 241-247.

British Columbia Centre for Disease Control (BCCDC). 2009. British Columbia Annual Summary of Reportable Disease. Provincial Health Services Authority. Vancouver, British Columbia.

Cacciò SM, Thompson RC, McLauchlin J and Smith HV. 2005. Unraveling *Cryptosporidium* and *Giardia* epidemiology. Trends in Parasitology. 21(9): 430-437.

Cacciò SM, De Giacomo M, Aulicino FA, and Pozio E. 2003. *Giardia* cysts in wastewater treatment plants in Italy. Applied and Environmental Microbiology. 69(6): 3393-3398.

Castro-Hermida JA, García-Presedo I, Almeida A, González-Warleta M, Correia Da Costa JM, Mezo M. 2008. Presence of *Cryptosporidium* spp. and *Giardia duodenalis* through drinking water. Science of the Total Environment. 405(1-3): 45-53.

Dorner SM, Anderson WB, Gaulin T, Candon HL, Slawson RM, Payment P and Huck PM. 2007. Pathogen and indicator variability in a heavily impacted watershed. Journal of Water and Health. 5(2): 241-257.

Fayer R, Trout JM and Jenkins MC. 1998. Infectivity of *Cryptosporidium parvum* oocysts stored in water at environmental temperatures. Journal of Parasitology. 84(6): 1165-1169.

Hopkins RM, Meloni BP, Groth DM, Wetherall JD, Reynoldson JA and Thompson RC. 1997. Ribosomal RNA sequencing reveals differences between the genotypes of *Giardia* isolates recovered from humans and dogs living in the same locality. Journal of Parasitology. 83(1): 44-51.

Hunter PR and Thompson RC. 2005. The zoonotic transmission of *Giardia* and *Cryptosporidium*. International Journal for Parasitology. 35(11-12): 1181-1190.

Gilbert ML, Levallois P and Rodriguez MJ. 2006. Use of a health information telephone line, Info-santé CLSC, for the surveillance of waterborne gastroenteritis. Journal of Water Health. 4(2): 225-232.

Goldstein ST, Juranek DD, Ravenholt O, Hightower AW, Martin DG, Mesnik JL, Griffiths SD, Bryant AJ, Reich RR and Herwaldt BL. 1996. Cryptosporidiosis: an outbreak associated with drinking water despite state-of-the-art water treatment. Annals of Internal Medicine. 124(5): 459-468.

Karanis P, Kourenti K and Smith HV. 2007. Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. Journal of Water and Health. 5(1): 1–38.

Kistemann T, Classen T, Koch C, Dangendorf F, Fischeder R, Gebel J, Vacata V and Exner M. 2002. Microbial load of drinking water reservoir tributaries during extreme rainfall and runoff. Applied and Environmental Microbiology. 68(5): 2188-2197.

Lalle M, Pozio E, Capelli G, Bruschi F, Crotti D and Cacciò SM. 2005. Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. International Journal for Parasitology. 35(2): 207-13.

Morgan-Ryan UM, Fall A, Ward LA, Hijjawi N, Sulaiman I, Fayer R, Thompson RC, Olson M, Lal A and Xiao L. 2002. *Cryptosporidium hominis* n. sp. (Apicomplexa: Cryptosporidiidae) *from Homo sapiens*. Journal of Eukaryotic Microbiology. 49(6): 433-440.

Mor SM, DeMaria A Jr, Griffiths JK and Naumova EN 2009. Cryptosporidiosis in the elderly population of the United States. Clinical Infectious Diseases. 48(6): 698-705.

Miller DN, Bryant JE, Madsen EL, Ghiorse WC. 1999. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Applied and Environmental Microbiology. 65(11):4715-4724.

Naumova EN, Egorov AI, Morris RD, Griffiths JK. 2003. The elderly and waterborne *Cryptosporidium* infection: gastroenteritis hospitalizations before and during the 1993 Milwaukee outbreak. Emerging Infectious Diseases. 9(4): 418-425.

O'Handley RM, Olson ME, Fraser D, Adams P and Thompson RC. 2000. Prevalence and genotypic characterisation of *Giardia* in dairy calves from Western Australia and Western Canada. Veterinary Parasitology. 90(3): 193-200.

Olsen ME, Goh J, Phillips M, Guselle N and McAllister T. 1999. *Giardia* cyst and *Cryptosporidium* oocyst survival in water, soil and cattle feces. Journal of Environmental Quality. 28: 1991-1996.

Public Health Officer. 2001. Drinking water quality in British Columbia: the public health perspective. Officer of the Public Health Officer of British Columbia, Victoria, Canada.

Rajal VB, McSwain BS, Thompson DE, Leutenegger CM, Kildare BJ and Wuertz S. 2007. Validation of hollow fiber ultrafiltration and real-time PCR using bacteriophage PP7 as surrogate for the quantification of viruses from water samples. Water Research. 41(7): 1411-1422.

Rodgers MR, Flanigan DJ, and W Jakubowski. 1995. Identification of algae which interfere with the detection of *Giardia* cysts and *Cryptosporidium* oocysts and a method for alleviating this interference. Applied and Environmental Microbiology. 61(10): 3759-3763.

Ruecker NJ, Bounsombath N, Wallis P, Ong CS, Isaac-Renton JL and Neumann NF. 2005. Molecular forensic profiling of *Cryptosporidium* species and genotypes in raw water. Applied and Environmental Microbiology. 71(12): 8991-8994.

Schreier H, Hall K, Brown S, Wernick B and Berka C. 1999. Agriculture: an important non-point source of pollution. In: C. Gray and T. Tuominen (eds.) Health of the Fraser River Aquatic Ecosystem, Environment Canada, DOE-FRAP, Vancouver. 2: 83-99.

Smith HV, Cacciò SM, Cook N, Nichols RA, Tait A. 2007. *Cryptosporidium* and *Giardia* as foodborne zoonoses. Veterinary Parasitology. 149(1-2): 29-40.

Straif-Bourgeois S, Ratard R. Infectious Diseases. In: Ahrens W, Pigeot I (Eds) Handbook of Epidemiology. Springer, Germany.

Thomas KM, Charron DF, Waltner-Toews D, Schuster C, Maarouf AR and Holt JD. 2006. A role of high impact weather events in waterborne disease outbreaks in Canada, 1975 - 2001. International Journal of Environmental Health Research. 16(3): 167-180.

Thompson RC and Monis PT. 2004. Variation in *Giardia*: Implications for taxonomy and epidemiology. Advances in Parasitology. 58: 69-137.

Thompson, RC. 2000. Giardiasis as a re-emerging infectious disease and its zoonotic potential. International Journal for Parasitology. 30: 1259-1267.

United States Environmental Protection Agency (USEPA). 2005. Method 1623: *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA. Office of Water, U.S. Environmental Protection Agency, Washington, DC.

Ware MW, Wymer L, Lindquist HD and Schaefer FW 3rd. 2003. Evaluation of an alternative IMS dissociation procedure for use with Method 1622: detection of *Cryptosporidium* in water. Journal of Microbiological Methods. 55(3): 575-583.

Wernick B, Cook E and Schreier H. 1998. A GIS evaluation of land-use and streamwater nitrate-N dynamics in a watershed at the urban/rural fringe. Journal of the American Water Resources Association. 34(3): 639-650.

Wilkes G, Edge T, Gannon V, Jokinen C, Lyautey E, Medeiros D, Neumann N, Ruecker N, Topp E, Lapen D. 2009 Seasonal relationships among indicator bacteria, pathogenic bacteria, *Cryptosporidium* oocysts, *Giardia* cysts, and hydrological indices for surface waters within an agricultural landscape. Water Research. 43(8): 2209-2223.

Wilson IG. 1997. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology. 63(10): 3741-3751.

Xiao L and Fayer R. 2008. Molecular characterisation of species and genotypes of *Cryptosporidium* and *Giardia* and assessment of zoonotic transmission. International Journal for Parasitology. 38(11): 1239-1255.

Xiao L, Fayer R, Ryan U and Upton SJ. 2004. *Cryptosporidium* taxonomy: recent advances and implications for public health. Clinical Microbiology Reviews. 17(1): 72-9

Xiao L, Morgan UM, Limor J, Escalante A, Arrowood M, Shulaw W, Thompson RC, Fayer R and Lal AA. 1999. Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species. Applied and Environmental Microbiology. 65(8): 386-391.

Chapter 4

Comparative analysis of *Giardia lamblia* occurrence and genotype prevalence in two Canadian watersheds of differing geography, land-use and climate type[3]

4.1 Introduction

Protozoan agents of disease, such as *Giardia*, continue to challenge drinking water treatment facilities. This is largely due to their low concentrations, low infectious dose and resistance (full or partial) to traditional drinking water treatment approaches. Amongst waterborne protozoa, *Giardia lamblia* is the most environmentally ubiquitous and most frequent cause of documented waterborne outbreaks in Canada and the United States (Krewski *et al*., 2002; Yoder *et al*., 2007). It is also described as the most common parasitic cause of diarrhea worldwide (Adam, 2001; Xiao and Fayer, 2008).

Giardia lamblia is a flagellated, binucleated eukaryote that causes giardiasis, a gastrointestinal illness commonly referred to as beaver-fever. (Adam, 2001). It has a simple, two-staged life cycle, consisting of a metabolically active and replicative form called a trophozoite, and a metabolically dormant, environmentally resistant form called a cyst. It is the cyst form of the organism that is responsible for its transmission, which occurs via the fecal-oral route. The most common *G.lamblia* transmission modes occurs through contaminated water (drinking or recreational) and person-to-person transmission. Foodborne outbreaks have been documented and direct animal-to-person transmission has been suggested, but both are less common routes of transmission (Mintz *et al*., 1993; Quick *et al*., 1992; van Keulan *et al*., 2002; Palmer *et al*., 2008). *G.lamblia* causes symptomatic infections in a large range of animals, including domestic pets such as cats and dogs, livestock species such as cattle and sheep, and wildlife, such as deer and muskrat.

---

[3] A version of this chapter will be submitted to the Journal of Environmental Health for publication. Prystajecky N, Isaac-Renton JL and Huck PM.., Comparative analysis of *Giardia lamblia* occurrence and genotype prevalence in two Canadian watersheds of differing geography, land-use and climate type

Giardiasis is characterized as a self-limiting gastrointestinal disease. Infection can occur following the ingestion of as few as 10 cysts. Cyst excystation occurs in the upper small intestine, where trophozoites emerge and cause infection. Clinical evidence suggests a typical incubation period lasting 12-20 days. Symptoms include nausea, abdominal cramps, diarrhea and flatulence. The infection may lead to weight loss and malabsorption. In most patients, the infection is self-limiting but treatment with metronidazole or tinidazole reduces the duration, severity and nutritional impacts of infection. Cyst formation occurs as the trophozoites move through the colon; shedding from infected hosts can be as high as 10 million cysts per gram of stool.

Genotyping analysis has revealed two distinct 'types' of *G.lamblia* isolates: zoonotic isolates and host-adapted isolates (Thompson and Monis, 2004). Host-adapted isolates infect specific animal hosts and have not crossed the animal-human barrier, thus having no impact on human health. In contrast, zoonotic isolates are infective to humans and animal hosts, thus posing a threat to human health. Of particular concern is animal shedding of zoonotic isolates into drinking water source supplies (Hunter and Thompson, 2005). While agricultural best management practices (BMPs) may reduce agricultural loading, these practices are difficult to enforce and potential pathogen loadings may occur nonetheless (Trask *et al*., 2004; Atwill *et al*., 2002; Bishop *et al*., 2005). In addition, there is still the possibility of loading of *G.lamblia* isolates from human sources. Cysts originating from human sources may come from leaking septic fields, as a result of incomplete wastewater treatment, recreational water vehicles and cross-connections between sewage and drinking water pipes (Robertson *et al*., 2006; Chauret *et al*., 1999). Because zoonotic isolates can originate from humans or animals, when a contamination event is identified, it may be difficult to attribute the contamination as coming from a specific source.

The recent development of genotyping tools allows further characterization of all *G.lamblia* isolates into Assemblages, a term that is mostly synonymous with genotype or subtype, thus reflecting the species-complex nature of *G.lamblia* (Thompson and Monis, 2004). Under the current taxonomic nomenclature there are seven Assemblages (A through G). Assemblages A and B include zoonotic isolates, while Assemblages C through G contain host-adapted isolates. While genotyping cannot always link *G.lamblia* to a contamination source, it is an invaluable component during outbreak investigations. Genotyping is also useful in understanding contamination sources and risk assessments related to *G.lamblia* in watersheds. This is particularly true since there are five *G.lamblia*

Assemblages that do not infect humans and this distinction cannot be made by the gold-standard *Giardia* detection methods (US Environmental Protection Agency Method 1623) currently used to detect parasites in drinking water supplies.

In British Columbia, *G.lamblia* is the most common etiological agent in documented waterborne outbreaks identified since 1980 (PHO, 2001). While rates of giardiasis have declined in British Columbia since their peak in 1990, British Columbians continue to experience higher rates of giardiasis compared to Canadian averages. In 2004, the rate of giardiasis in Ontario was 12.69 cases per 100,000 individuals, while the rate in British Columbia was 17.56 cases per 100,000 individuals. This study characterizes and compares waterborne *Giardia* prevalence in two Canadian watersheds, the British Columbia watershed separated from the Ontario watershed by 4250 kilometers. Specifically, we aimed to identify any differences in the *G.lamblia* Assemblages present in each watershed and to better understand how climate may impact *G.lamblia* loadings in watersheds with differing geographic and land-use characteristics.

4.2 Methods and Materials

4.2.1 Study Sites

4.2.1.1 Salmon River Watershed, Township of Langley, British Columbia

The Salmon River watershed is located in southwestern British Columbia, 50 km east of Vancouver (Figure 4-1, Figure 4-2). The watershed occupies 80 square kilometers and is considered an important spawning site for Coho salmon. Unlike other watersheds in British Columbia, the elevation change between the headwaters and the river's end is only 140 meters (Wernick *et al*., 1998). This has allowed for significant development within the watershed, although urban and industrial development intensity has been limited by the inclusion of this area in the British Columbia Agricultural Land Reserve. The watershed is best described as a mixed urban-rural watershed, with approximately 50% of land-use oriented towards agricultural activities (Wernick *et al*., 1998). Land-use for residential purposes has increased by 22% between 1986 and 2005 (Schreier, unpublished data).

Approximately 13,000 inhabitants of the Township of Langley (pop 91,000) live within the Salmon River watershed in a variety of residence types, including acreages and farms (Ross, 2006). Depending on the location within the watershed, some residences are serviced by municipal drinking water and sewer, while others rely on groundwater and septic systems. It is estimated that there are 550 farms and over 4,000 septic systems within the watershed (Ross, 2006). Agricultural activities include cranberry operations, cattle and chicken farms, along with smaller hobby farms.

Figure 4-1. Map of Canada showing the provincial boundaries and distance between British Columbia and Ontario.

Water quality in the Salmon River watershed has been of considerable concern. Within the Salmon River watershed is the Hopington aquifer, an unconfined aquifer, making it vulnerable to contamination. Persistent high nitrate concentrations, exceeding Health Canada's guideline of 10mg/L have led to the classification of the Hopington Aquifer as a vulnerable aquifer (Schreier *et al*., 1999). The elevated nitrate levels have been attributed to contamination from agriculture and septic systems (Wernick *et al*., 1998). While residents and the Township of Langley do not take drinking water directly from the Salmon River, there is a significant groundwater-surface water interaction (Naugler, 2006). This is of particular concern to public health because of private groundwater users who consume untreated groundwater.

The Salmon River watershed is located in a temperate rainforest climate zone. Summer temperatures average 20 degrees Celsius while winter temperatures average two degrees Celsius. The average annual rainfall is 1400 mm, with most rainfall falling between October and April. There are approximately 200 frost-free days each year.

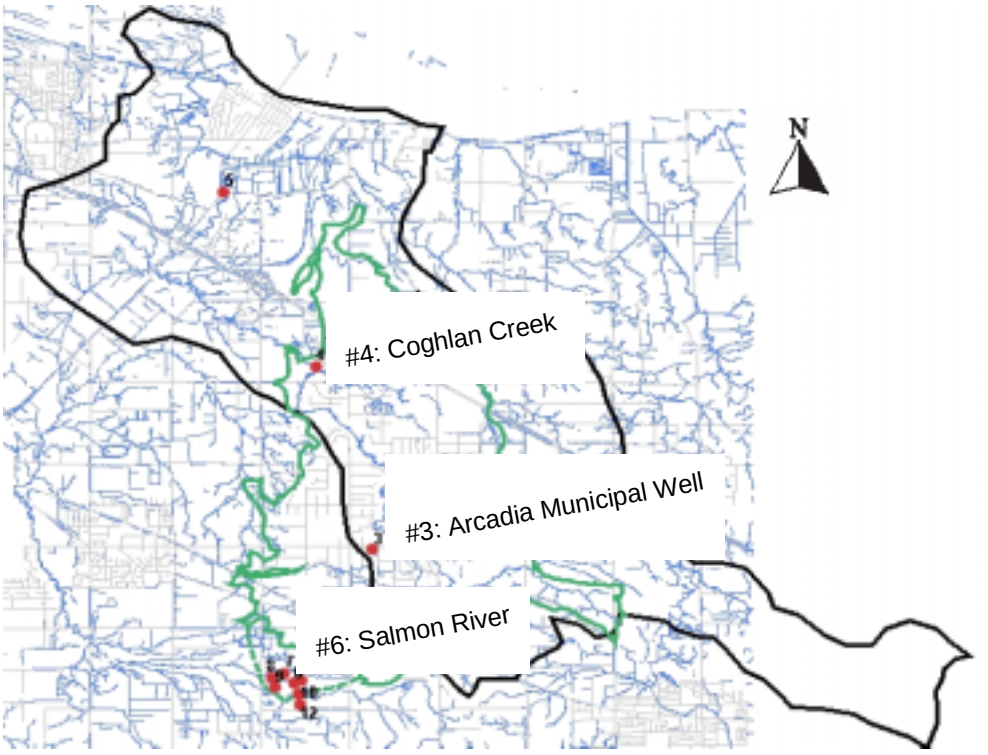


Figure 4-2. Sampling locations within the Salmon River watershed, British Columbia.

4.2.1.2 Grand River Watershed, Southern Ontario

The Grand River watershed is located in southern Ontario, 90 km west of Toronto (Figure 4-3). The watershed occupies 70 square kilometers and empties into the north shore of Lake Erie. Within the confines of the watershed are five large municipalities: Waterloo (97,475), Kitchener (204,668), Cambridge (120,371), Guelph (114, 943) and Brantford (90,192). Water resources in the Grand River watershed provide drinking water for approximately 800,000 residents. Approximately 115,000 residents use private groundwater wells while the remaining population relies on municipal drinking water supplies. The Grand River serves as a receiving water for effluents from numerous municipal wastewater treatment plants. In addition, the Grand River is used for recreational activities, such as fishing, boating and swimming.

The Grand River watershed is much more intensely developed than the Salmon River watershed; the land-use in the watershed is dominated by agricultural and dense urban development. Urban development, including residential, industrial and commercial development, is dispersed throughout the watershed in multiple municipalities (GRCA, 2008). Surrounding the municipalities are areas of intensive agricultural activities. There are approximately 6,400 farms in the watershed, representing approximately 67% of land-use. There are 12 municipal wastewater treatment plants that discharge treated effluent into the Grand River. The intensity of agricultural land-use, combined with urban impacts such as wastewater discharges and storm runoff, have placed significant pressures on the watershed. These impacts are expected to intensify, as agricultural density is expected to increase along with population growth of approximately 300,000 people over the next 20 years.

The climate in the Grand River watershed is moderate to cool temperate, demonstrating distinct seasons including winters with snow and hot humid summers. Extreme temperatures can reach as low as -35 degrees Celsius in the winter and as high as 40 degrees Celsius in the summer. Depending on the location within the watershed, average rainfall is 892 mm to 940 mm while snowfall averages are 101mm to 169 mm (converted to rainfall equivalents), annually.

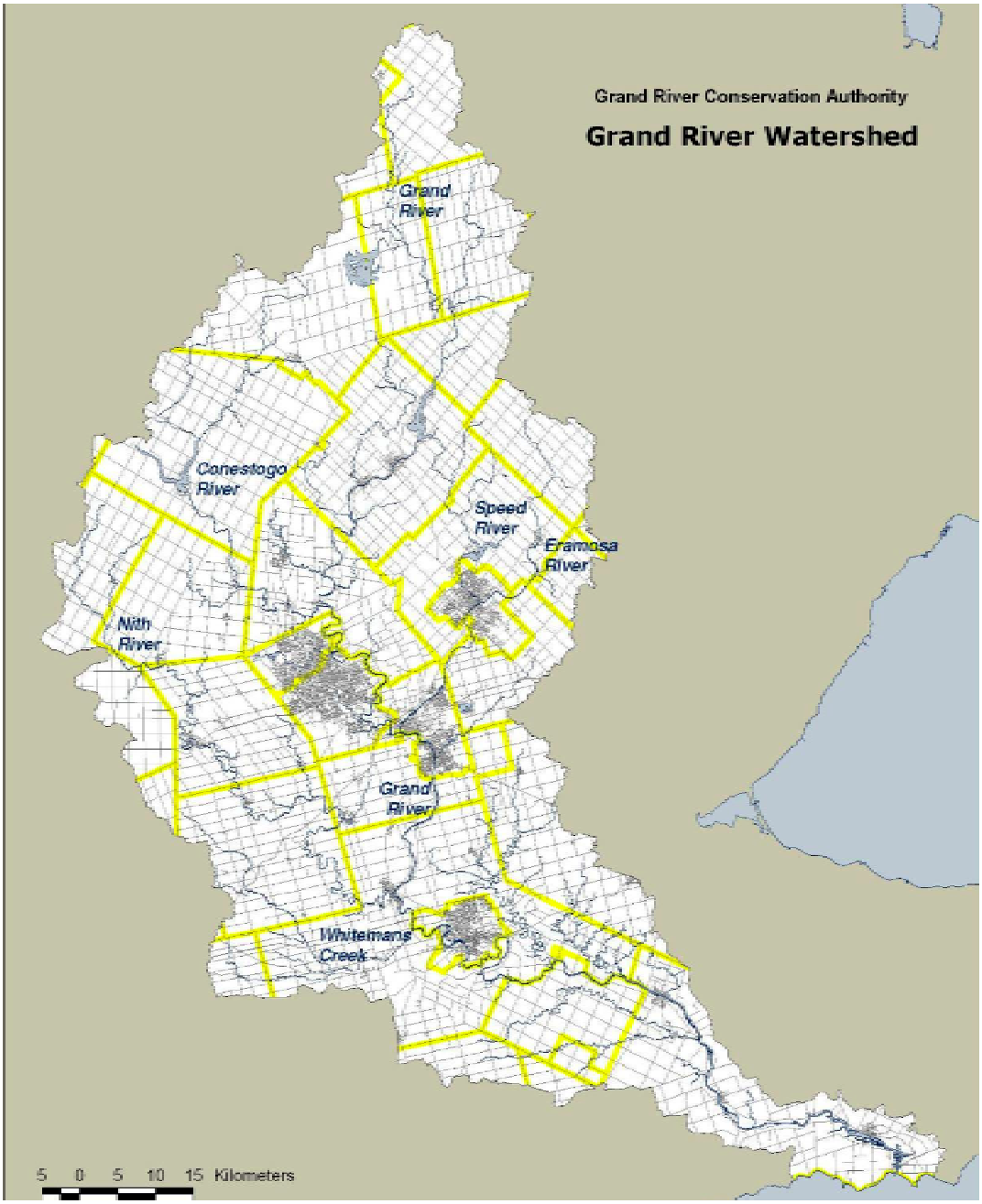


Figure 4-3. Map of the Grand River watershed. Source: Grand River Conservation Authority.

