UNIVERSITY OF CALGARY

The prevalence of *Giardia duodenalis* and *Cryptosporidium* spp., and the molecular characterization of *Cryptosporidium* spp. isolated from human, wildlife and agricultural sources of the North Saskatchewan River basin in Alberta, Canada.

by

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Abstract

The environmental distribution of *Giardia* and *Cryptosporidium* is dependent upon human, agriculture, and wildlife sources. The significance of each source on the presence of parasites in the environment is unknown. This study examined parasite prevalence in sewage influent, wildlife and agricultural sources of the North Saskatchewan River basin. Samples were collected from cow-calf, dairy, and hog operations, wildlife, and sewage treatment facilities in the watershed. *Giardia* and *Cryptosporidium* were detected in all sources. The lowest prevalence of *Giardia* (2.25%) and *Cryptosporidium* (0.36%) were found in wildlife. Sewage had the highest prevalence of *Giardia* (48.80%) and *Cryptosporidium* (5.42%), however the concentration of both parasites was less than that detected in agriculture ($p \leq 0.01$). Cow-calf sources contained the highest concentration of *Giardia* and dairy sources the highest concentration of *C. parvum*. PCR-based characterization of *Cryptosporidium* isolates revealed differences between human, pig, calf, cow, and cat isolates. The information presented will contribute to the epidemiology of waterborne parasites in Canada.

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β	regression model coefficient
µg/ml	micro gram per milli litre
µm	micro metre
°C	degrees Celsius
18S	small subunit ribosomal gene
AIDS	acquired immune deficiency syndrome
bp	base pair(s)
C-C	cow-calf operation
Cl^-	chloride