4.2.2 *Giardia* Sampling

Samples were collected every other week over a two-year study period, according to US Environmental Protection Agency's Method 1623 protocols (2005 to 2006). Samples were collected from the Salmon Watershed from site in the Salmon River and Coghlan Creek, a tributary of the Salmon River from 2005 to 2006. Samples were collected in the Grand River at the intake of the Mannheim drinking water treatment plant in Kitchener, Ontario (2005 to 2007).

Up to 50L of surface water samples was collected, using a pump with a flow rate of 0.2 – 4.5L/minute and an IDEXX Filta-Max foam-disc filters in a standard filtration unit (IDEXX Filta-Max, Westbrook ME, USA) Filters were transported by courier, on ice, to the Environmental Microbiology Laboratory at the British Columbia Centre for Disease Control (BCCDC) Public Health Microbiology Laboratories, stored at 4°C and processed within 96 hours.

4.2.3 Sample Processing

Samples contained within the IDEXX filters were processed using automatic wash station (IDEXX, Westbrook, ME, USA), allowing for consistent filter elution and sample concentration. The wash station elutes sediment by repeated expansions and compressions of the foam disc using a proprietary wash buffer and concentrated using a concentrator tube fitted with a 3µm membrane to remove non-target debris and to reduce sample volume**.**

4.2.4 IMS Procedure

Immunomagnetic separation (IMS) was used for further sample processing (Dynabeads-GC Combo Kit, Dynal Biotech, Brown Deer, WI, USA; Invitrogen). Antibodies directed against *Giardia* and *Cryptosporidium* were conjugated to magnetic beads and mixed with sample. Using a magnet, the organisms were removed from the sample matrix, thus separating the organisms from interfering organisms and chemicals. IMS purification followed the manufacturer's procedure with the exception of the dissociation step; the suggested acid dissociation was replaced with heat dissociation to yield greater organisms recovery (Ware *et al*., 2003)

4.2.5 Microscopic Examination

The resulting IMS-purified sample was then used for an immunofluorescence assay (IFA) for the detection and enumeration of *Giardia* cysts. The IMS-purified sample was applied to a Dynal Spot-On glass microscope slide and dried overnight in a refrigerator at 4°C. If the volume of the IMS-purified sample exceeded 0.5ml, the sample was set-up on two microscope slides. The slides were then methanol-fixed and stained with a FITC conjugated antibody (Easy-Stain, Biotechnology Frontiers, North Ryde, Australia) and 4'6'-diamidino-2-phenylindole (DAPI). Presumptive positive cysts were confirmed by DAPI staining and differential interference contrast (DIC) microscopy.

4.2.6 Slide Scraping Procedure

The microscope slides were then temporarily stored at 4°C until further processing. For long-term storage of the slides, the cover-slips were removed and the slides were gently washed with warmed PBS solution to remove the DABCO-glycerol. Slides were dried at room temperature and then stored at -20°C. To remove the methanol-fixed cysts from the slides for further analysis, slides were removed from the freezer and processed immediately as described by Ruecker *et al*. (10). A total volume of 180µl of tissue lysis buffer (Buffer ATL) was added to the microscope slide well (Qiagen, Mississauga, ON) in 45µl aliquots, alternating between rinsing the slide and scraping the slide using a pipette tip. Between each step the buffer was transferred to a sterilized microcentrifuge tube and kept on ice. Samples were stored at -20°C until further processing. The detection limit for the recovery of cysts from the Spot-On slides was determined by spiking slides with up to 30 cysts per slide (Waterborne Inc, New Orleans, LA, USA)

4.2.7 DNA Extraction

Genomic DNA was extracted by freeze-thaw with proteinase K digestion. Six freeze-thaw cycles were used, alternating between a two minute freeze in liquid nitrogen and a five minute thaw at 65°C, followed by the addition of 400µg of proteinase K (Qiagen) and an overnight digestion in a thermomixer at 56°C to further degrade the cyst wall and to inactivate DNase activity. The resulting lysates were then purified using a QiaAMP DNA Micro Kit (Qiagen), following the manufacturer's protocol for tissue lysis for samples with low numbers of target DNA. Carrier RNA was added to

Qiagen's Buffer AL (lysis buffer) at a concentration of 10µg/L (both supplied in the QiaAMP DNA Micro Kit) before processing each batch of lysates. After the samples were applied to the MinElute columns and two wash steps were performed, DNA was eluted from the column using 50µl of distilled molecular grade water. For samples in which two heat dissociation steps were used, each slide resulting from each heat dissociation step was treated as a separate sample until the elution step on the MinElute column, at which point the samples were each eluted with 25µl of water and combined for a final volume of 50µl. Samples were stored at -20°C for genotyping. A spiked positive control and a negative control were also included in the genomic extraction step.

4.2.8 Nested PCR

Nested polymerase chain reaction (PCR) protocols were used for the amplification of *Giardia lamblia* DNA to provide adequate target sequences for genotyping purposes. For genotyping of *G.lamblia*, the 18s rRNA gene was amplified in a nested PCR reaction as previously described (Appelbee *et al*., 2005; Hopkins *et al*., 1997). For primary PCR, primers Gia2029 (5'-AAGTGTGGTGCAGACGGACTC-3') Gia2150c (5'-CTGCTGCCGTCCT-TGGATGT-3') were used to amplify a target sequence of 497bp. The PCR reaction mix was comprised of 25µl HotStarTaq MasterMix (Qiagen, Mississauga, ON) containing 1X PCR Buffer, 1.5mM $MgCl_2$, 2.5 U of modified Taq polymerase, 200µM each deoxynucleoside triphosphate (dNTP), 25pmoles of each primer, 5% DMSO, 10mg/ml BSA and 2.5 - 5µl of purified genomic lysate in a 50µl reaction volume. The primary PCR was conducted using a 15 minute hot-start at 95ºC, followed by 35-40 cycles of PCR (96ºC for 45s, 55ºC for 30s, and 72ºC for 45s) and a 4 minute extension at 72ºC. PCRs were carried out on either a Perkin-Elmer GeneAmp 9600 or an Eppendorf Mastercycle thermocycler.

For the secondary PCR, primers RH11 (5'-CATCCGGTCGATCCTGCC-3') and RH4 (5'-AGTCGAACCCTGATTCTCCGCCAGG-3') were used to amplify a target sequence of 292bp product using the same PCR reaction mix with the addition of 2-5µl of primary PCR product. The secondary PCR cycle conditions were identical to the primary PCR except the annealing temperature was increased from 55°C to 59°C for the annealing period. Negative controls and positive controls using genomic DNA extracted from commercially obtained cysts (Waterborne Inc, New Orleans, LA, USA) were used for each PCR batch.

4.2.9 Sequencing

Secondary PCR products were visualized using agarose gel electrophoresis and ethidium bromide staining under UV light. PCR-positive samples were purified using QiaQuick PCR Purification Kit according to the manufacturer's instructions (Qiagen) and quantified using a spectrophotometer (Ultrospec 3100 Pro, BioChrom, Ltd., Cambridge, United Kingdom) and diluted to a final volume of 10ng/µl. The samples were sequenced using the BigDye Terminator Version 3.1 Cycle Sequencing Kit (Perkin-Elmer, Boston, MA, USA) on an ABI 3100 automated sequencer (Perkin-Elmer, Boston, MA, USA). Both forward and reverse sequencing of the secondary product were performed for increased sequence accuracy. Sequencing reactions comprised of 2µl of BigDye Terminator sequencing reagents, 6µl of BigDye Terminator sequencing Buffer, 1, 1.6 µl of 2 µM concentrations of primers. Sequencing reactions consisted of 2 µl of BigDye terminator cycle sequencing reagents, 6 µl of BigDye terminator cycle sequencing buffer, 3.2 pmoles of primer,1-2µl purified PCR product and molecular grade distilled water to make a 20µl reaction volume. The cycle sequencing was performed for 25 cycles (96°C for 10 s, 50°C for 5 s, and 60°C for 4 min) on a 9600 GeneAmp Thermocycler (Perkin-Elmer, Boston, MA, USA). Sequence products were ethanol-acetate precipitated and dried, followed by reconstitution in Hi-Di formamide and loaded into the ABI 3100 sequencer.

4.2.10 Climate Data

Climate data were obtained from Environment Canada's Climate Archives. Weather station selection was determined by proximity to the study site and completeness of precipitation data. For the Township of Langley site, the Abbotsford A weather station was used (Climate ID 1100030; 49° 1.517' N, 122° 21.633' W). For the Grand River Sites, complete precipitation data were not available for climate stations located in Kitchener, therefore the Roseville weather station was used instead (Climate ID 6147188; 43° 21.217' N, 80° 28.418' W). Daily data were used at both weather stations.

4.2.11 Data Analysis

All statistical analyses were performed using Microsoft Excel (2003). Sequence analysis was performed using DNAStar SeqMan and MegAlign Programs. Bootstrap analyses were performed using Geneious DNA software.

4.3 Results and Discussion

For the comparative analysis, data from the two main river sites from each watershed were used. The most comparable sampling sites were the intake to the Mannheim drinking water treatment plant, in the Grand River, and the Salmon River sampling site in the Salmon River.

4.3.1 *Giardia* Detection in Raw Water

Samples were collected from the intake at the Mannheim plant from August 2005 to January 2007. *Giardia* was detected in 94.5% samples (n=38) (Figure 4-4). Concentrations of *Giardia* ranged from no parasites detected (NPD) to 486 cysts per 100L, with an average concentration of 88 cysts/100L (SD=102.5). Detection limits ranged from 4 cysts to 14.8 cysts (average 4.4 cysts/100L), depending on the volume of water that was filtered.

Concentrations peaked in January and were elevated during winter months, while NPDs were most common in mid-spring until late-summer. The elevated concentrations of *Giardia* in the winter months is consistent with lower water volumes in winter months resulting in elevated pathogen detection levels (concentration effect). During winter months in the Grand River, pathogen loading from overland flow is less likely due to limited rainfall and snowmelt (due to low temperatures). However, the winters during the study period (August 2005 to January 2007) experienced periods of unseasonable warmness, which could contribute to atypical snow melt and pathogen loading. Analysis of the relationship between maximum daily temperature and *Giardia* concentration are shown in Appendix C. While some *Giardia* loading events in winter months occurred in concert with maximum daily temperatures above zero (thus snowmelt occurring and contributing to pathogen loading), this relationship is not consistent throughout the study period (Appendix C, Figure C-1). Pearson correlation between maximum daily temperature and *Giardia* concentration showed a moderate negative relationship (r = - 0.53) between the variables, indicating that pathogen loading is higher at lower temperatures. Giardia loading did occur in the absence of rainfall and daily temperatures above zero, suggesting that a source of Giardia that is not related to overland flow. It is possible that this contribution is due to sewage, either from sewage effluent or septic tank leakage into the Grand River or its tributaries.

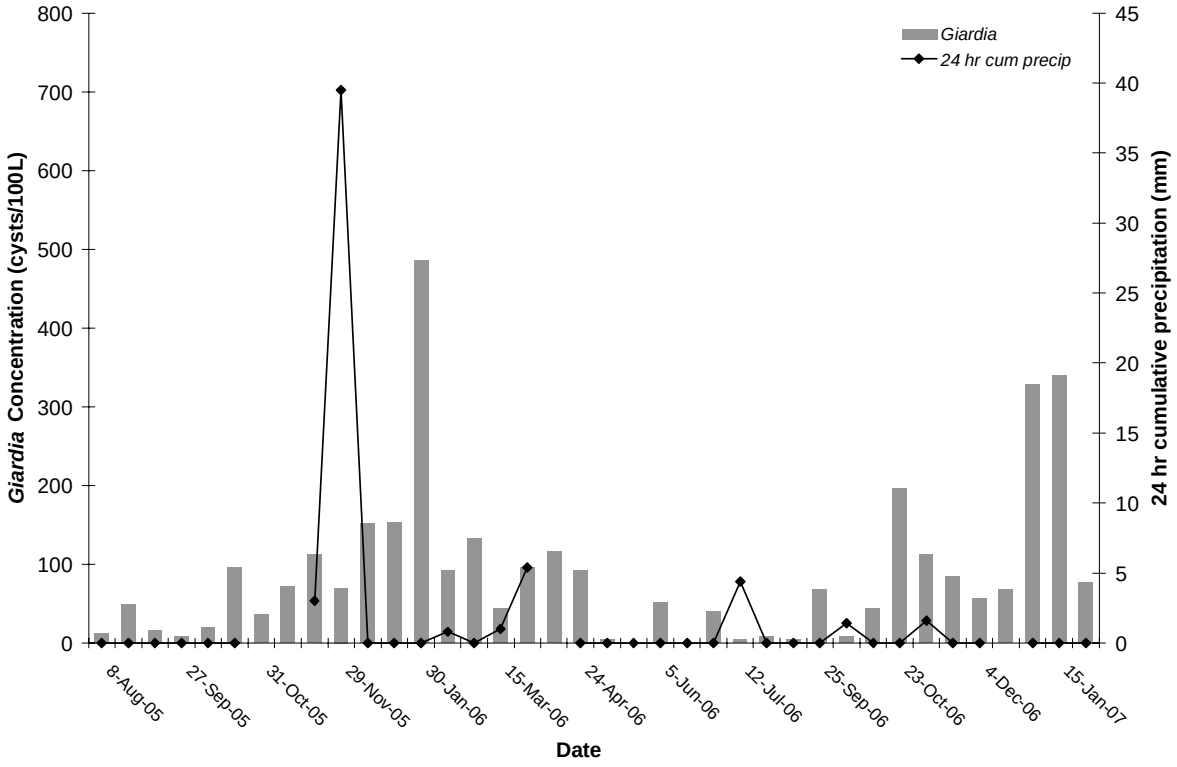


Figure 4-4: *Giardia* concentrations and 24-hour cumulative precipitation for the intake at the Mannheim drinking water plant sampling location, Grand River watershed.

Samples were collected in the Salmon River from February 2005 until November 2006. *Giardia* was detected in 77.6% samples (n=49) (Figure 4-5). Concentrations of *Giardia* ranged from no parasite detected (NPD) to 730 cysts per 100L, with an average concentration of 31.8 cysts/100L (SD=103.7). Detection limits ranged from 2.6 cysts to 14.5 cysts, dependent of filtration volume, with an average detection limit of 4.3 cysts/100L.

Concentrations of *Giardia* in the Salmon River watershed peaked in fall months and were detected at lower concentration in the summer months. This is consistent with first flush phenomena, where contaminants accumulate over dry months and are carried in high concentration during the first rainfall event after a dry period. First flush appears to be an important source of pollutants in the Salmon River watershed in the fall months, when rainfall commences after prolonged pollutant

accumulation (including fecal material) during the dry summer. Because of the change in the hydrograph in the summer months, *Giardia* concentrations may appear higher than predicted loadings may indicate; this is likely due to the changes in the river hydrograph in summer months when flows decrease substantially and higher quality groundwater contributes to surface flows in the Salmon River.

There was one very high peak in *Giardia* concentration in the Salmon River on March 15, 2005. There was no rainfall in the 96 hours preceding sampling, suggesting that parasite loading did not occur via overland flow. Elevated *Cryptosporidium* concentrations accompanied this spike (126 oocysts/100L, data not shown), however, total and fecal coliforms were extremely suppressed (five and one colony forming unit (CFU)/100mL compared to averages during the study period of 6295 and 737 CFU/100mL, respectively) (data not shown). This is consistent with unique loading of parasites, emphasizing previous observations that coliforms counts do not correlate reliably with elevated *Giardia* concentrations.

Comparatively, *Giardia* was detected more frequently and at higher concentrations in the Grand River watershed than in the Salmon River watershed. Periods of peak concentrations differed between the two watersheds, with peak concentrations of *Giardia* occurring in the Grand River site in January, while concentrations are elevated in the Salmon River in the fall months. It should be noted that there are 12 municipal wastewater treatment plants that discharge treated sewage effluent into the Grand River, while there are no municipal wastewater treatment in the Salmon River watershed. Both watersheds have a high volume of septic tank users.

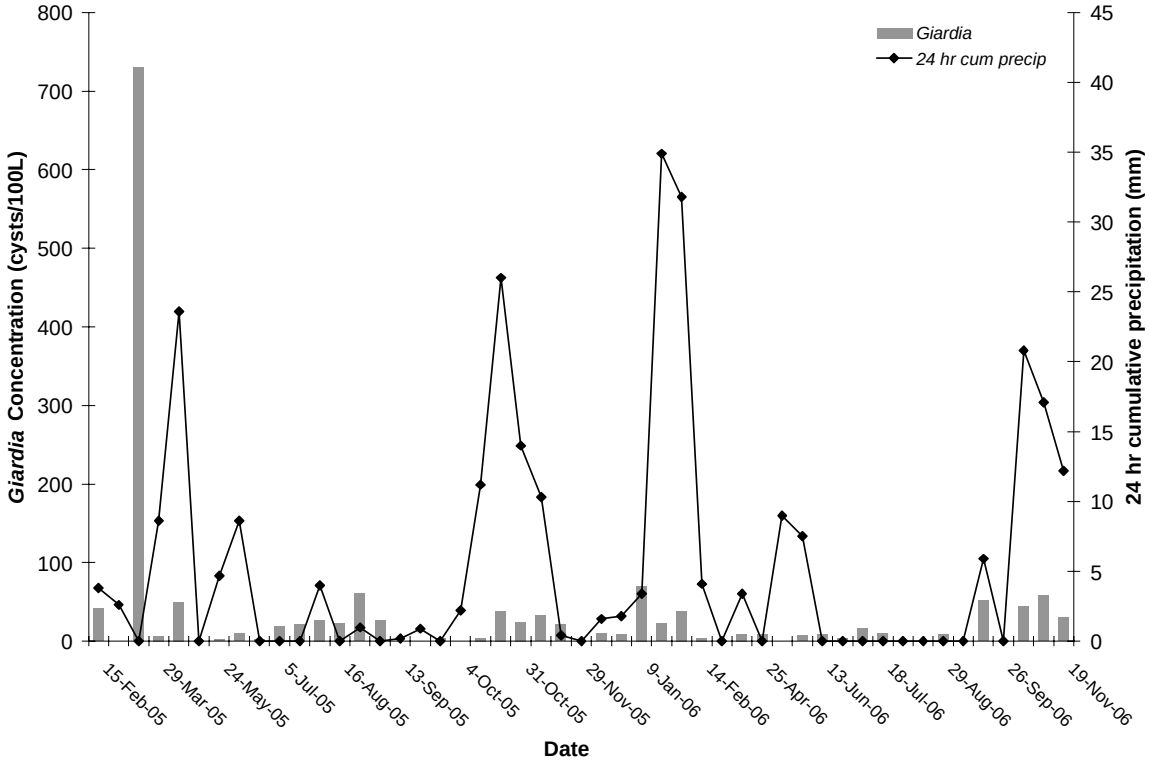


Figure 4-5: *Giardia* concentrations and 24-hour cumulative precipitation for the intake at the Salmon River drinking water plant sampling location, Salmon River watershed.

4.3.2 Rainfall Analysis

The two watersheds analyzed are located in different climatic zones and as such, analysis was performed to determine if differences precipitations patterns could account for the patterns of *Giardia* concentrations observed. Three types of rainfall analysis were performed. First, correlational analysis was performed to determine the relationship between *Giardia* concentration and 24-, 48-, 72- and 96-hour cumulative rainfall for each watershed. Second, correlational analysis was performed to determine the relationship between *Giardia* concentrations and number of precipitation-free days preceding the sampling event. This analysis was performed to complement the cumulative rainfall analysis and addressing the possibility that precipitation-free days may allow the accumulation of

fecal material on-land and could contribute to greater pathogen loadings during a precipitation event. Lastly, analysis of significant rainfall events was conducted.

4.3.2.1 Rainfall Correlation

In the Salmon River data set, a single outlier (March 15, 2005) was removed for correlational analysis. Analysis indicated no relationship between precipitation (rainfall or snowfall) and *Giardia* detection in the Grand River (Table 4-1). In fact, there was a negative relationship between rainfall and *Giardia* concentrations in the Grand River. However, a moderate relationship was observed in the Salmon River watershed between *Giardia* concentration and rainfall (R-values ranging from 0.46 to 0.53). The 48-hour cumulative rainfall was a better indicator than the 24-hour rainfall. There was no relationship between the number of precipitation-free days preceding a sampling event and the concentration of *Giardia* detected.

Table 4-1: R-values (correlation coefficient) from Pearson correlation analysis comparing cumulative rainfall and precipitation-free days to *Giardia* concentrations at two study sites, in the Grand River watershed and Salmon River watershed. An r-value of 0.4 – 0.7 was considered a moderate positive correlation.

	24-hr	48-hr	72-hr	96-hr	Precipitation free days
Grand River	0.05	0.03	0.11	0.18	0.1
Salmon River	0.46	0.53	0.52	0.48	0.22

The lack of correlation between rainfall and *Giardia* concentrations in the Grand River watershed (r-values of 0.18 and lower) is not surprising, as the predicative relationship between climate variables and pathogen concentration likely varies from watershed to watershed. There is no consensus on the predictive capacity of climate variables on pathogen loading in the literature (Thomas *et al*., 2005; Auld *et al*., 2004; Wilkes *et al*., 2009).

However, a negative relationship between rainfall and loadings in this watershed, along with the highest concentrations observed in January in the absence of rainfall or snow-melt, suggests that precipitation serves as a diluting factor, rather than providing a pathogen-loading factor. This differs from the observations in the Salmon River watershed, where there was a correlation, albeit weak, between cumulative precipitation and *Giardia* loading. It is unclear if this difference between the watersheds arises from differences in land-use, geology and topography, or that there is simply such a high baseline loading of *Giardia* into the Grand River (i.e. treated sewage effluent, septic tanks or other sources of fecal contamination that provide consistent pathogen loading), that changes in loadings cannot be accounted for by single variable statistical analyses.

4.3.2.2 Significant Rainfall Events

Detailed rainfall analyses were conducted to determine if the magnitude of a precipitation event, compared to historical averages, could account for pathogen loading (i.e. a "significant rainfall event"). In a retrospective study of Canadian waterborne outbreak occurrence, a relationship between waterborne outbreak occurrence and precipitation in the 93rd percentile was observed (Thomas *et al*, 2005). At this intensity of rainfall, the relative odds of an outbreak increased by a factor of 2.28. Further evidence demonstrating linkages between weather events and disease have been shown in the United States and the United Kingdom as well (Nichols *et al*., 2009; Curriero *et al*., 2001l MacKenzie *et al*., 1993)

An analysis of 'significant rainfall events' was conducted to determine if there was a relationship to pathogen loading in this study. Precipitation percentiles were determined seasonally (using equinoxes and solstices for seasonal definitions), to account for rainfall events in the summer that would contribute significantly to pathogen loadings but would be lost if an annual seasonal analysis was performed. Rainfall statistics were gathered using a ten-year period preceding the start of the study for each watershed. Summary results are indicated below (Table 4-2).

Table 4-2: Summary of historical precipitation statistics for the Grand River and Salmon River watersheds, using a 10-year period from 1995-2005.

	GRAND RIVER				SALMON RIVER			
	Spring	Summer	Fall	Winter	Spring	Summer	Fall	Winter
	mm	mm	mm	mm	mm	mm	mm	mm
MAX	53.50	81.10	43.00	35.20	78.20	55.50	93.80	84.60
MIN	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MEAN	2.50	2.51	2.58	2.26	4.04	1.78	5.49	6.17
ST DEV	5.70	6.28	5.73	4.72	7.50	5.08	10.72	9.73
90th percentile	8.74	8.20	8.51	8.00	12.40	5.80	16.44	17.80
93rd percentile	12.30	12.41	11.36	10.00	14.60	8.20	20.20	21.61
96th percentile	16.16	17.85	16.50	13.88	19.00	12.78	26.03	27.57

Using 93rd percentile precipitation, it was determined that four out of the 38 samples (10.5%) were collected in the Grand River during 'significant rainfall events'. Two of these four of the events were associated with above average *Giardia* concentrations and were both occurred in the fall. However, there were six events with above average *Giardia* concentrations (greater than 31.8 cysts/100L) in the absence of significant rainfall events (all in fall and winter months) (Table 4-3).

Table 4-3: Sample dates in the Grand River association with 93rd percentile rainfall, with associated *Giardia* concentrations.

	Precipitation	*Giardia*
Date	mm	cysts/100L
28-Nov-05	28.8	112
17-Jan-06	15.4	153.6
10-Oct-06	23.5	8
23-Oct-06	11.8	44
Average	3.31	88.04
St Dev	7.16	102.55

Of the 49 samples collected in the Salmon River watershed, five (10.2%) were collected on sampling dates where precipitation exceeded the 93rd percentile precipitation and therefore considered to be a significant rainfall events. These events occurred in the winter (two events), fall (two events and spring (one event) (Table 4-4). Four out of the five events were associated with *Giardia* concentrations that were above average concentrations during the study period. On only three sampling dates were *Giardia* concentrations elevated compared to study averages (greater than 88 cysts/100L), in the absence of significant rainfall events.

Table 4-4: Sample dates in the Salmon River association with 93rd percentile rainfall, with associated *Giardia* concentrations.

	Precipitation	*Giardia*
Date	mm	cysts/100L
12-Apr-05	23.6	49.8
17-Oct-05	26	38.7
17-Jan-06	34.9	23.1
30-Jan-06	31.8	38.5
16-Oct-06	20.8	43.8
Average	3.31	31.80
St Dev	7.16	103.60

These results indicate that precipitation data alone cannot be used to predict *Giardia* loading, nor can it be used to account for differences in pathogen concentrations in two watersheds of differing climatic patterns. It does appear, although not significant statistically, that rainfall is a larger contributor to pathogen loading in the Salmon River watershed, compared to the Grand watershed. It should be noted that the basis of the rainfall analysis (a paper by Thomas and colleagues who studied the relationship between rainfall and waterborne outbreaks of disease) did not account for other variables related to pathogen loading (2005). For example, local soil type, river hydrology and storm intensity may impact pathogen loading, amongst other variables (Dorner *et al.*, 2007; Dorner *et al.*, 2006; Ferguson *et al.*, 2005). Further analyses of these factors would aid in understanding the difference in Giardia occurrence between these two watersheds.

4.3.3 Genotyping

Nested PCR and genotyping were conducted on each positive IFA slide from the Salmon River and Grand River watersheds (Table 4-5). Because multiple slides may be set-up based on IMS-volumes and two heat-dissociation steps, the number of PCRs conducted exceeds the number of samples collected. In the Salmon River watershed, 81% of samples positive by IFA were also positive by at least one PCR. In the Grand River watershed, 65% of samples positive by IFA were also positive by at least one PCR. PCRs were conducted in duplicate to account for heterogeneity of genomic DNA distribution in genomic extracts. The higher number of negative PCRs in the Grand River may reflect either false positives by IFA or false negatives by PCR. While it has been shown that the immunofluorescent antibodies used in Method 1623 (various distributors) may cross react with algal species (Rodgers, 1995), all the positives in the Grand River study were confirmed to be *Giardia* based on fluorescence, size/shape, DAPI staining and DIC microscopy. It is more likely that false-negative PCRs occurred. False negative PCRs may be due to an increased presence of PCR inhibitors (Wilson, 1997) found in the Grand River, which is a much more impacted watershed compared to the Salmon River. Addition of more BSA and dilution of genomic DNA did not resolve this presumed PCR inhibition (data not shown). Furthermore, it is possible that cysts were damaged and contained poor quality DNA, thus impacting the sensitivity of the PCR assay. Lastly, it is also possible that non-*G.lamblia* species were detected by IFA and were not amplified by the primers used in the PCR assay.

Table 4-5: PCR results for the Salmon River and Grand River watersheds, showing the results when performing PCR replicates

PCR Replicates	Salmon River # Reps (%)	Grand River # Reps (%)
0/2	19 (19.4)	31 (35.2)
1/2	16 (16.3)	19 (21.6)
2/2	63 (64.3)	38 (43.2)
TOTAL	**98**	**88**

In the Salmon River watershed, Assemblage A was more predominant (45.5%) than Assemblage B (34.5%) (Figure 4-6). With the exception of Assemblage E, the non-zoonotic, host-adapted strains were not detected in the Salmon River watershed. Assemblage E (livestock associate) was detected infrequently (1.5%), despite moderate sized agriculture activities in the area. No seasonal trends in genotype prevalence were observed. The predominance of zoonotic genotypes in the Salmon River watershed, year-round, suggests a risk to human health through recreational contact and surface water exchange with groundwater in the Hopington Aquifer (Naugler, 2007). It is also suggestive of a unique ecology in British Columbia that supports greater zoonotic transmission of *Giardia lamblia* through Assemblage A, the Assemblage with the greatest host range. Sequence analysis revealed that sequence homology amongst Salmon River isolates was 88.9%.

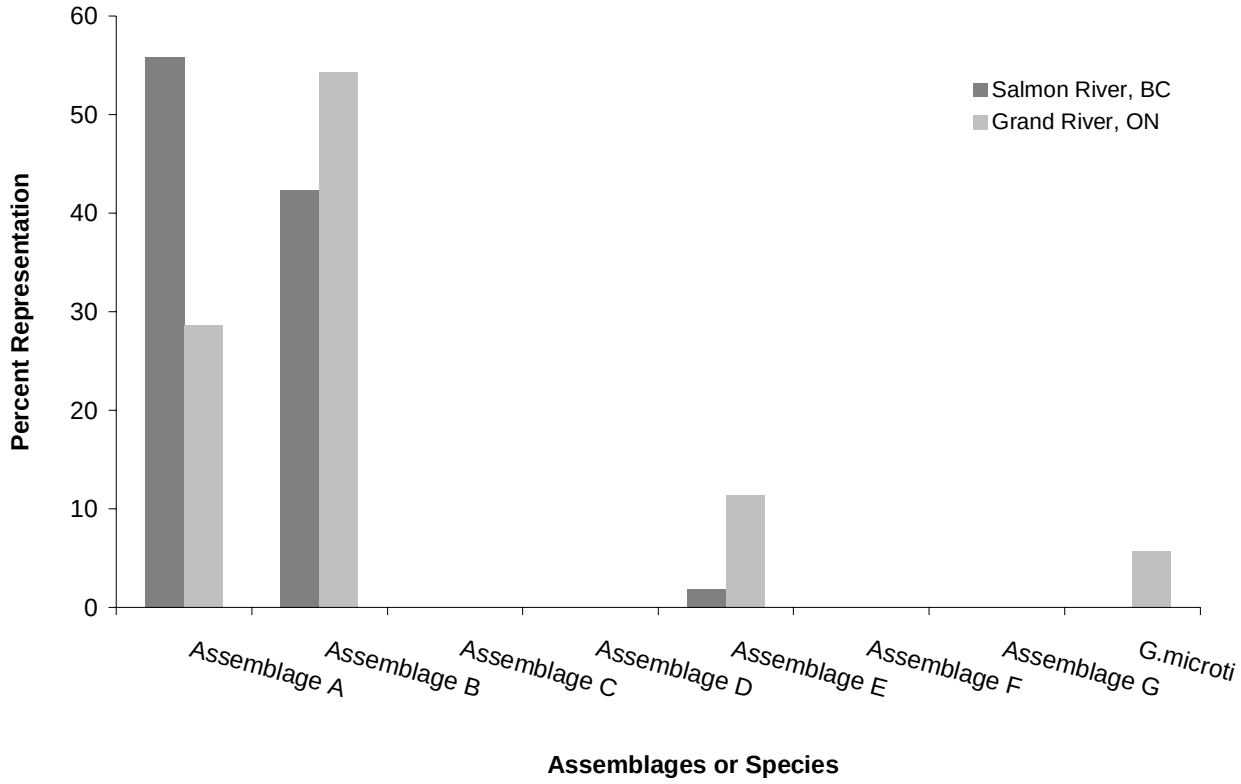


Figure 4-6: Percent representation of *G.lamblia* Assemblages and other *Giardia* species in the Salmon River and Grand River watersheds

In the Grand River watershed, Assemblage B was detected most frequently (54.3%) compared to Assemblage A (28.6%). Assemblage E was detected more frequently in the Grand River (11.4%) than in the Salmon River and *G.microti* (vole and rodent associated) was detected only in the Grand River (5.7%). This indicates that 17% of isolates detected and characterized pose no known risks to human health. The majority of isolates isolated belonged to Assemblages that are known to be infectious to humans and are therefore a public health risk. A non-zoonotic transmission cycles appears to be more important in the Grand River than the Salmon River.

In the early months of the investigation, Assemblage B predominated in the Grand River (August 2005 to March 2006), at which point greater genotype variety was observed. Numerous Assemblage B isolates collected in the Grand River watershed had 99-100% sequence homology to a published sequence previously isolated from aquatic birds (FJ984567), North American beaver (DQ789112) and coyotes (DQ385547) (Pultzer and Tomor, 2009; Fayer *et al*., 2006 and Trout *et al*., 2006). Despite a large number development types and potential sources of pollution, sequence analysis revealed grater sequence homology amongst Grand River isolates was 90.6% than the Salmon River isolates (88.9%). This suggests that ecology of *Giardia lamblia* transmission in the Grand River differs from the ecology in the Salmon River. It is possible that there are fewer dominant strains in the Grand River, based on the proximity of humans, wildlife and livestock. It is also possible that there are fewer sources of *G.lamblia* in the Grand River, loading the parasite in higher concentrations. Further studies are needed to elucidate these possibilities.

Phylogenetic analysis demonstrated no consistent grouping of Grand River versus Salmon River isolates (Figure 4-7). Assemblage B isolates demonstrated the greatest amount of sequence variability, both within watersheds and between watersheds (Figures 4-7 and 4-8). Assemblage A isolates demonstrated very little sequence variability, as indicated in the phylogenetic tree. In cases where two slides were set up for each filter (when pellet size after IMS exceeding 0.5mL), both slides were scrapped and genomic DNA preparation were done separately for each slide. In some cases, such as with filter GR_5658, each slide yielded a different sequencing result (GR_5658_a1 and GR_5658_b1), confirming the presence of mixed genotype in water samples.

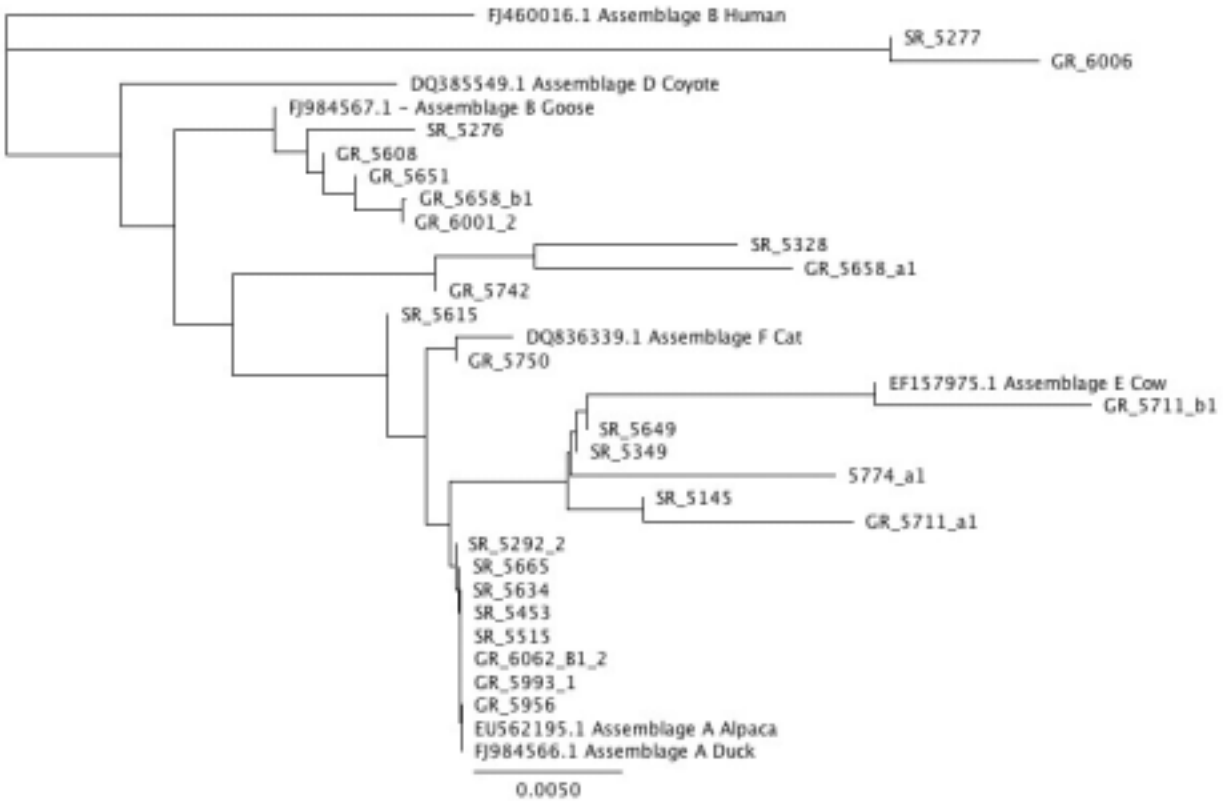


Figure 4-7: Phylogenetic analysis of Grand River and Salmon River *Giardia lamblia* isolates, constructed with Geneious Tree Builder, using Jukes-Cantor genetic distance model with neighbour-joining. The prefix SR is indicative of isolates collected from the Salmon River while GR indicates Grand River.

```
GR_5608      1 CCCCAAGGACACAAGCCATGCATGCCCGCGCACCCGGGAGGCGGCGGACGGCTCAGGACA  60
SR_5711      1 CCCCAAGGACG-AAGCCATGCATGCCCGCTCACCCGGGACGCGGCGGACGGCTCAGGACG  59

GR_5608     61 ACGGTTGCACCCCCCGCGGCGGTCCCTGCTAGCCGGACACCGCTGGCAACCCGGCGCCAA 120
SR_5711     60 ACGGTTGCACCCCCCGCGGCGGTCCCTGCTAGCCGGACACCGCTGGCAACCCGGCGCCAA 119

GR_5608    121 GACGTGCGCGCAAGGGCGGGCGCCCGCGGGCGAGCAGCGTGACGCAGCGACGGCCCGCCC 180
SR_5711    120 GACGTGCGCGCAAGGGCGGGCGCCCGCGGGCGAGCAGCGTGACGCAGCGACGGCCCGCCC 179

GR_5608    181 GGGCTTCCGGGGCATCACCCGGTCGG 206
SR_5711    180 GGGCTTCCGGGGCATCACCCGGTCGG 205
```

Figure 4-8: Sequences of two Assemblage B isolates originating from the Salmon River (SR_5711) and the Grand River (GR_5608). Base changes are indicated in green.

There has been increasing evidence that suggests that Assemblages A and B are two different *Giardia* species, rather than two different Assemblages, based on their host range and genome sequence (Frazen *et al*., 2009; Monis *et al*., 2009 and Monis *et al*, 2004). Assemblage A tends to have a wider host range, including livestock and wild mammals, while Assemblage B tends to be more host restricted, infecting dogs and some wild mammals. Both Assemblages infect humans and other primates. The predominance of Assemblage B in the Grand River watershed is suggestive of *Giardia lamblia* contributions from either wildlife such as beavers, coyotes and birds, or contributions from human sources. The contribution from human sources is plausible, due to the number of wastewater treatment plants upstream. This is confirmed by detection of *Cryptosporidium hominis* in the Grand River, a species that is infective only to humans (data not shown). The predominance of Assemblage A in the Salmon River is consistent with a zoonotic transmission cycle with a wider host range, creating greater difficulties in pathogen source tracking efforts.

Comparisons of genotypes that were recovered during significant rainfall events to those that occurred during regular non-significant rainfall events and to those that occurred during dry periods did not reveal any consistent trends in either watershed. The failure to observe any kind of relationship could reflect limitations of the study rather than a lack of a trend. Because sampling did not occur over a long period of time, seasonal genotype trends could not be established for this comparison to be made reliably. Furthermore, PCR-based characterization of *Giardia* will amplify the

predominant genotype in a sample, thus preventing to observation of minor variations genotype prevalence.

4.4 Conclusions

This study demonstrated high concentrations of *Giardia lamblia* occurring in two Canadian watersheds with differing land-use and climate patterns. There were no statistical relationships between rainfall and *Giardia* loading in either watershed, despite evidence that waterborne outbreaks of disease in Canada tend to be associated with significant rainfall events. It appears that there would be no benefit of changing parasite sampling protocols to reflect significant rainfall events. Genotype analysis demonstrated different types of *Giardia lamblia* Assemblages in the different watersheds, suggesting that a combination of land-use impacts and transmission ecology impact the types of *G.lamblia* Assemblages found in each watershed. As such, it may be possible in the future, with further study, to relate human health risks to specific land-use activities. This would have a significant impact on water resource management and public health interventions in Canada and worldwide.

4.5 References

Adam, R. 2001. Biology of *Giardia lamblia.* Clinical Microbiology Reviews. 14(3): 447-475.

Appelbee A, Frederick L, Heitman T and Olson M. 2003. Prevalence and genotyping of *Giardia duodenalis* from beef calves in Alberta, Canada. Veterinary Parasitology. 112(4): 289-294.

Atwill E, Hou L, Karle B, Harter T, K Tate and Dahlgren R. 2002. Transport of *Cryptosporidium parvum* oocysts through vegetated buffer strips and estimated filtration efficiency. Applied and Environmental Microbiology. 68(11): 5517-5527.

Auld H, MacIver D and Klaassen J. 2004. Heavy rainfall and waterborne disease outbreaks: the Walkerton example. Journal of Toxicology and Environmental Health A. 67(20-22): 1879-1887.

Bishop R, Hively W, Stedinger J, Rafferty M, Lojpersberger J and Bloomfield J. 2005. Multivariate analysis of paired watershed data to evaluate agricultural best management practice effects on stream water phosphorus. Journal of Environmental Quality. 34: 1087-1101.

British Columbia Centre for Disease Control (BCCDC). 2009. British Columbia Annual Summary of Reportable Disease. Provincial Health Services Authority. Vancouver, British Columbia.

Chauret C, Springthorpe S and Sattar S. 1999. Fate of *Cryptosporidium* oocysts, *Giardia* cysts, and microbial indicators during wastewater treatment and anaerobic sludge digestion. Canadian Journal of Microbiology. 45(3): 257–262.

Curriero FC, Patz JA, Rose JB and Lele S. 2001. The association between extreme precipitation and waterborne disease outbreaks in the United States, 1948-1994. American Journal of Public Health. 91(8): 1194-9.

Dorner SM, Anderson WB, Gaulin T, Candon HL, Slawson RM, Payment P and Huck PM. 2007. Pathogen and indicator variability in a heavily impacted watershed. Journal of Water and Health. 5(2): 241-257.

Dorner SM, Anderson WB, Slawson RM, Kouwn N and Huck PM. 2006. Hydrologic modeling of pathogen fate and transport. Environmental Science and Technology. 40(15): 4746-4753.

Fayer R, Santín M, Trout J, DeStefano S, Koenen K, Kaur T. 2006. Prevalence of *Microsporidia*, *Cryptosporidium* spp., and *Giardia* spp. in beavers (*Castor canadensis*) in Massachusetts. Journal of Zoo Wildlife Medicine. 37(4): 492-497.

Ferguson CM, Croke B, Ashbolt NJ and Deere DA. 2005. A deterministic model to quantify pathogen loads in drinking water catchment; pathogen budget for the Wingecarribee. Water Science and Technology. 52(8): 191-197.

Franzén O, Jerlström-Hultqvist J, Castro E, Sherwood E, Ankarklev J, Reiner DS, Palm D, Andersson J, Andersson B, Svärd S. 2009. Draft genome sequencing of *Giardia intestinalis* assemblage B isolate GS: is human giardiasis caused by two different species? PLoS Pathogens. 5(8): In Press.

Hopkins RM, Meloni BP, Groth DM, Wetherall JD, Reynoldson JA and Thompson RC. 1997. Ribosomal RNA sequencing reveals differences between the genotypes of *Giardia* isolates recovered from humans and dogs living in the same locality. Journal of Parasitology. 83(1): 44-51.

Hunter P and Thompson R. 2005. The zoonotic transmission of *Giardia* and *Cryptosporidium*. International Journal for Parasitology. 35(11-12): 1181-1190.

Krewski D, Balbus J, Butler-Jones D, Haas C, Isaac-Renton J, Roberts KJ and Sinclair M. 2002. Managing health risks from drinking water--a report to the Walkerton inquiry. Journal of Toxicology Environmental Health A. 65(21): 1635-1823.

Mac Kenzie WR, Hoxie NJ, Proctor ME, Gradus MS, Blair KA, Peterson DE, Kazmierczak JJ, Addiss DG, Fox KR, Rose JB, *et al*. 1993. A massive outbreak in Milwaukee of cryptosporidium infection transmitted through the public water supply. New England Journal of Medicine. 331(3):161-167.

Mintz ED, Hudson-Wragg M, Mshar P, Cartter ML and Hadler JL. Foodborne giardiasis in a corporate office setting. Journal of Infectious Diseases. 167(1):250-253.

Monis P, Caccio S and Thompson R. 2009. Variation in *Giardia*: towards a taxonomic revision of the genus. Trends in Parasitology. 25(2): 93-100.

Monis P, Andrews R, Mayrhofer G, Ey P. 2003. Genetic diversity within the morphological species *Giardia intestinalis* and its relationship to host origin. Infection, Genetics and Evolution. 3(1): 29-38.

Naugler, T. 2007. Groundwater - surface water interactions in the Salmon River watershed, B.C.: Integrating spectroscopy, isotopes, water quality and land use analyses. M.Sc. Thesis, Resource Management and Environmental Studies. University of British Columbia.

Nichols G, Lane C, Asgari N, Verlander NQ, Charlett A. 2009. Rainfall and outbreaks of drinking water related disease and in England and Wales. Journal of Water and Health. 7(1): 1-8.

Palmer CS, Traub RJ, Robertson ID, Devlin G, Rees R and Thompson RC. 2008. Determining the zoonotic significance of *Giardia* and *Cryptosporidium* in Australian dogs and cats. Veterinary Parasitology. 154(1-2): 142-147.

Plutzer J and B Tomor. 2009. The role of aquatic birds in the environmental dissemination of human pathogenic *Giardia duodenalis* cysts and *Cryptosporidium* oocysts in Hungary. Parasitology International. 58(3): 227-231.

Public Health Officer. 2001. Drinking water quality in British Columbia: the public health perspective. Officer of the Public Health Officer of British Columbia, Victoria, Canada.

Quick R, Paugh K, Addiss D, Kobayashi J, Baron R. 1992. Restaurant-associated outbreak of giardiasis. Journal of Infectious Diseases. 166(3): 673-676.

Robertson L, Hermansen L, Gjerde B, Strand E, Alvsvåg O and Langeland N. 2005. Application of genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn and winter 2004. Applied and Environmental Microbiology. 72(3): 2212-2217.

Rodgers M, Flanigan D and Jakubowski W. 1995. Identification of algae which interfere with the detection of *Giardia* cysts and *Cryptosporidium* oocysts and a method for alleviating this interference. Applied and Environmental Microbiology 61: 3759–3763.

Ross, J. 2006. Influence of climate and agricultural land use on nutrient and bacterial dynamics in surface waters of the Lower Fraser Valley, British Columbia. PhD Thesis, University of British Columbia. 246pp.

Ruecker NJ, Bounsombath N, Wallis P, Ong CS, Isaac-Renton JL and Neumann NF. 2005. Molecular forensic profiling of *Cryptosporidium* species and genotypes in raw water. Applied and Environmental Microbiology. 71(12): 8991-8994.

Schreier H, Hall K, Brown S, Wernick B and Berka C. 1999. Agriculture: an important non-point source of pollution. In: C. Gray and T.Tuominen (eds.) Health of the Fraser River Aquatic Ecosystem, Environment Canada, DOE-FRAP, Vancouver, Vol. 2: 83-99.

Thomas KM, Charron DF, Waltner-Toews D, Schuster C, Maarouf AR and Holt JD. 2006. A role of high impact weather events in waterborne disease outbreaks in Canada, 1975 - 2001. International Journal of Environmental Health Research. 16(3): 167-180.

Thompson R and Monis P. 2004. Variation in *Giardia*: Implications for taxonomy and epidemiology. Advances in Parasitology. 58: 69-137.

Trask J, Kalita P, Kuhlenschmidt M, Smith R and Funka T. 2004. Overland and near-Surface transport of *Cryptosporidium parvum* from vegetated and nonvegetated surfaces. Journal of Environmental Quality. 33(3): 984-993.

Trout J, Santín M and Fayer R. 2006. *Giardia* and *Cryptosporidium* species and genotypes in coyotes (*Canis latrans*). Journal of Zoo and Wildlife Medicine. 37(2): 141-144.

United States Environmental Protection Agency (USEPA). 2005. Method 1623: *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA. Office of Water, U.S. Environmental Protection Agency, Washington, DC.

van Keulen H, Macechko PT, Wade S, Schaaf S, Wallis PM and Erlandsen SL. 2002. Presence of human *Giardia* in domestic, farm and wild animals, and environmental samples suggests a zoonotic potential for giardiasis. Veterinary Parasitology. 108(2): 97-107.

Ware M, Wymer L, Lindquist H and Schaefer F 3rd. 2003. Evaluation of an alternative IMS dissociation procedure for use with Method 1622: detection of *Cryptosporidium* in water. Journal of Microbiological Methods. 55(3): 575-583.

Wernick B, Cook E and Schreier H. 1998. A GIS evaluation of land-use and streamwater nitrate-N dynamics in a watershed at the urban/rural fringe. Journal of the American Water Resources Association. 34(3): 639-650.

Wilkes G, Edge T, Gannon V, Jokinen C, Lyautey E, Medeiros D, Neumann N, Ruecker N, Topp E, Lapen D. 2009 Seasonal relationships among indicator bacteria, pathogenic bacteria, *Cryptosporidium* oocysts, *Giardia* cysts, and hydrological indices for surface waters within an agricultural landscape. Water Research. 43(8): 2209-2223.

Wilson, I. 1997. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology. 63(10): 3741-3751.

Xiao L and Fayer R. 2008. Molecular characterisation of species and genotypes of *Cryptosporidium* and *Giardia* and assessment of zoonotic transmission. International Journal for Parasitology. 38(11):1239-1255.

Yoder JS, Beach MJ and Centers for Disease Control and Prevention (CDC). 2007. Giardiasis surveillance-United States, 2003-2005. MMWR Surveillance Summary. 56(7): 11-18.

Chapter 5
Discussion, Contributions and Conclusions

5.1 New Knowledge Regarding Waterborne *Giardia lamblia*

Since its description as a waterborne pathogen, *Giardia lamblia* has been implicated as an etiological agent in the majority of outbreaks worldwide. It is described as the most commonly detected parasitic cause of diarrhea and perhaps the most common cause of diarrhea in the developing world (in the developed world, norovirus is likely the most common cause of diarrhea). It is frequently found in surface water supplies worldwide and is found, but less frequently, in groundwater and treated water supplies. The overall impact of *G.lamblia* on human populations globally is significantly underestimated.

The first study in this thesis, **Retrospective Analysis of Giardiasis Specimens Collected in British Columbia**, was conducted to develop optimal molecular epidemiological genotyping methods, for routine use in the BCCDC Environmental Microbiology Laboratory and for use in future water quality studies. The purpose of this work was to develop tools to enable research that would answer research questions and provide new knowledge regarding risk analysis, molecular surveillance tools and public health policy development with respect to parasites in drinking water supplies. Following optimization of the molecular testing and analyses of the isolates (n = 78), the study showed that the majority of the archived isolates collected over time from various BC sources belonged to Assemblage A, one of two *G.lamblia* Assemblages capable of infecting humans. Assemblage A has been demonstrated to have the greatest host-range but causes milder illness, while Assemblage B has a more limited host range but is found more frequently in symptomatic patients. It was initially thought that the predominance of Assemblage A during this first study could have arisen from the culturing method used initially for sample amplification (TYI-33 media), as it has been demonstrated that this method preferentially amplifies Assemblage A over Assemblage B (Nash and Mowatt 1992; Monis *et al*., 1996). However, in the subsequent study in Langley, British Columbia, the observation that Assemblage A was the predominant genotype as well suggests that the phenomenon observed in the historical study is a unique feature of British Columbia. The study was crucial in establishing optimal molecular tools to further study this parasite and was useful in creating a geographically relevant sequence database to which comparisons in current studies could be made.

The second study in this thesis, **Occurrence of *Giardia lamblia* and *Cryptosporidium* species in a mixed urban-rural watershed**, while there was a focus on *Giardia lamblia, Cryptosporidium* data also generated as part of the study were included in the paper to create a complete picture of parasite occurrence and to provide comparisons between *Giardia* and *Cryptosporidium*. The purpose of the study was to determine the relationships between classical drinking water indicators and the presence of (oo)cysts. It was also to determine the prevalence of human-infectious *Giardia lamblia* and *Cryptosporidium* species in a mixed urban-rural watershed. It was determined that *Giardia* (86.3%) and *Cryptosporidium* (63%) were detected in the majority of water samples. *Giardia* was detected at much higher concentrations than *Cryptosporidium* year-round, emphasizing the importance of this organism in BC water supplies. There was no consistent relationship between traditional water quality indicators, including rainfall and parasite occurrence, highlighting the need for pathogen specific testing of parasites in drinking water supplies. We found that half of the *Cryptosporidium* isolates and almost all *G.lamblia* isolates (98%) that were genotyped were potentially infectious to humans, based on taxonomic classifications. *Giardia* genotyping revealed a predominance of Assemblage A, the *G.lamblia* assemblage with the greatest host range, while *Cryptosporidium* genotyping revealed a predominance of *C.andersoni*, *C.baileyi*, *C.parvum* and *C.hominis*. *C.andersoni* and *C.baileyi* are restricted to non-human animal hosts, while *C.parvum* and *C.hominis* are infectious to human, indicating that some *Cryptosporidium* samples detected in the study did pose a potential risk to public health. The predominance of Assemblage A in this study and the previous study emphasize the role of this Assemblage in the local ecology in British Columbia. This predominance is most likely a reflection of the large host range of Assemblage A (compared to Assemblage B)

Both organisms demonstrated increased concentrations in the summer, without rainfall, suggesting that events other than precipitation were important factors in parasite loading in the watershed. Previous studies have shown an association between rainfall and outbreaks (Thomas *et al*., 2006). This is a particular concern because some sampling did occur in the Williams Park, a recreational area in the Salmon River watershed that includes swimming in the summer. As described below in Section 6.2.2, the Salmon River watershed is not the most ideal location to make direct links between surface water and human health. However, this link between recreational water use and parasite loads in summer loads should be explored further, particularly since the classical indicators for recreational water use total and fecal coliforms, which were shown in the study to have no relationship to the detection of *Giardia* cysts and *Cryptosporidium* oocysts in the water source.

The third study, Comparative analysis of *Giardia lamblia* occurrence and genotype prevalence in two Canadian watersheds of differing geography, climate type and land-use was conducted to determine differences in prevalence and *Giardia* genotype occurrence in two mixed-urban watersheds. The watersheds of similar size differed with respect to their land-use and climatic conditions. The Salmon River watershed, also studied in the second paper, has moderate urban and rural development and is located in the temperate rainforest climate zone of British Columbia. The Grand River watershed is located in Ontario, has intense urban and rural development and is located in a moderate to cool temperate zone. *Giardia* was detected more frequently (94.5% vs 77.6%) and at higher concentrations (mean concentration, 88 cysts, standard deviation of 102.5 cysts/100L compared to a mean of 31.8 cysts/100L, standard deviation of 103.7 cysts/100L) in the Grand River compared to the Salmon River. Despite significant differences in climatic patterns between the watersheds, differences in precipitation alone could not account for different loading patterns in the two watersheds. Genotyping revealed considerable differences in *G.lamblia* isolates from each watershed. The Salmon River watershed was dominated by a zoonotic Assemblage A; the cattle-specific assemblage, Assemblage E, was only detected once. In contrast, the Grand River watershed was dominated by Assemblage B, with Assemblage E was detected more frequently than in the Salmon watershed. Assemblage B, while still zoonotic, demonstrates a more restricted host range than Assemblage A and cannot infect livestock. Therefore, the prevalence of Assemblage B genotypes in the Grand River must originate from either human, wildlife or dog sources. Combined with the increased loading of these genotypes in winter months (in the absence of precipitation or snow-melt), it is suggested that contamination is occurring from either human sources (wastewater effluent or leaking septic tanks) or wildlife defecating directly into the river. With the current 18s genotyping approach, it is not possible to distinguish human versus animal Assemblage B strains. The proposal that sewage or septic tank contamination in the Grand River contributes to *Giardia* concentrations is supported by the presence of *C.hominis*, a *Cryptosporidium* species known to infect only humans (data not shown). Further analyses would be required for more detailed source attribution of Assemblage B sources in winter months in the Grand River. Suggested analyses include continued testing for *G.lamblia*, *Cryptosporidium* with the addition of *Bacteriodes* sp. and caffeine detection, which together could suggest the presence of human contamination.

5.2 Pitfalls and Opportunities for Improvement

5.2.1 Detection Limits, False Positives and False Negatives

A limitation of this research, which is a common limitation of many environmental molecular epidemiological studies, was the limit of detection for molecular analyses. Low levels of pathogens occur more frequently in the environment than high levels of pathogens. Furthermore, the presence of inhibitors of PCR, such as humic acids and sediments, can greatly reduce the sensitivity of PCR, thus greatly diminishing detection capabilities.

In a clinical laboratory, reproducible sensitivity is required for validation of any clinical assay. Due to the large variability in the characteristics of environmental matrices, there tends be greater variation with respect to exact reproducibility of molecular laboratory methods. However, if molecular tests were to be incorporated into legislated methods such as Method 1623 in the United States (USEPA, 2005) or Water Supply Regulations in England (DWI, 2007), better reproducibility is required. This test reproducibility, while not possible at the matrix level must however be well studied for test optimization at the laboratory procedure level. Likewise, if genotyping data are to be incorporated into Quantitative Microbial Risk Assessments (QMRA), more reproducible data will also be required.

The improved PCR sensitivity in the Salmon River watershed (80%) over the Grand River watershed (67%) may reflect that the PCR assays were validated and troubleshooting conducted using water samples from the Salmon River watershed. Initially, PCR assays were conducted only once (not in duplicate), yielding poor numbers of positives. Trouble shooting work was done to investigate the low PCR sensitivity. This led to increasing the amount of dimethyl sulfoxide (DMSO) from three percent to five percent and bovine serum albumin (BSA) was added as well. DNA volumes were also adjusted. Experiments were also conducted to determine the optimum number of PCR replicates. It was determined that performing a second duplicate increased the number of positive samples but did not benefit from a third replicate for *Giardia* PCR, but was beneficial for *Cryptosporidium* PCR. This likely reflects the observation that the majority of slides examined for *Cryptosporidium* had very few oocysts (less than 5 oocysts), which necessitated a greater number of PCR replicates. By performing PCRs in replicate, assay sensitivity was increased by addressing the heterogeneous nature of DNA in genomic preparations with low amounts of target DNA (Smieja *et al*., 2001). Grand River samples were not validated for optimization of PCR replications because early samples demonstrated high detection levels.

These procedural improvements were performed using genomic DNA extracts collected from Salmon River in the Fall of 2004. Fall months in this watershed are characterized by increasing number of storms and mobilization of accumulated contaminants. Towards the end of the fall, rainfall becomes increasingly consistent in intensity and duration; it is expected there is less mobilization of "contaminants" (pathogens, sediment, inhibitors) at this time. Furthermore, the increased volume of precipitation is expected to changes in the river hydrograph, increasing the river's discharge and thus creating a dilution effect. Therefore, the samples collected in early Fall likely contained the greatest amount of PCR inhibitors present in the Salmon watershed and therefore the most accurate period for performing validation and troubleshooting studies.

In contrast, PCR assays were not re-evaluated before commencing tests for the Grand River watershed. It was assumed that the higher concentration of cysts would allow for a greater detection limit. However, lower than expected PCR detection levels were observed. This could arise from a greater number of false positives IMS/IFA (organisms that are falsely identified and enumerated counted as *Giardia*, or false negatives (organisms that fail to be detected by PCR).

False positives occur when the antibodies used in the IMS and IFA steps of purification and identification of *Giardia* cysts from concentrated drinking water samples bind with antigens that are not *Giardia*. This is a type I error. It has been shown that the antibody from IFA kits can cross react with a variety of algal species (Rogders *et al*., 1995; Nieminski *et al*., 1995). In the study by Rodgers *et al*., it was demonstrated that 24 out of 54 (44%) of algal species tested cross-reacted with a commercial immunofluorescence kit used for the detection go *Giardia* cysts. Two of the algal species examined demonstrated similar intensity apple-green fluorescence while the other species showed lesser fluorescence. One diatom species*, Navicula minima*, showed similar size and fluorescence to *Giardia* cysts.

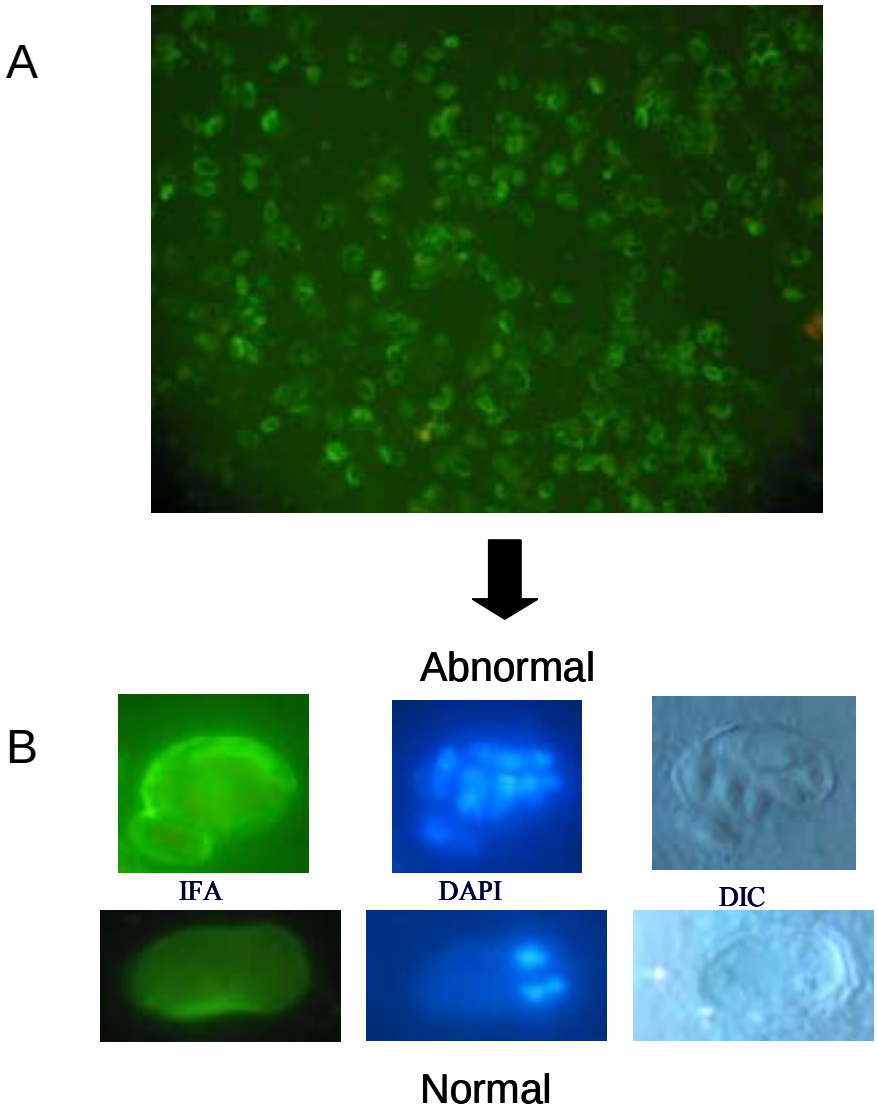


Figure 5-1: Incidence of cross-reactivity between antibodies used during IMS and IFA and a presumed algal species in the Salmon watershed on April 26, 2005. A: 100x magnification. B: 1000x magnification. Top row of images in B show the abnormal features observed using IFA, DAPI and DIC, while bottom row of images show typical structures observed u

An event of substantial cross-reactivity was observed on April 26, 2005 in Coghlan Creek in the Salmon River watershed (Figure 5-1). Hundreds of organisms that were a similar size and shape to *Giardia* were observed and demonstrated similar apple-green fluorescence characteristic of the IFA test used in Method 1623, under 100X magnification. However, upon examination under 1000X magnification, the cells were inconsistent with typical *Giardia* cells. Cells were often associated with smaller 'daughter' cells (Figure 6-1) and flourescein isothiocyanate (FITC) staining was often brighter than usual. DAPI staining revealed more nuclei than actually present in *Giardia*; these nuclei were not localized to a single axis of the cell. Furthermore, there was an appearance of distinct daughter cells within the larger cell, also not consistent with *Giardia*. Lastly, DIC microscopy, which is used to evaluated internal morphology of cells, confirmed the presence of distinct daughter cells within the larger cell, a morphology that is more consistent with algae than *Giardia*.

Communication with Dr. Susan Watson (University of Calgary, National Water Research Institute (NWRI)) suggested that the cells were consistent in appearance with *Oocystis* (Figure 5-2). Oocystis is a genus of green algae belonging to the Order *Chlorococcales*. This algae is ubiquitous but thrives specifically in areas with high nitrogen phosphorous ratio (P:N), such as from sewage or manure contamination.

Figure 5-2: *Oocystis* cells. Source: Dr. Susan Watson, University of Calgary and NWRI.

Due to the morphological features of the *Oocystis* cells, to eliminate most cross-reacting cells was possible. However, when the cross-reacting cells demonstrate fewer atypical features, microscopy is a much greater challenge in correctly identifying *Giardia* in environmental samples. In theory, the internal morphology (as shown by DIC) and the presence of distinct nuclei should differ between *Giardia* and an algal species. However, in practice, identification of *Giardia* by microscopy is not straightforward. Distinguishing features of a cyst are rarely all observed under DIC microscopy (such as a median body or the axonemes). Yet objects are still classified as cysts unless they contain abnormal features such as appendages, crystals or stalks. Likewise, examination of DAPI staining rarely reveals four well-staining nuclei; more frequently two or less nuclei are observed.

It is likely that false negatives also occurred. When PCR fails to detect a *Giardia* cyst, even when the appropriate target is present, a type II error occurs. In water microbiology, this occurs most frequently due to the presence of inhibitors of the PCR reaction in the water matrix. The most commonly described PCR inhibitor in water are humic acids. Humic acids are the acid insoluble fraction of humus soil and presence in even minute quantities can result in inhibition of PCR. As little as one µl of humic acid in a 100µI reaction volume can result in complete inhibition of the PCR reaction (Tsai and Olson, 1992). Inhibition by humic acids occurs by the phenolic groups in the humic acids forming bonds with DNA and therefore physically preventing the attachment of polymerase to DNA (Young *et al*., 1993).

Other sources of interference in environmental matrices include other phenolics, plant polysaccharides, fulvic acids, non-target DNA and heavy metals (Wilson, 1997). A less common described inhibitor of PCR is the presence of suspended solids such as sediments, which may physically prevent the interaction of DNA and polymerase. This can occur via general interference, or when positively charged sediments are present such as silica, can bind to DNA and thus cause physical interference between DNA and polymerase. There are many strategies to counteract PCR inhibition; the most commonly used strategies are additives such as BSA and dilution of genomic DNA preparations to reduce the quantity of inhibitors in a reaction. Because water matrices changes so frequently, it is difficult to optimize PCR for all water conditions.

In the case of Grand River watershed, there was a period from April 24, 2006 to September 25, 2006, where 12 out of 16 PCRs were negative (no parasites detected, NPD). Cyst counts were variable during this period but the negative PCRs did include a sample that contained 23 cysts, which definitely should

be detectable by PCR. This could be due to either a false positive or a false negative. The increased hours of sunlight and elevated water temperature may result in algal blooms, resulting in false positives. Likewise, lower water levels in the summer can result in increased concentrations of inhibitors, resulting in false negatives.

Samples that were expected to be positive were infrequently retested, using both larger volumes and diluted DNA, as well as spiking with known quantities of DNA to evaluate PCR inhibition. When performing research, such an approach is acceptable, but should PCR be incorporated into a legislated method or a QMRA model, a more vigorous algorithm for confirming false positives or false negatives needs to be established.

5.2.2 Site Selection

For research at both the Salmon River site and the Grand River site, detection and characterization of *Giardia* was a sub-project of larger research programs. Both sites are well characterized, having been included in many previous research activities (Naugler, 2007; Ross, 2006; Zandbergen and Hall, 1998, Wernick *et al*., 1998; Hall and Schreier, 1996; Wernick, 1996; Cook, 1994; Watts, 1993). The Grand River site is an ideal site to characterize protozoa due to the intensity of land-use, presence of wastewater treatment plants and potential impacts of contaminated water on human health, through both contamination of groundwater and use of surface water. It has also been the site of many research activities (Cheyne *et al*., 2009; Dorner *et al*., 2007; Jyrkema *et al*., 2007; Lissemore *et al*., 2005; Jamieson *et al*., 2005; Plummer *et al*., 2005; Dorner *et al*., 2004; Krause *et al*., 2001).

In contrast, the Salmon River watershed has a lower intensity of land-use and there is not a direct risk to public health through the consumption of contaminated water. In addition to being well characterized, the Salmon River site was considered a desirable research site due to the mixed land-use, its increasing urbanization and the intensity of agriculture due to its inclusion in the Agricultural Land Reserve of British Columbia. There are also a large range of agricultural activities in the area, including large-scale commercial farms and smaller hobby farms raising llamas and other unique animal species.

The features of the groundwater also create an interesting research site; the Hopington Aquifer, which underlies the watershed, is a composite of three layered aquifers. In the Township of Langley, there are a

total of 13 aquifers from which water is drawn for drinking water purposes. Furthermore, there is evidence of persistent nitrate contamination of groundwater in the area, suggesting persistent agricultural contamination of groundwater. However, using the Salmon River in a Source-to-Tap study, to examine inputs of pathogens and outputs of human health impact was less than ideal, since surface water is not used directly for human consumption. There is evidence of complex groundwater-surface water interactions, in which groundwater feeds river water in summer months and vice versa in the winter months, as determined using nitrate isotopes ($\delta^{18}0$ and $\delta^{15}N$) (Naugler, 2007).

There is, however, substantial evidence demonstrating that *Giardia* is rarely found in deep aquifers. The natural filtration process of water and contaminants percolating from the surface into groundwater removes larger particles; much like a slow-sand filter would remove particles in a drinking water treatment plant. The efficiency of pathogen removal depends largely on the size of the organisms and the characteristics of the subsurface. Larger microorganisms such as *Giardia* and other parasites such as *Cryptosporidium* and *Toxoplasma gondii* are removed more easily than viruses. Aquifers overlaid with sandy soil, gravel or bedrock containing many fissures are more vulnerable to contamination. Therefore, it is expected that groundwater in then Township of Langley would be infrequently contaminated with *Giardia*. This was confirmed by sampling raw Arcadia Municipal groundwater and never detected *Giardia* in a one-year study period.

While the Salmon River site was not ideal for examining direct health effects, it did provide an opportunity to study a moderately developed watershed and to develop an understanding of how land use and climate could impact pathogen loading. Furthermore, it provided a reference to compare loadings and characteristics of *Giardia* in a heavily impacted watershed in Ontario.

5.3 Implications

5.3.1 Implication of Findings for Public Health Officials, For Drinking Water Purveyors and For Watershed Managers

The purpose of monitoring raw water supplies for *Giardia* and other parasites is two fold; first, to identify transmission and loading patterns in the and secondly, to identify and quantify the potential impacts on public health. Using the Method 1623 without the incorporation of molecular methods gives

public health officials, drinking water purveyors and watershed managers very little information regarding actual health risks. The use of Method 1623 without molecular methods is useful only in understanding loading patterns of all members of the *Giardia* genus, and more importantly, drinking water treatment approaches necessary to achieve 99% reduction. Given the considerable costs and labour of performing Method 1623 (up to $800 at private laboratories), it seems wasteful to perform a test that cannot provide health risk data.

The purpose of this study was to develop new knowledge regarding *Giardia lamblia*, to have implications on public health risk assessments, microbial surveillance and ultimately, on development of scientifically sound public policy. *Giardia* was selected as the main organism of investigation for this thesis, in part due to its elevated number of laboratory-confirmed cases in British Columbians. More importantly, however, is that this pathogen has a significant impact on global populations and is relatively under-examined compared to its more popular protozoan relative *Cryptosporidium*. There is a need, internationally, to develop molecular epidemiological tools specifically to generate more meaningful data regarding *Giardia lamblia* occurrence and health risks.

However, the study of *Giardia* can be particularly challenging for several reasons. First, because *Giardia* is studied less frequently, the number of published *Giardia* sequences is limited (compared to *Cryptosporidium*) and covers less animal hosts and a smaller geographic range. Furthermore, because *Giardia* most likely reproduces asexually (or currently reproduces asexually, as per recent publications), there are less opportunities for genetic recombination to create greater diversity of genotypes and as such, *Giardia* sequences tend to be more stable. Lastly, the greater host specificity of *Cryptosporidium* species has allowed for more specific pathogen source tracking and a more black-and-white determination of potential human health risks of specific isolates. When an Assemblage A isolate is collected, the range of potential hosts to which the isolate could be attributed includes most mammalian species. *Cryptosporidium*, with the exception of *C.parvum*, has a much better defined host range. While not proven, it is possible that this difference in host specificity arises from *Giardia* being an extracellular parasite while *Cryptosporidium* is an intracellular parasite. *Cryptosporidium* requires specific ligands for attachment and internalization into cells, which would differ from host to host. However, since *Giardia* remains extracellular, it would appear that this interaction is less important in determining host range.

The environmental research focused on amplification and characterization the 18s rRNA gene, because it is stable and because it is a multi-copy gene, thus improving sensitivity significantly. While it has not be determined definitively, *Giardia* is considered to have five copies of the 18s rRNA gene (like *Cryptosporidium*), thus increasing sensitivity of the assay five-fold over an assay that targets a single copy gene. Initially, the β-giardin gene was selected as the ideal target for genotyping of environmental samples, because it is a surface cytoskeletal protein, rather than a component of an extremely stable organelle, such as the ribosomal small subunit (SSU = 18s rRNA). It was also presumed to be slightly antigenic and therefore more likely to be under evolutionary pressure and expected to demonstrate greater sequence variability. However, it did not demonstrate levels of sensitivity that would be required in an environmental assay. During its use in the early stages of the Salmon River watershed study, less than 50% of samples were positive by repeated PCRs, despite high concentration of cysts. It was replaced with the 18s rRNA assay, which showed much higher sensitivity (80%). In this case, however, there was a trade-off between sensitivity and genotyping resolution. Resolution is defined as the ability to distinguish between two different genotypes. β-giardin was a better target for identifying subtle changes in the gene sequence that could distinguish one isolate from another, while 18s rRNA was a better target for sensitivity and reproducibility of results.

In the Salmon River study site, 98% of isolates were determined to belong to Assemblage A or B and therefore could potentially infect human population. In the Grand River study site, 83% of isolates were determined to belong to Assemblage A or B and therefore could infect human populations. From the perspective of public health officials, these data suggest that drinking water purveyors (suppliers) should assume that all *Giardia* cysts in these watersheds could infect humans; water treatment should be carried out at a level to ensure 99.99% removal of *Giardia* cysts. Furthermore, if there were ever a break-down in any step of the drinking water treatment process, the population would be at risk for being infected by *Giardia lamblia.* Lastly, from the public health perspective, the concentration of *Giardia* and the known Assemblage present, may impact the suitability of these waters for recreational use. From a drinking water purveyor's perspective, despite the possibility that Method 1623 overestimates the number of potential infectious cysts in a raw water supply, in both these watersheds the presence of *Giardia* poses a real health threat. Therefore, purveyors should treat water to ensure 99.9% removal of *Giardia*. From the perspective of a watershed planner, the predominance of the zoonotic transmission cycle suggests that all hosts of zoonotic *G.lamblia* strains could be or become infected with *G.lamblia*. Therefore, watershed best management practices should attempt to reduce loadings of any fecal material into water sources,

whenever possible. This could include the implementation of buffer zones to reduce surface runoff into the water source, as well as situation intensive animal operations away from water sources.

5.3.2 New Knowledge Application: Integration of molecular data into current methods and regulations and interpretation of results.

Molecular analyses are increasingly being incorporated into the current gold-standard for detection of *Giardia* and *Cryptosporidium* in water supplies, Method 1623 (Lobo *et al*., 2009; Yang *et al*., 2008; Plutzer *et al*., 2008; Di Giovanni *et al*., 2006; Ruecker *et al*., 2005; Jiang *et al*., 2005). PCR with sequencing or RFLP is being incorporated into both research and routine testing laboratories, without the standardization that is tenant of Method 1623. Furthermore, it has been suggested that molecular data could be incorporated into Quantitative Risk Assessments (QMRA), in which case data of an extremely high quality is necessary. Successful integration of molecular epidemiological into routine parasite monitoring will have great significance to regulators, drinking water purveyors and laboratories performing drinking water monitoring.

Success of incorporation of molecular analyses into Method 1623 varies from study to study, likely due to the nature of the water matrix, DNA extraction method, PCR and sequencing methods and how the data was analyzed. Of great concern is that due to the nature of environmental matrices (low target concentration and presence of PCR inhibitors), results are not always reproducible. Furthermore, when using PCR, there are no guidelines for confirming or refuting false positives and false negatives. For examples, if a water sample was positive by IFA but negative by PCR, what are the necessary steps to confirm that the IFA was falsely positive, or that the PCR was falsely negative. In the studies described in this thesis, IFA was considered the gold-standard and if duplicate PCR failed, that it was a resulted from matrix effects. When duplicate PCRs failed, the filtration and IFA records were scrutinized to provide insight into why the PCR may have failed. Furthermore, if other assays have already been performed (i.e. if *Cryptosporidium* testing was conducted before *Giardia* testing), those records were also analyzed to identify trends in PCR failures. Lastly, if those records could not provide insight into PCR failure, additional testing was occasionally conducted if genomic DNA volumes permitted.

In a clinical or chemical laboratory testing facility such as a public health laboratory or a commercial laboratory where QA/QC is very important, the assay sensitivities in *Giardia* and *Cryptosporidium*

environmental testing would be unacceptable and would likely fail validation procedures. For Method 1623, the gold-standard *Giardia/Cryptosporidium* assay, matrix spikes for validation allows recoveries ranging from 13-110%. Such recoveries would almost never be observed in any clinical microbial assay. Likewise, the challenges in recoveries for molecular analyses cannot be addressed with typical QA/QC procedures, but there does need to be an effort towards producing high quality, reliable data that can be incorporated into public health policy decisions.

5.3.3 Quality Assurance and Quality Control in *Giardia* Molecular Analyses and Confirming Negative Results

Quality assurance (QA) is a planned and systematic approach to provide confidence in an assay's suitability for its intended testing purpose. The main paradigm of QA is the Shewhart cycle, which is a Plan-Do-Check-Act (PDCA) approach. Quality control (QC) is a process of ensuring a certain level of quality to a specific assay. In molecular biology, this is most commonly addressed by incorporating a series of positive controls (PCR positive control, method positive control and matrix spike) and negative controls (PCR negative control and method blank).

Under typical QA/QC approach, which was followed whenever possible in method development and employment, there should be a deliberate corrective action approach for any errors identified during the PDCA approach. The approach employed for dealing with the high number of negative PCRs was not methodological and routine. The following flow chart recommends a more rigorous approach at managing the large number of negative result, typical of environmental samples (Figure 5-3). Due to increased number of tests, it is suggested that negatives that need to be retested be grouped and retested together.

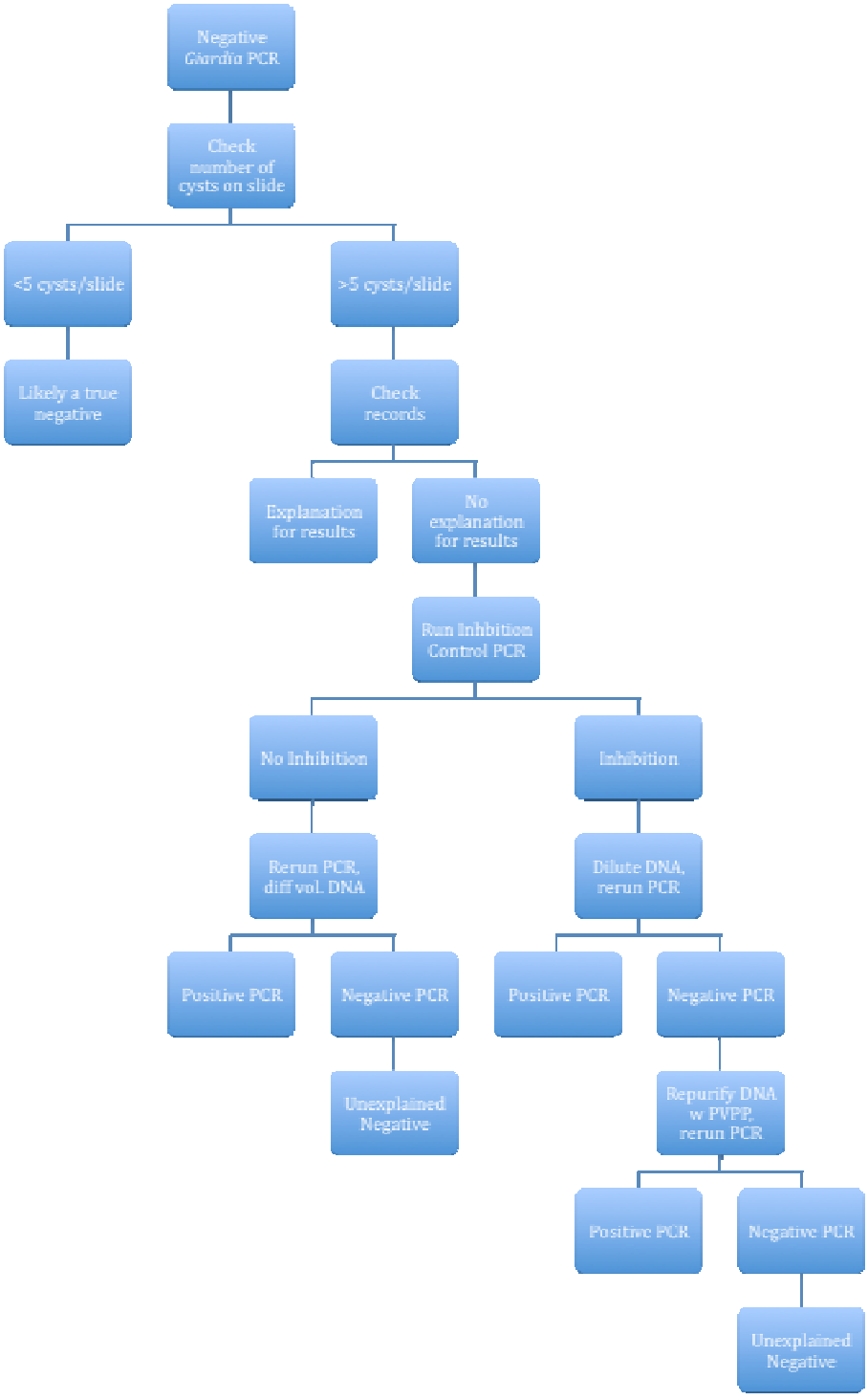


Figure 5-3: Proposed flow-chart of confirming negative PCRs

It is suggested that when there are less than five cysts per IFA slide, that those negative results should be considered true negatives, due to the analytical sensitivity of the test. While the sensitivity of the assay in laboratory water was a single cyst, when performing PCRs in real samples that below five cysts, recoveries were inconsistent. Above five cysts, it is recommended that analytical records be reviewed to determine if there were explanations for poor molecular recoveries. This could include examining records of other molecular analyses such as *Cryptosporidium* PCR. If those PCRs were negative as well in presence of high oocyst counts, it may suggest issues of inhibition and the inhibition control step could be skipped. Next, if the records to not reveal any explanation, an inhibition control test should be conducted. This can be conducted by spiking a known quantity of *Giardia lamblia* DNA or cloned and engineered18s rRNA target of *G.lamblia*. If the inhibition control demonstrates no inhibition, the PCR should be repeated with different volumes of DNA, if sufficient DNA remains, to determine if it is a quantity effect. If there is inhibition, it is suggested that samples first be diluted and to rerun the PCR to dilute out the inhibitors. If this is unsuccessful and sufficient DNA remain, the DNA preparation should be re-purified using a PVPP spin column, which preferentially removes humic acids from the specimen, which are the most common inhibitors in environmental samples. If all corrective action still results in a negative PCR, then the result should be considered a true negative (without clear explanation). In this situation, it is possible that the target contained within the genomic preparation is not *Giardia* (false-negative IFAs). If this situation persists, it is suggested that a universal 18s rRNA PCR with sequencing be performed to determine the organism that is causing the false-positive results.

5.3.4 Interpreting Results: What Happens When an Unexpected Genotype is Detected?

The basic biology of *Giardia* and *Cryptosporidium* are quite well understood. While changes in taxonomy are occurring as new sequence data is generated, the host range of different Assemblages (*Giardia*) and species/genotypes (*Cryptosporidium*) are well defined. It is possible, however, that unexpected results may arise where a genotype is recovered from an unexpected source. For example, what is an Assemblage E (cattle) sequence was isolated from a protected watershed? What if a *Cryptosporidium hominis* (human) was isolated from the same watershed?

While neither of these two scenarios was encountered during the studies of the Salmon River or Grand River, a plan should be in place as part of a rigorous QA/QC program for handling such rarities. If such an event were to occur, a review of all documentation should be performed to ensure there was no

possibility of cross-contamination with controls or other samples. Then, the PCR and sequencing should be performed in duplicate by another technician. If the result still shows the rarity and further investigation is warranted, the result should be reported to the client and additional testing/investigations should be conducted into the source of the isolate.

5.3.5 Developing Bioinformatic Tools for In-House Use

Another consideration for performing molecular analyses in routine environmental microbiology is having well developed bioinformatic tools available. A localized BLAST search can be developed in-house using bioinformatic software such as Geneious Pro or when the database exceeds the threshold in the software tools, local BLAST search engines can be developed with a programmer. A localized *Giardia* BLAST library was constructed in Geneious Pro consisting of the most common/high quality sequences from GenBank along with sequences generated during this study. However, this library is connected to a single user on a single computer, based on license requirements and current network setup. For future use, multi-user copy, shared localized BLAST libraries are suggested.

Better tools for storing and sharing data are also required for routine molecular testing. Currently, sequences are saved in a dedicated sequence drive as *.seq* files, analysis is performed and phylogenetic trees are saved in Geneious Pro under a single license and genotype designations are saved in an Excel file. It is suggested that better data management is required as sequence volumes increase with ongoing testing. An example of such a program is Bionumerics (Applied Maths, Austin, Texas), which also allows multiple users to deposit sequence data.

5.3.6 Collected Data with Nowhere To Go – How to Ensure Enumeration and Genotyping Data Has Meaning.

Giardia and *Cryptosporidium* data is being collected in a number of watersheds and water utilities across Canada, for both routine and research purposes. Drinking water utilities in Canada are not under any legislated requirements to test for these organisms in drinking water at a federal level. Some utilities choose to enumerate these organisms voluntarily, such as Metro Vancouver, while others such as Epcor facilities in Calgary and Edmonton are required to perform periodic testing as part of their operating

permits. For all three of these utilities, molecular testing are performed as a value-added service (in-kind) by the Provincial Health Laboratory. Furthermore, research is being conducted at numerous Universities, Public Health Laboratories and several private laboratories in Canada. In most cases, molecular analyses are being conducted as part of academic activities. Enumeration and molecular data is being collected nationwide and with the exception of publications, this data is rarely being shared. This is particularly true for water quality data collected by drinking water utilities.

At a local level, data can be made meaningful by incorporated both enumeration data and molecular data into Quantitative Microbial Risk Assessments (QMRA). A QMRA model has been developed by Health Canada, specifically for Canadian drinking water municipalities. It is not yet publicly available, but both advanced and simplified models are expected to be available following a consultation process. QMRA has been applied to water quality data extensively, including models specifically developed for *Giardia* and *Cryptosporidium* (Bryan *et al*., 2009; Petterson *et al*., 2007; Mons *et al*., 2007; Smeets *et al*., 2007; Astrom *et al*., 2007; Signor and Ashbolt 2006; Haas 2002). Current *Giardia* and *Cryptosporidium* models do not include a parameter for percentage of human-infectious genotypes detected in a watershed. However, such a parameter could easily be incorporated into current models and would provide meaning to genotyping data that is frequently collected but rarely utilized.

At a national level, it would be beneficial to share prevalence and genotyping data. If only raw water quality were shared and no operational data, this would have no privacy issues for utilities. However, due to the sensitivity of such data and in particular, pollution contributors upstream (such as wastewater treatment plants), this data could be anonymized or aggregated geographically. It would be extremely useful to have a national database of environmental *Giardia* and *Cryptosporidium* sequences, particularly as the practice of genotyping isolates becomes part of routine practice. Compared to studies in the United States and Europe, there are very few publications regarding Canadian environmental sources of these organisms.

5.4 Future Directions

5.4.1 Methodological Improvements

5.4.1.1 Deficiencies with the Current Approach

While Method 1623 remains the current gold-standard for the detection of *Giardia* in water supplies, it is not without deficiencies. Our research confirmed many of the challenges of the gold standard method and demonstrated that without molecular analyses; very little information could have been generated regarding the impact of parasites on the water supplies under investigation. The following are the most commonly described problems with the current approach:

1. Costly ($500 - $700 CND, depending on the sample turbidity)
2. Labour intensive (up to 12.5 hours, without molecular)
3. Requires highly specialized staff, particularly for microscopy
4. **Identifies only to the genus level, therefore detecting species and subtypes not infectious to humans**
5. Identifies both viable and non-viable cells
6. Sampling only captures a snapshot of water quality

We describe the incorporation of molecular analyses post-IFA in these studies, a novel approach improving the level of identification (item number four above) which is extremely important in identifying if isolates could pose a risk to human health by knowing the host range of different isolates. This study demonstrated that genotyping could easily be incorporated into the work flow of Method 1623, essentially adding a fourth step (Characterization) to the three existing Method 1623 steps (Filtration, Purification and Enumeration).

However, we suggest, based on these studies, further improvements to the methods by incorporating new technologies into each of four steps for the detection and characterization of parasites All of these improvements could be applied to *Cryptosporidium* testing as well and to other encysted parasites such as *Toxoplasma gondii* or *Cyclospora cayetanensis,* should they be incorporated into routine testing.

The current approach to sampling for *Giardia* is filtration of the largest volume possible, as limited by the sediment load and the manufacturer's recommendations. Filtration is accomplished using one of two approved filters; either IDEXX's FiltaMax or Pall's Envirochek capsule. The Envirochek filter is a one micron filter folded like an accordion to increase surface area, while the Filtamax filter is a nominal pore sized filter, made of multiple foam layers again to increase the surface area. Both filters clog easily, particularly when samples exceed five NTU, which greatly affects the detection limit under turbid conditions. When filtering large volumes of water to concentrate low levels of parasites, this also results in the concentration of other materials that can interfere with all steps of Method 1623 and related molecular analyses. Furthermore, these filtration units can only be used for the detection of parasites; pore sizes are too small to concentrate bacteria or viruses. This work suggests that an approach where all three microbial types are studied (viruses, bacteria and protozoa), three different concentration steps would be extremely beneficial. Furthermore, in our laboratory using FiltaMax, we have found that very turbid samples can result in poor elution of organisms from during washing steps. This appears to be due to improper expansion of the filter when inundated with sediments.

Results from this research suggest that an improvement to the current filtration approach is the use of ultrafiltration (UF). Ultrafiltration is increasingly being applied, in a research context, to evaluate the microbial quality of drinking water because it can filter large volumes of water and simultaneously concentrates parasites, bacteria and viruses (Hill *et al*., 2009; Morales-Morales *et al*., 2003). The greatest benefit of simultaneous concentration would be the availability of virus method. Because enteric viruses can be tightly adapted to a specific animal host, in addition to the health risk data provided by studying viruses, they could also be used in a source-tracking capacity for identifying sources of fecal contamination. The main limitation of such an approach is that is more costly and labour intensive than Method 1623.

I propose that another alternative to filtration is to apply the concept of dielectrophoresis (DEP) to isolate pathogens from a complex matrix. It is described in more details in Section 6.4.1.3 below, as it can be applied to both the concentration (Step 1) and separation (Step 2) aspects of Method 1623.

The current approach to separation of parasites from the resulting eluate after filtration is immunomagnetic separation (IMS). This step, incorporated into Method 1623 in 1999, vastly improved specificities of the method. Without the IMS, enumeration was extremely difficult, with interference of

both fluorescing and non-fluorescing particles under FITC staining, and further difficulties with DAPI staining and DIC microscopy. There is, however, only one manufacturer of anti-*Giardia*, anti-*Cryptosporidium* IMS kit approved for Method 1623, Invitrogen. The IMS beads were previously manufactured by Dynal Biotech but the company was purchased by Invitrogen in 2005. Since then, our laboratory has observed a considerable decline and variability in the recoveries of both *Giardia* and *Cryptosporidium* during spiking experiments. Recoveries are evaluated upon receiving a new batch of IMS beads and recoveries have been reported as low as 30%. This, however, is still within the acceptable parameters of Method 1623. These results of our reagent quality control (QC) are not incorporated into our routine results. However, based on this variability of the reagents, results may vary depending on the lot of IMS beads used.

Furthermore, IMS is the most costly step of the entire procedure. Excluding QA/QC procedures, it costs $74.16 CND per sample if the filtration pellet volume is less than 0.5 mL. If the pellet volume exceeds 0.5mL (for turbid samples), a second IMS reaction must be set up and the total cost for a turbid sample is $147.80 CND. I suggest that DEP, as mentioned above, could serve as a concentration and separation step to replace IMS.

DEP occurs when a force is applied to a neutral charged particle in the presence of a non-uniform electric field. The difference in dielectric properties of a particle and the surrounding fluid, along with creating specific electric fields that best capture a particle of interest, can be used to select specific particles from a complex matrix. Thus, a flow-through cell can be created with a non-uniform electric field designed to preferentially capture *Giardia* or *Cryptosporidium*. The dielectric characteristics of *Giardia* and *Cryptosporidium* have already been identified and early studies have been conducted in collaboration with Drs. Eric Legally and Karen Cheung at the University of British Columbia to determine the suitability of this approach for drinking water matrices (Dalton *et al*., 2004 and Dalton *et al*., 2001). The greatest benefit of DEP is that samples would be simultaneously filtered and purified, eliminated the need for Step 2 in Method 1623, purification by IMS. Furthermore, DEP should eliminate other particles such as humic acids and sediments, known to impact downstream molecular analyses. This method is also beneficial because it can differentiate between viable and non-viable (oo)cysts (Dalton *et al*., 2004) Currently, the usefulness of DEP is greatly limited by the small sample volumes on which most DEP platforms were developed; typical volumes have been in the microlitre to millilitre range. There is

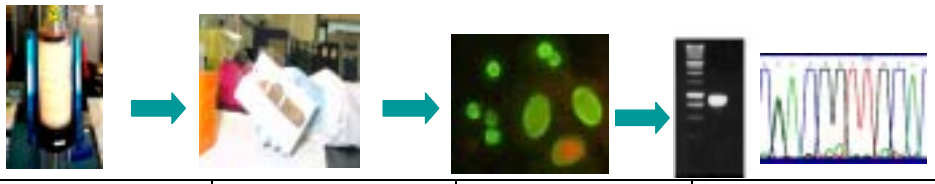
evidence that running many DEP cells in parallel could be used to scale up the volumes to more applicable volumes (Gadish and Voldman, 2006).

The current approach for enumeration of *Giardia* is through an indirect immunofluorescence assay (IFA), with further confirmation using DAPI and DIC microscopy. This approach is fairly inexpensive, but is labour intensive and requires well trained technologists or scientists with considerable microscopy experience. A combination of improved separation using DEP and improved quantitative detection of *Giardia* by molecular methods could eliminate the need for labour-intensive microscopy. However, it is recommended that until molecular methods are improved to a level where false-negatives rarely occur, enumeration of *Giardia* by IFA microscopy continue to be the gold-standard for enumeration of *Giardia*.

In the studies described in this thesis, molecular analyses were incorporated into Method 1623 by scraping IFA slides, performing a routine DNA extraction, followed by nested-PCR and sequencing. The current method is labour intensive and vulnerable to PCR inhibition, resulting in false-negatives. DNA quality would improved by better the better separation method described above (DEP) but could also be improved by a better DNA purification method. A method that was explored, but could not be incorporated into routine testing during the course of this thesis due to cost and the experimental nature of the method, is Synchronous Coefficient of Drag Alteration (SCODA). Developed by engineers at the University of British Columbia and using two-dimensional non-linear electrophoresis, this novel nucleic acid purification approach is capable of purification of DNA at zeptomolar levels (Marziali *et al*., 2005). This method preferentially concentrates nucleic acids over salts, proteins and other inhibitory compounds, therefore producing a genomic DNA preparation of greater quality. The method was tested using low number of (oo)cysts spiked into a turbid post-filtration, pre-IMS pellet. PCR was successful for these samples but not for samples purified by Qiagen silica-spin columns, thus demonstrating that the SCODA method is more effective at removing PCR inhibitors. However, the method is still in development and still requires a cost-down before it would be suitable for research or routine work.

An improvement to labour intensity aspect of PCR and sequencing, which would also allow for the testing of multiple targets simultaneously, is the Luminex® suspension array (BioRad). This approach is liquid microarray and can be used to simultaneously enumerate and genotype a sample. Focusing on single nucleotide polymorphisms (SNPs) that are unique to each *Giardia* Assemblage, probes are designed to distinguish between each Assemblage. These probes are conjugated to proprietary

polystyrene microsphere, which are internally dyed with varying ratios of two spectrally unique fluorochromes, which allows for the differentiation amongst microspheres using a flow cytometer. These microsphere/probes are added to amplified nucleic acids. The interaction of the capture probe with the target nucleic acid is detected using a third fluorochrome, which is incorporated into the target nucleic acid. The beads conjugated to probes are mixed with the labeled nucleic acid and this mixture is then processed by flow cytometry inside the Luminex® 100 analyzer. Laser excitation at two different wavelengths determines probe identity and quantification of the target. This approach not only allows genotyping and quantification simultaneously, the ratios of different genotypes can be determined, which is not possible by the current genotyping approach (which only sequences the predominant genotype). The main limitation to this approach is the costs of the polystyrene beads, however, this cost would be offset by decreased labour costs for sequencing and sequence analysis.



Step	Concentration	Purification	Enumeration	Characterization
Current	FiltaMax	IMS	IFA	PCR/Sequencing
Proposed	UF or DEP	DEP	Unchanged	Luminex™

Figure 5-4: Areas where novel technologies could be incorporated into routine *Giardia* testing (Method 1623 and molecular). UF = ultrafiltration, DEP = dielectrophoresis

5.5 Research Significance and Conclusions

This research is the first study focusing specifically on the molecular epidemiology of *Giardia* isolates in British Columbia water supplies, using current molecular tools. The aim of this research was to generate new knowledge regarding *Giardia lamblia* that could be applied at local, national and international levels for disease prevention. It was demonstrated that without molecular analyses, it is extremely difficult to ascertain the real risk of parasites to public health. Molecular epidemiological data is therefore extremely

important in information public health risk assessment, pathogen monitoring scheme and informing public health policy.

This research demonstrated a predominance of *G.lamblia* Assemblage A in a British Columbia watershed and Assemblage B in an Ontario watershed. It appears that this difference in predominant Assemblage could be a reflection of difference in land-use and differences in transmission ecology of the parasite.

The research was expected to reveal that the current gold-standard method for detection of *Giardia* would overestimate the potential health risk of *Giardia* in water supplies, because it detects both human infectious and non-human infectious species and Assemblages of *Giardia*. However, in the watershed in British Columbia under investigation, the Salmon River watershed, almost all isolates collected and analyzed were determined to be zoonotic and therefore a potential risk to human health. In a comparison study in a more developed watershed in Ontario of similar size (Grand River watershed), more non-infectious isolates were detected, but still only represented 17% of all *Giardia* isolates analyzed. In contrast, half of *Cryptosporidium* isolates analyzed belonged to species or genotypes groups that were infectious to humans. Therefore, not only does *Giardia* occur more frequently and at higher concentrations than *Cryptosporidium* in both watershed, but poses a greater health risk public health. Not only does *Giardia* occur more frequently in surface water supplies, but also is a more frequent cause of gastrointestinal illness in British Columbians.

In addition to the data generated regarding human health risks, association between *Giardia* occurrence and water quality indicators and patterns of *Giardia* loadings, this research also provides insight into the incorporation of new technologies into current laboratory testing procedures. While PCR and sequencing are not novel technologies, they have yet to be incorporated into routine methods for assessing drinking water quality. This study identified some major barriers to incorporating such methods into routine testing and makes suggestions on how best to incorporate and utilize *Giardia* sequencing data.

This study addresses gaps in knowledge regarding the prevalence and genotype prevalence of *Giardia lamblia* in both British Columbia and Ontario. This study has generated new knowledge regarding the risks of *G.lamblia* in Canadian surface supplies, how it related to climate and land-use, and the overall risk to water supplies. This data will be important and useful in developing new drinking water policy

with respect to risk assessments, surveillance of source water and providing scientific data for sound policy decisions.

5.6 References

Aström J, Petterson S, Bergstedt O, Pettersson TJ and Stenström TA. 2007. Evaluation of the microbial risk reduction due to selective closure of the raw water intake before drinking water treatment. Journal of Water Health. 5(Suppl 1): 81-97.

Bryan BA, Kandulu J, Deere DA, White M, Frizenschaf J and Crossman ND. 2009. Adaptive management for mitigating Cryptosporidium risk in source water: a case study in an agricultural catchment in South Australia. Journal of Environmental Management. 90(10): 3122-3134.

Cheyne BM, Van Dyke MI, Anderson WB and Huck PM. 2009. An evaluation of methods for the isolation of *Yersinia enterocolitica* from surface waters in the Grand River watershed. Journal of Water and Health. 7(3): 392-403.

Cook, K. 1994. An evaluation of water quality and Land use in the Salmon River Watershed in Langley using GIS techniques. MSc. Department of Soil Sciences, UBC, 250 pp.

Dalton C, Goater AD, Burt JP and Smith HV. 2004. Analysis of parasites by electrorotation. Journal of Applied Microbiology. 96(1): 24-32.

Dalton C, Goater AD, Pethig R and Smith HV. 2001. Viability of *Giardia intestinalis* cysts and viability and sporulation state of *Cyclospora cayetanensis* oocysts determined by electrorotation. Applied and Environmental Microbiology. 67(2): 586-590.

Di Giovanni GD, Betancourt WQ, Hernandez J, Assadian NW, Flores Margez JP and Lopez EJ. 2006. Investigation of potential zooanthroponotic transmission of cryptosporidiosis and giardiasis through agricultural use of reclaimed wastewater. International Journal of Environment Health Research. 16(6): 405-418.

Dorner SM, Anderson WB, Gaulin T, Candon HL, Slawson RM, Payment P and Huck PM. 2007. Pathogen and indicator variability in a heavily impacted watershed. Journal of Water and Health. 5(2): 241-257.

Dorner S, Huck P, Slawson R, Gaulin T and Anderson W. 2004. Assessing levels of pathogenic contamination in a heavily impacted river used as a drinking-water source. Journal of Toxicology and Environmental Health A. 67(20-22): 1813-1823.

Dorner SM, Huck PM and Slawson RM. 2004. Estimating potential environmental loadings of *Cryptosporidium* spp. and *Campylobacter* spp. from livestock in the Grand River Watershed, Ontario, Canada. Environmental Science and Technology. 38(12): 3370-3380.

Drinking Water Inspectorate (DWI). 2007. Water Supply Regulations (2000) Amendment Regulations 2007. Statutory Instrument 2734. United Kingdom.

Gadish N and Voldman J. 2006. High-throughput positive-dielectrophoretic bioparticle microconcentrator. Analytical Chemistry. 78(22): 7870-7876.

Haas CN. 2002. Progress and data gaps in quantitative microbial risk assessment. Water Science and Technology. 46(11-12): 277-284.

Hall KJ and Schreier H. 1996. Urbanization and agricultural intensification in the Lower Fraser River Valley: impacts on water Use and quality. GeoJournal. 40(1-2): 135-146.

Hill VR, Polaczyk AL, Kahler AM, Cromeans TL and Hahn D. 2009. Comparison of hollow-fiber ultrafiltration to the USEPA VIRADEL technique and USEPA Method 1623. Journal of Environmental Quality. 238(2): 822-825

Jamieson RC, Joy DM, Lee H, Kostaschuk R and Gordon RJ .2005. Resuspension of sediment-associated *Escherichia coli* in a natural stream. Journal of Environmental Quality. 34(2): 581-589.

Jiang J, Alderisio KA and Xiao L. 2005. Distribution of *Cryptosporidium* genotypes in storm event water samples from three watersheds in New York. Applied and Environmental Microbiology. 71(8): 4446-4454

Jyrkama MI, Sykes JF and Normani SD. 2002. Recharge estimation for transient ground water modeling. Ground Water. 40(6): 638-648

Krause P, Smith A, Veale B and Murray M. 2001. Achievements of the Grand River Conservation Authority, Ontario, Canada. Water Science and Technology. 43(9): 45-55.

Marziali A, Pel J, Bizzotto D and Whitehead LA. 2005. Novel electrophoresis mechanism based on synchronous alternating drag perturbation. Electrophoresis. 26(1): 82-90.

Monis P, Mayrhofer G, Andrews R, Homan W, Limper L and Ey P. 1996. Molecular genetic analysis of *Giardia intestinalis* isolates at the glutamate dehydrogenase locus. Parasitology.112 (Pt 1): 1-12.

Lissemore, L Haob C, Yang P, Sibley PK, Mabury S and Solomon KR. 2006. An exposure assessment for selected pharmaceuticals within a watershed in Southern Ontario. Chemosphere. 64(5): 717-729.

Lobo ML, Xiao L, Antunes F and Matos O. 2009. Occurrence of *Cryptosporidium* and *Giardia* genotypes and subtypes in raw and treated water in Portugal. Letters in Applied Microbiology. 48(6): 732-737.

Mons MN, van der Wielen JM, Blokker EJ, Sinclair MI, Hulshof KF, Dangendorf F, Hunter PR and Medema GJ. 2007. Estimation of the consumption of cold tap water for microbiological risk assessment: an overview of studies and statistical analysis of data. Journal of Water and Health. 5(Suppl 1): 151-170.

Morales-Morales HA, Vidal G, Olszewski J, Rock CM, Dasgupta D, Oshima KH and Smith GB. 2003. Optimization of a reusable hollow-fiber ultrafilter for simultaneous concentration of enteric bacteria, protozoa, and viruses from water. Applied and Environmental Microbiology. 69(7): 4098-4102.

Nash TE and Mowatt MR. 1992. Identification and characterization of a *Giardia lamblia* group-specific gene. Experimental Parasitology. 75(4): 369-378

Naugler, T. 2007. Groundwater - surface water interactions in the Salmon River watershed, B.C.: Integrating spectroscopy, isotopes, water quality and land use analyses. M.Sc. Thesis, Resource Management and Environmental Studies. University of British Columbia.

Nieminski EC, Schaefer FW 3rd and Ongerth JE. 1995. Comparison of two methods for detection of *Giardia* cysts and *Cryptosporidium* oocysts in water. Applied and Environmental Microbiology. 61(5): 1714-1719.

Petterson SR, Signor RS and Ashbolt NJ. 2007. Incorporating method recovery uncertainties in stochastic estimates of raw water protozoan concentrations for QMRA. Journal of Water and Health. 5(Suppl 1): 51-65.

Plummer R, Spiers A, FitzGibbon J and Imhof J. 2005. The expanding institutional context for water resources management: the case of the Grand River watershed. Canadian Water Resources Journal. 30(3): 227-244.

Plutzer J, Karanis P, Domokos K, Törökné A and Márialigeti K.2008. Detection and characterization of *Giardia* and *Cryptosporidium* in Hungarian raw, surface and sewage water samples by IFT, PCR and sequence analysis of the SSUrRNA and GDH genes. International Journal of Hygiene and Environmental Health. 211(5-6): 524-533.

Rodgers MR, Flanigan DJ and Jakubowski W. 1995. Identification of algae which interfere with the detection of *Giardia* cysts and *Cryptosporidium* oocysts and a method for alleviating this interference. Applied and Environmental Microbiology. 61(10): 3759-3763.

Ross, J. 2006. Influence of climate and agricultural land use on nutrient and bacterial dynamics in surface waters of the Lower Fraser Valley, British Columbia. PhD Thesis, University of British Columbia. Vancouver, British Columbia, Canada. 246pp.

Ruecker NJ, Bounsombath N, Wallis P, Ong CS, Isaac-Renton JL and Neumann NF. 2005. Molecular forensic profiling of *Cryptosporidium* species and genotypes in raw water. Applied and Environmental Microbiology. 71(12): 8991-8994.

Signor RS and Ashbolt NJ. 2007. Pathogen monitoring offers questionable protection against drinking-water risks: a QMRA (quantitative microbial risk analysis) approach to assess management strategies. Journal of Water and Health. 54(3): 261-268

Smeets PW, van Dijk JC, Stanfield G, Rietveld LC and Medema GJ. 2007. How can the UK statutory *Cryptosporidium* monitoring be used for Quantitative Risk Assessment of *Cryptosporidium* in drinking water? Journal of Water and Health. 5 (Suppl 1): 107-118.

Smieja M, Mahony JB, Goldsmith CH, Chong S, Petrich A and Chernesky M. 2001. Replicate PCR testing and probit analysis for detection and quantitation of *Chlamydia pneumoniae in* clinical specimens. Journal of Clinical Microbiology. 39(5): 1796-1801.

Thomas KM, Charron DF, Waltner-Toews D, Schuster C, Maarouf AR and Holt JD. 2006. A role of high impact weather events in waterborne disease outbreaks in Canada, 1975 - 2001. International Journal of Environmental Health Research. 16(3): 167-180.

Tsai YL and Olson BH. 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Applied and Environmental Microbiology. 58(7): 2292-2295.

United States Environmental Protection Agency (USEPA). 2005. Method 1623: *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA. Office of Water, U.S. Environmental Protection Agency, Washington, DC.

Watts, D. 1993. A GIS evaluation of Land Use dynamics and fish habitat in the Salmon River Watershed - Langley, B.C. MSc. Resource Management and Environmental Studies, UBC.

Wernick B, Cook E and Schreier H. 1998 A GIS evaluation of land-use and streamwater nitrate-N dynamics in a watershed at the urban/rural fringe. Journal of the American Water Resources Association. 34(3): 639-650.

Wernick, B.G. 1996. Land use and water quality dynamics on the urban-rural fringe: A GIS evaluation of the Salmon River Watershed, Langley, B.C. M.Sc. Thesis. Resource Management and Environmental Studies, UBC. 217 pp.

Wilson IG. 1997. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology. 63(10):3741-3751.

Yang W, Chen P, Villegas EN, Landy RB, Kanetsky C, Cama V, Dearen T, Schultz CL, Orndorff KG, Prelewicz GJ, Brown MH, Young KR and Xiao L. 2008. *Cryptosporidium* source tracking in the Potomac River watershed. Applied and Environmental Microbiology. 74(21): 6495-6504.

Young CC, Burghoff RL, Keim LG, Minak-Bernero V, Lute JR, Hinton SM. 1993. Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. Applied and Environmental Microbiology. 59(6):1972-1974.

Zandbergen PA and Hall KJ. 1998. Analysis of the British Columbia water quality index for watershed managers: A case study of two small watersheds. Water Quality Research Journal of Canada. 33: 519-549.

Appendix A - Appendix to Chapter 3

Table A-1: List of isolates used in Chapter 3 and isolation source.

NAME	SOURCE
HUMAN ISOLATES	
VANC/89/UBC/37	BC Outbreak
VANC/90/UBC/41	BC Outbreak
VANC/90/UBC/42	BC Outbreak
VANC/90/UBC/43	BC Outbreak
VANC/90/UBC/44	BC Outbreak
VANC/90/UBC/71	BC Outbreak
VANC/85/UBC/1	BC Endemic - Local
VANC/85/UBC/2	BC Endemic - Local
VANC/86/UBC/3	BC Endemic - Local
ANC/85/UBC/5	BC Endemic - Local
VANC/87/UBC/8	BC Endemic - Local
VANC/87/UBC/22	BC Endemic - Local
VANC/87/UBC/23	BC Endemic - Local
VANC/87/UBC/25	BC Endemic - Local
VANC/89/UBC/33	BC Endemic - Local
VANC/89/UBC/36	BC Endemic - Local
VANC/89/UBC/38	BC Endemic - Local
VANC/93/UBC/39	BC Endemic - Local
VAN/90/UBC/47	BC Endemic - Local
VANC/89/UBC/48	BC Endemic - Local
VANC/90/UBC/60	BC Endemic - Local
VANC/90/UBC/64	BC Endemic - Local
VANC/93/UBC/70	BC Endemic - Local
VANC/91/UBC/73	BC Endemic - Local
VANC/87/UBC/24	BC Endemic - Travel
VANC/88/UBC/35	BC Endemic - Travel
VANC/92/UBC/107	BC Endemic - Travel
BAH15/30	International
BAH30/31	International
BAH106/32	International
VN B1/86	International
VN B3/87	International
VN B5/88	International
ANIMAL ISOLATES	
D3/18	AB - Dog
VANC/89/UBC/59	BC - Dog
SI/16	AB - Sheep
S2/17	AB - Sheep

NAME	SOURCE
WATER ISOLATES	
VANC/90/UBC/40	BC Water
VANC/90/UBC/49	BC Water
VANC/90/UBC/50	BC Water
VANC/90/UBC/58	BC Water
VANC/90/UBC/61	BC Water
VANC/90/UBC/62	BC Water
VANC/91/UBC/69	BC Water
VANC/91/UBC/74	BC Water
VANC/92/UBC/98	BC Water
VANC/92/UBC/99	BC Water
VANC/92/UBC/100	BC Water
VANC/92/UBC/102	BC Water
VANC/92/UBC/104	BC Water
VANC/92/UBC/105	BC Water
VANC/92/UBC/106	BC Water
VANC/94/UBC/121	BC Water
VANC/94/UBC/122	BC Water
VANC/94/UBC/123	BC Water
CB2/108	CAN Water
BTW/109	CAN Water
SL2/110	CAN Water
SM1/113	CAN Water
SL1/114	CAN Water
SL4/115	CAN Water
BEAVER ISOLATES	
VANC/85/UBC/7	BC - Beaver
VANC/85/UBC/9	BC - Beaver
VANC/87/UBC/26	BC - Beaver
VANC/87/UBC/29	BC - Beaver
VANC/90/UBC/52	BC - Beaver
VANC/90/UBC/53	BC - Beaver
VANC/90/UBC/54	BC - Beaver
VANC/90/UBC/55	BC - Beaver
VANC/90/UBC/57	BC - Beaver
BE-2/14	AB - Beaver
BE-1/15	AB - Beaver
B5/19	AB - Beaver
MONASTASHE/6	Intl - Beaver

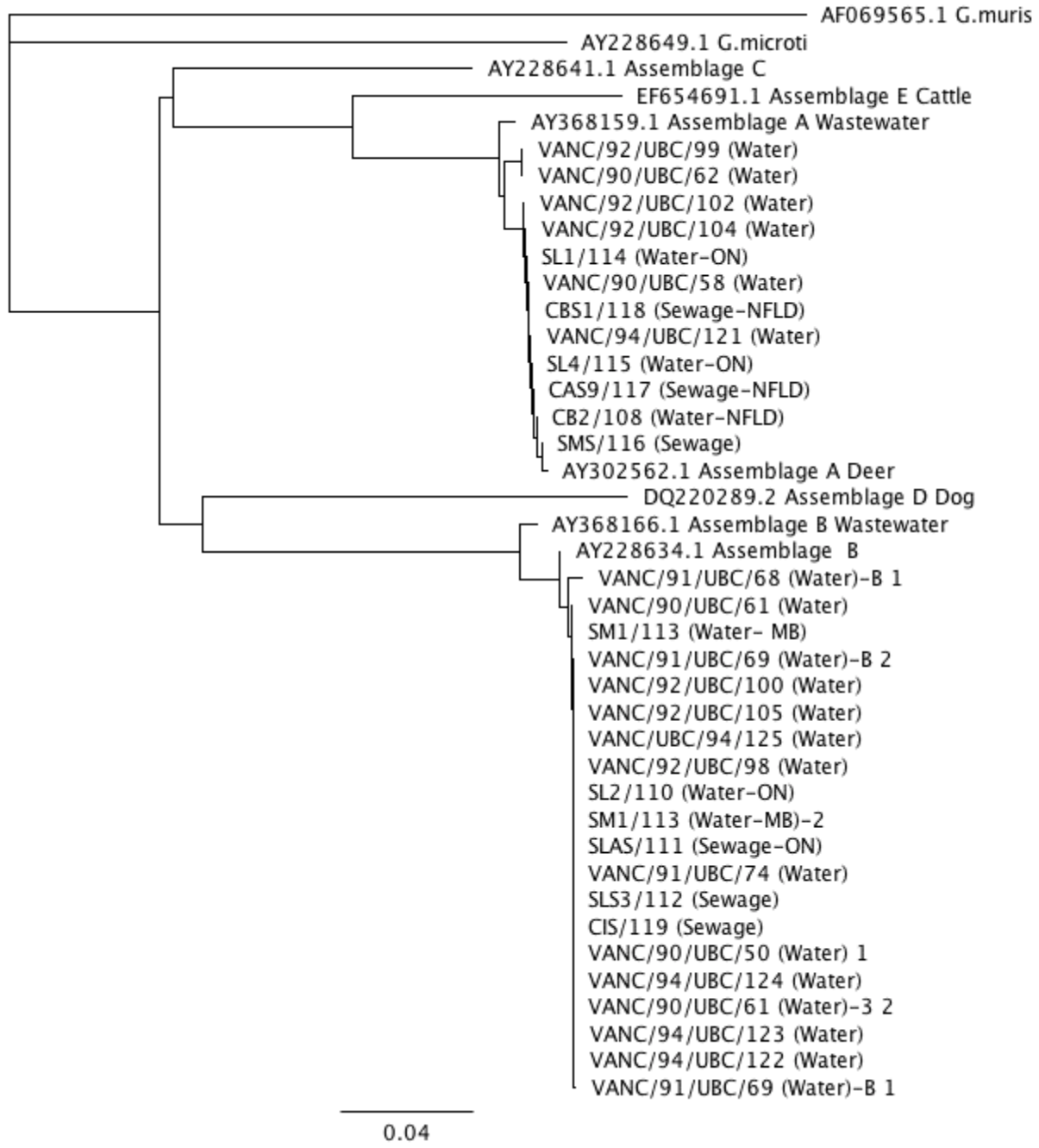


Figure A-2: Phylogenetic alignment of *tpi* sequences of isolates collected from water and sewage, using neighbor-joining tree, using Jukes-Cantor genetic distance model and *G.muris* as root.

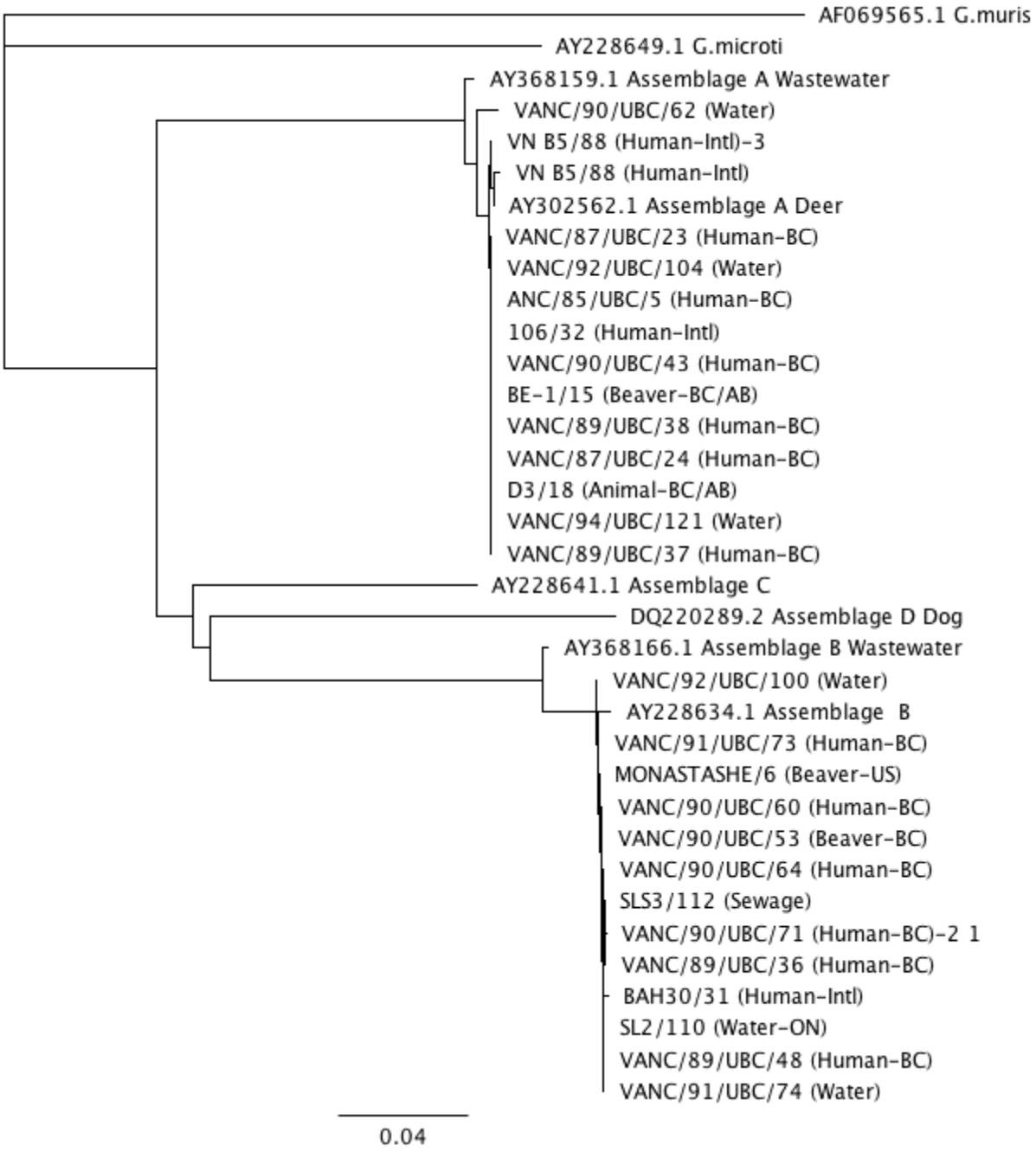


Figure A-3: Phylogenetic alignment of human, animal and environmental *tpi* sequences, using neighbor-joining tree, using Jukes-Cantor genetic distance model and *G.muris* as root.

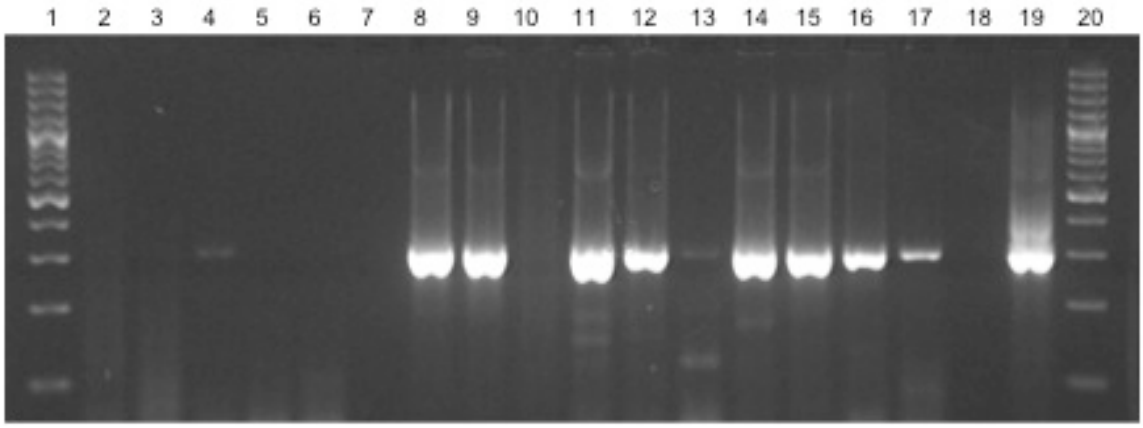


Figure A-1: Criteria for evaluating assay appropriateness for environmental specimens, with respect to PCR performance. Figure 1 shows variability in PCR results for *tpi* gene target. Lanes 2, 3, 5, 6, 7, 10 are negative, lane 4 shows a very weak positive, lane 13 shows false positive with non-specific bands. Lanes 11 and 14 also show non-specific bands, likely a result on non-specific amplification of non-*Giardia* DNA and the degenerate bases in the primers. Because of the weak positives and non-specific bands, nesting is required and there are concerns about the impact of non-specific DNA in environmental samples on PCR quality.

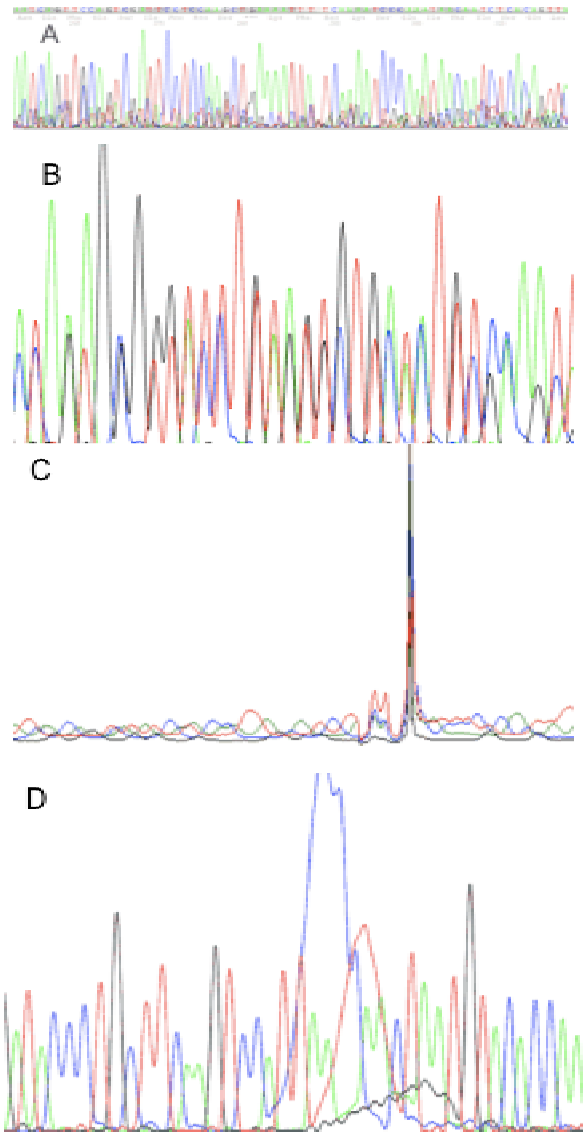
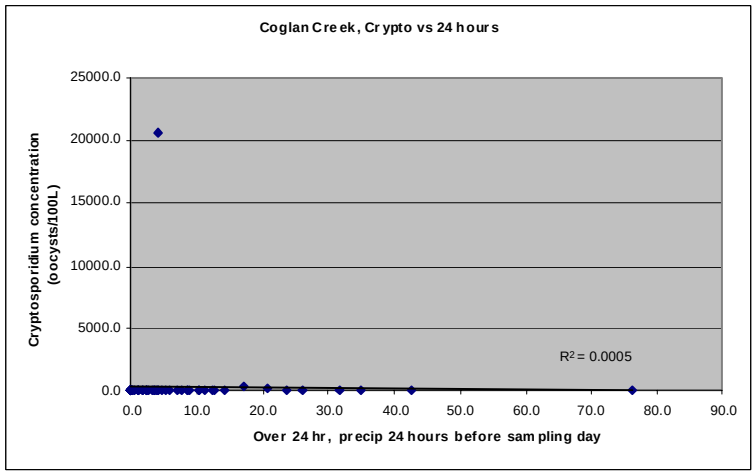
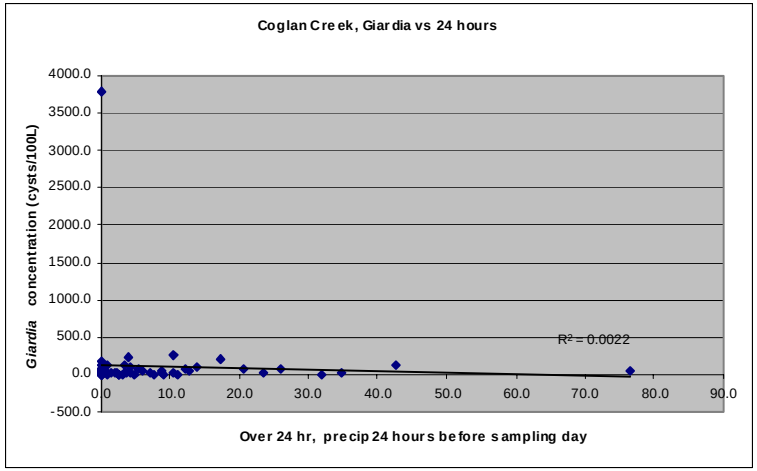


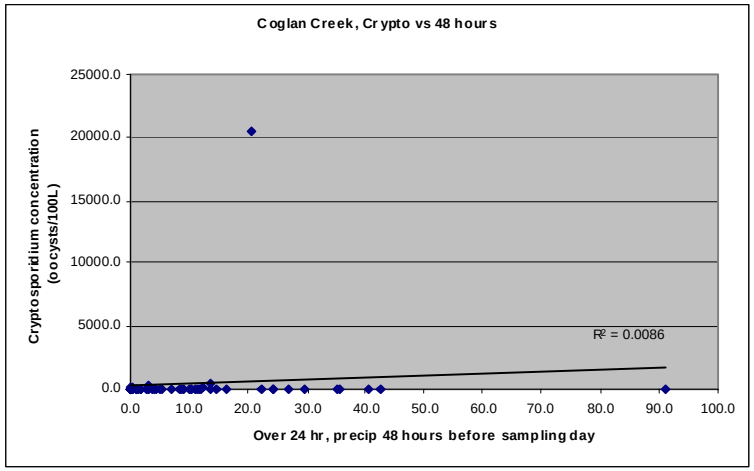
Figure A-2: Criteria for evaluating assay appropriateness for environmental specimens, with respect to sequencing performance. Criteria for evaluating assay appropriateness for environmental specimens, with respect to sequencing performance. of assay, while C and D are typical sequencing problems that can be resolved by further clean-up or repeat reaction. A: Failed reaction, likely due to poor template quality or challenges in sequencing. B: Good sequence strength but evidence of mixed template. C: Sharp spike, unknown cause, could be due to air bubbles in capillary or contamination of the template. D: Dye blob, likely due to poor clean-up or failed reaction.

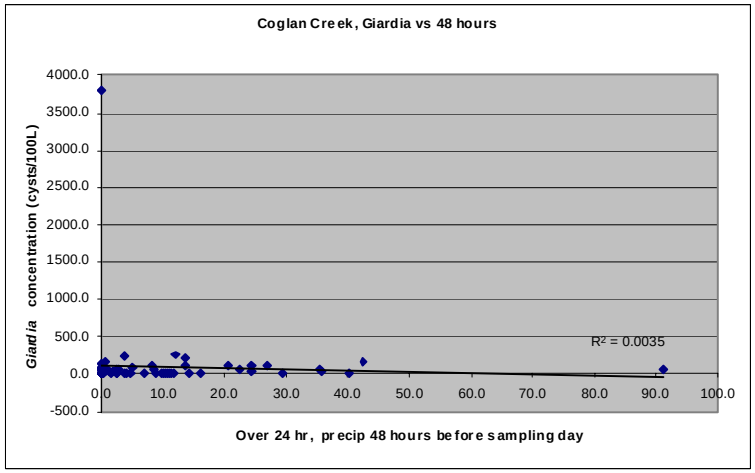
Appendix B - Appendix to Chapter 4

Figure B-1: Correlative analysis of rainfall and parasite concentration (cysts or oocysts per 100L) in Coghlan Creek.

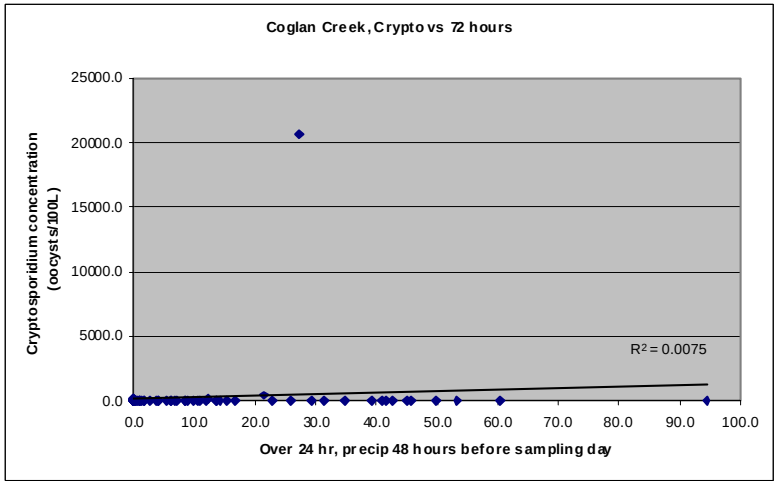


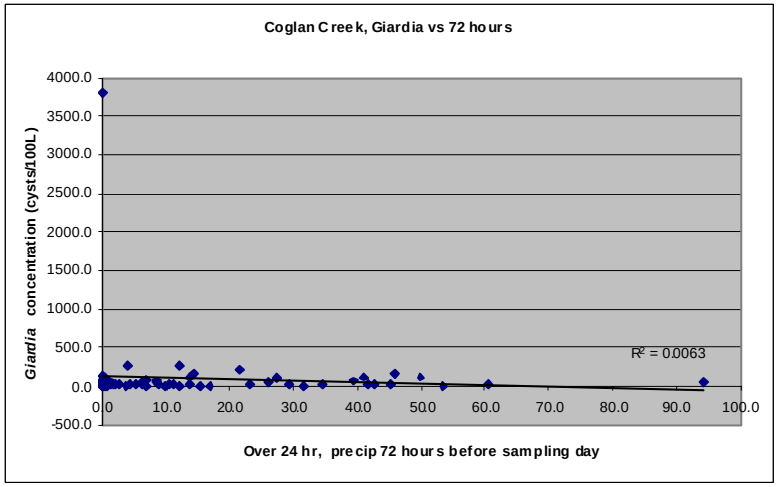


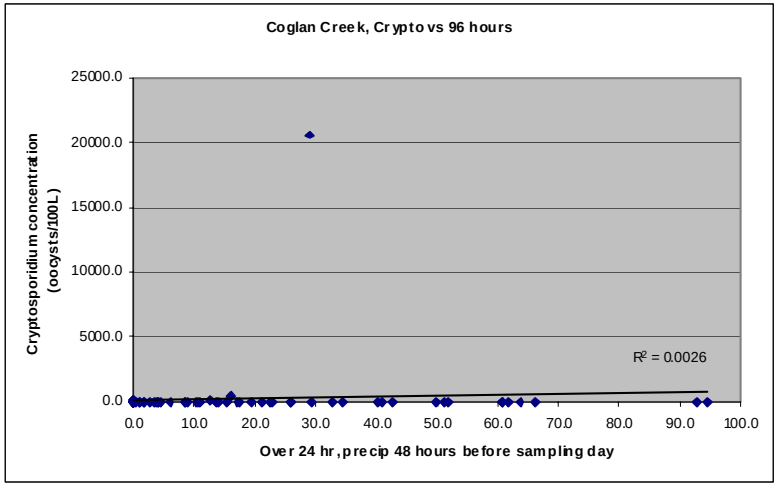




166


Coglan Creek, Crypto vs 72 hours
Cryptosporidium concentration (oocysts/100L)
25000.0
20000.0
15000.0
10000.0
5000.0
0.0
R² = 0.0075
0.0 10.0 20.0 30.0 40.0 50.0 60.0 70.0 80.0 90.0 100.0
Over 24 hr, precip 48 hours before sampling day


Coglan Creek, Giardia vs 72 hours
Giardia concentration (cysts/100L)
4000.0
3500.0
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
-500.0
R² = 0.0063
0.0 10.0 20.0 30.0 40.0 50.0 60.0 70.0 80.0 90.0 100.0
Over 24 hr, precip 72 hours before sampling day


Coglan Creek, Crypto vs 96 hours
Cryptosporidium concentration (oocysts/100L)
25000.0
20000.0
15000.0
10000.0
5000.0
0.0
R² = 0.0026
0.0 10.0 20.0 30.0 40.0 50.0 60.0 70.0 80.0 90.0 100.0
Over 24 hr, precip 48 hours before sampling day

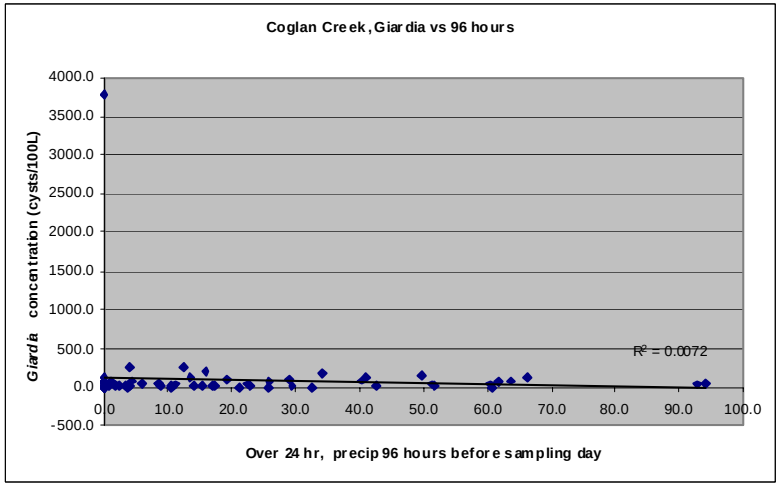
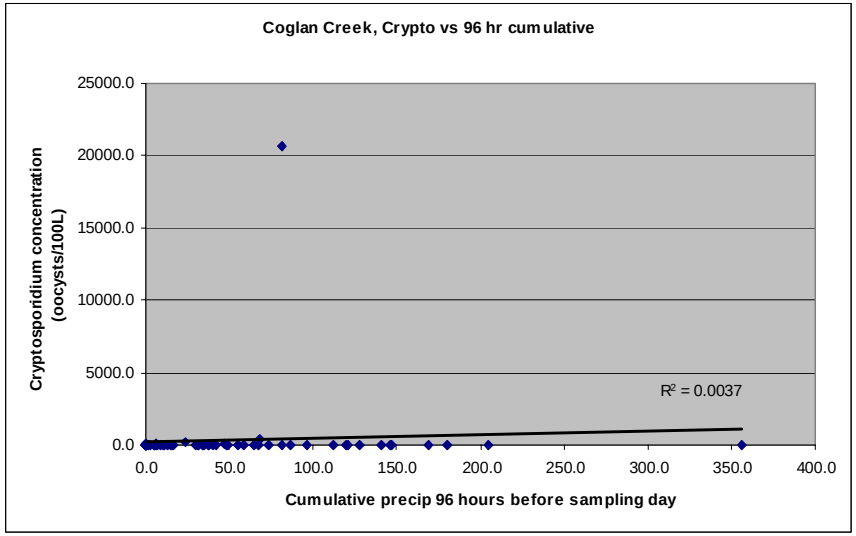

Coglan Creek, Giardia vs 96 hours
Giardia concentration (cysts/100L)
4000.0
3500.0
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
-500.0
R² = 0.0072
0.0 10.0 20.0 30.0 40.0 50.0 60.0 70.0 80.0 90.0 100.0
Over 24 hr, precip 96 hours before sampling day

Figure B-2: Correlative analysis of cumulative rainfall and parasite concentration (cysts or oocysts per 100L) in Coghlan Creek.



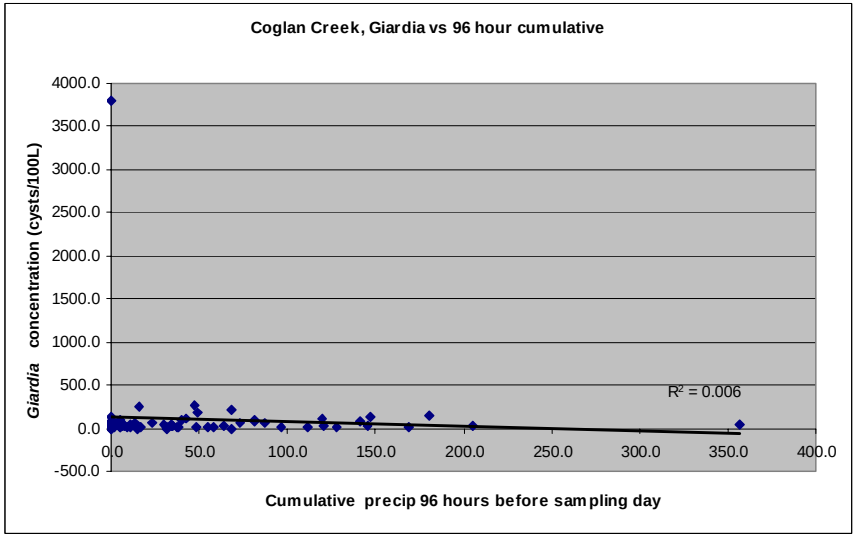


Figure B-2: Correlative analysis of cumulative rainfall and parasite concentration (cysts or oocysts per 100L) in Coghlan Creek.

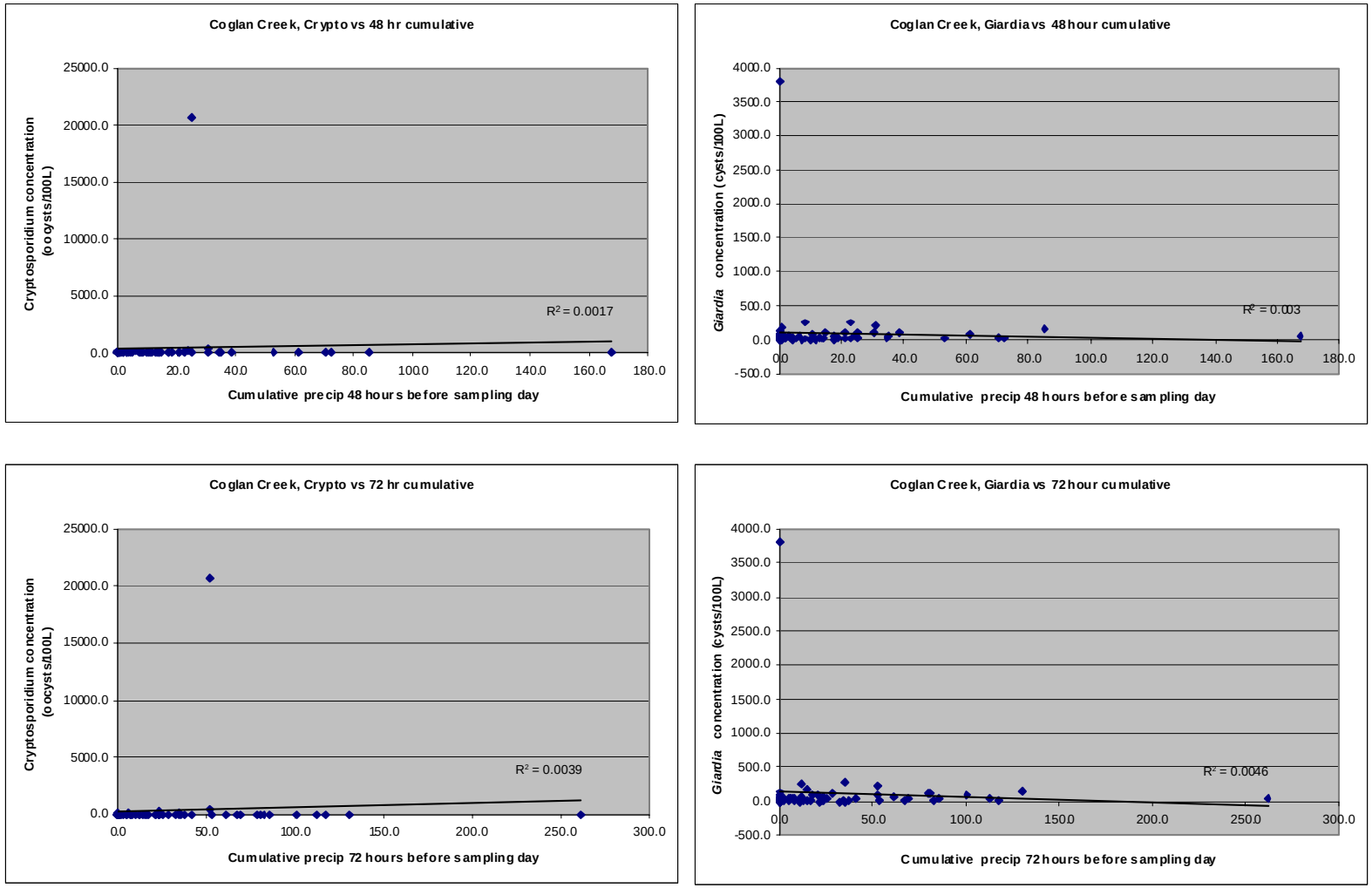


Table B-1. T-test and U-test (Mann Whitney) comparing each parasite occurrence between the two sampling sites

T-test Coglan vs Salmon		U-test Coglan vs Salmon	
Cryptosporidium	***Giardia***	***Cryptosporidium***	***Giardia***
0.3444	0.2803	0.0414*	0.0105*

(*significant difference)

Table B-2: T-test and U-test (Mann Whitney) comparing the occurrence of each parasite at the two sampling sites.

T-test *Cryptosporidium* vs *Giardia*		U-test *Cryptosporidium* vs *Giardia*	
Coghlan	**Salmon**	**Coghlan**	**Salmon**
0.4826	0.1692	<0.0001*	0.0424*

(*significant difference)

Appendix C - Appendix to Chapter 4

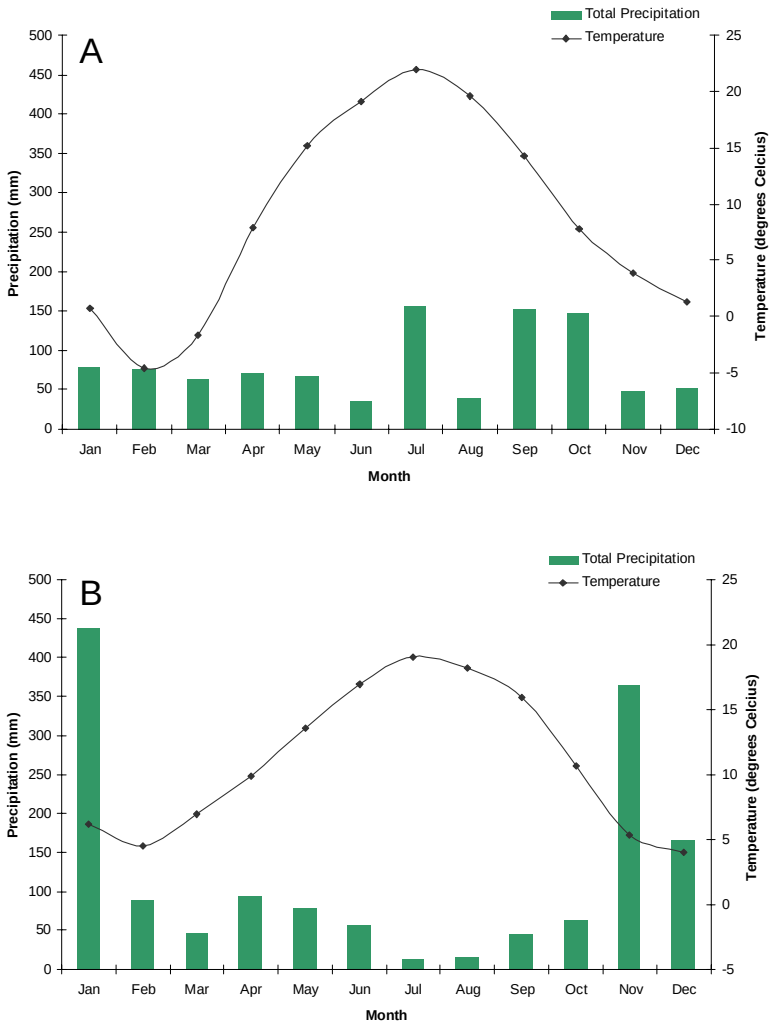


Figure C-1: Differences in monthly mean temperature and total precipitation between the two study sites, based on ten-year averages. Graph A: Grand River watershed data trends. Graph B: Salmon River Watershed data trends. Lowest mean temperatures were observed in both watersheds in February. Peak precipitation occurred in the Salmon River watershed in July, September and October, while peak precipitation occurred in the Grand River watershed in November, December and January.

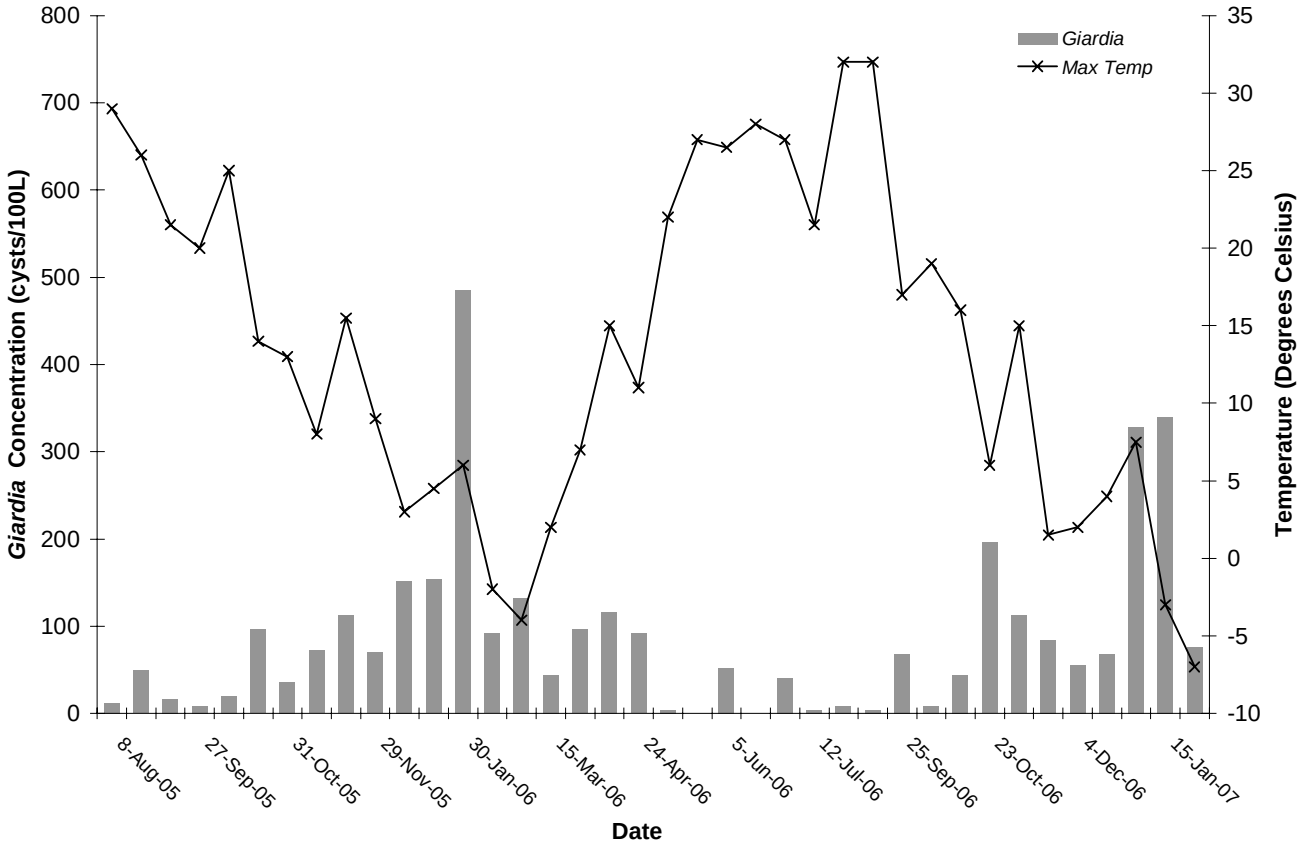


Figure C-2: *Giardia* concentrations and maximum daily temperatures in the Grand River sampling site, indicating maximum daily temperatures above zero on but four sampling dates.

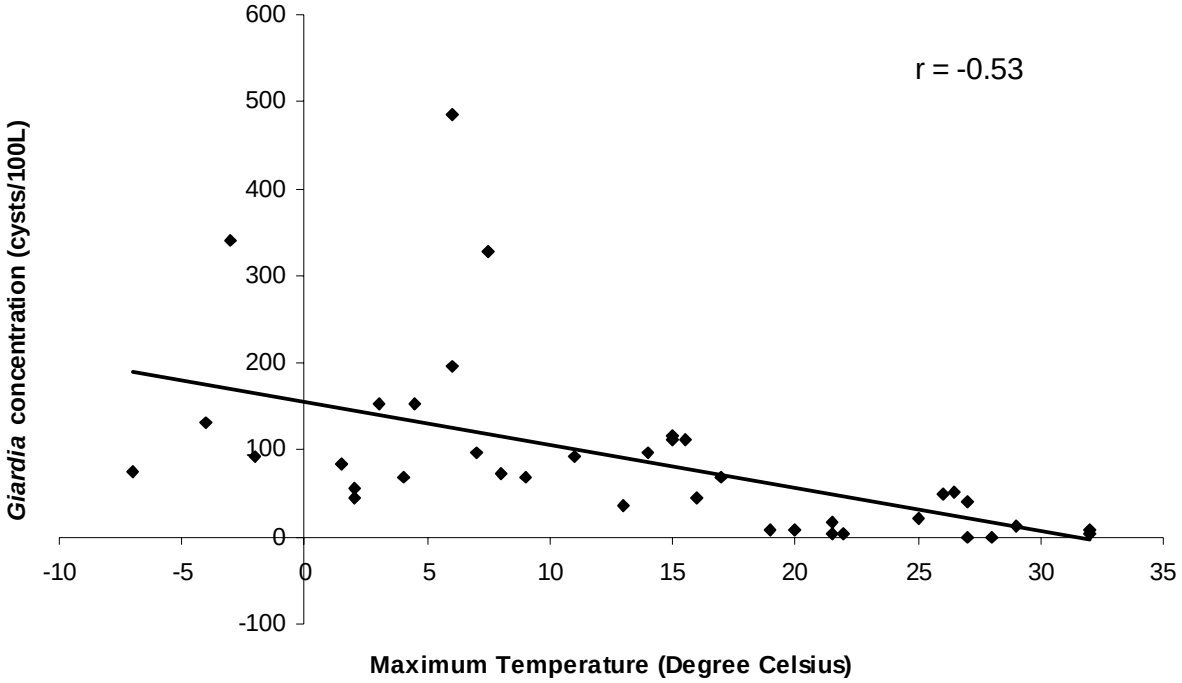


Figure C-3: Pearson correlation demonstrating the relationship between maximum temperature on each sampling date and *Giardia* concentration. *Giardia* concentrations are highest at temperatures below 15 degrees Celsius. The r-value of -0.53 indicates a negative, moderately correlated relationship between *Giardia* concentrations and maximum daily temperature.

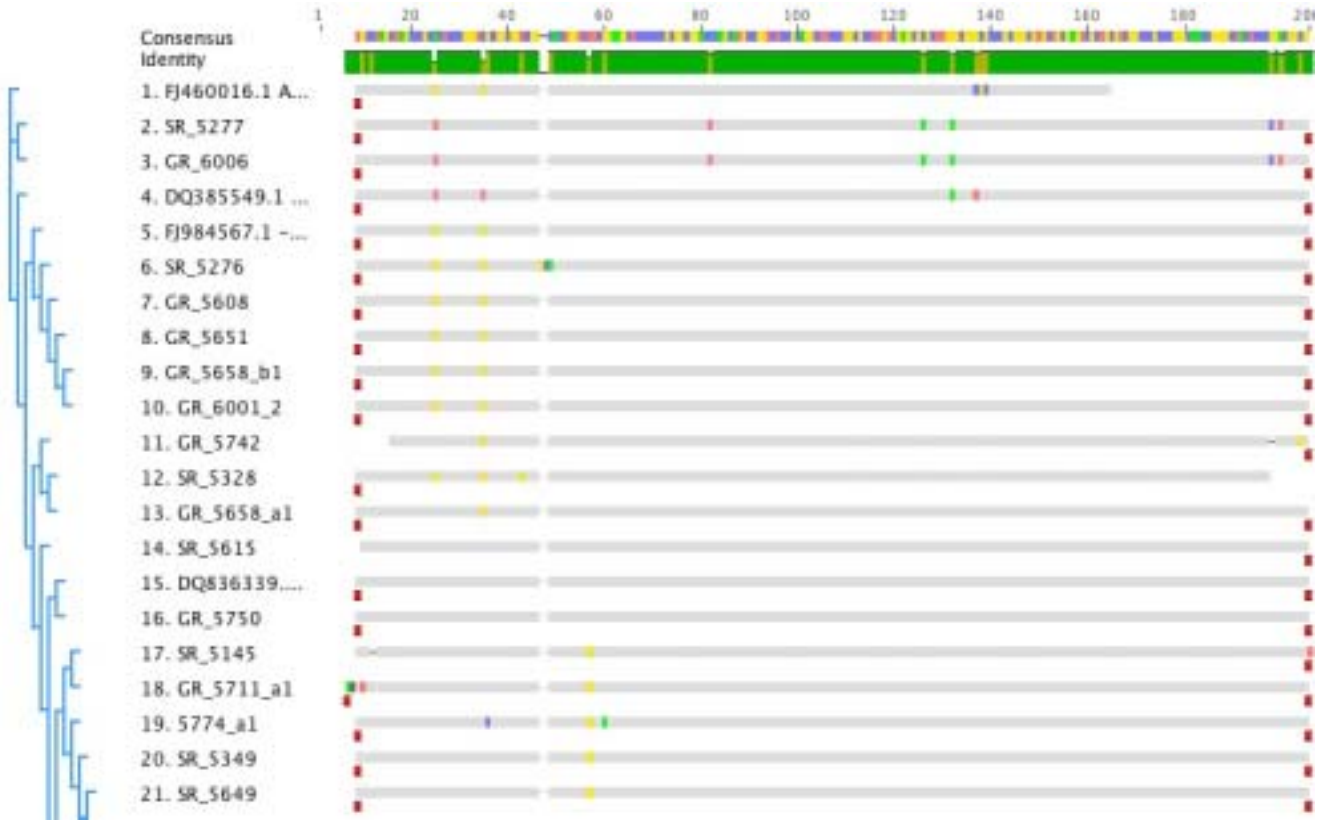


Figure C-2: Combination phylogenetic tree and alignment view that corresponds to Figure 5-7 in Chapter 5. This tree highlights the SNPs observed amongst isolates, shown in colors. SNPs in the first 40 base pairs are characteristic of *G.lamblia* 18s rRNA. Those later on in the sequence are more unique. Of particular interest is the 2bp insertion in SR_5276.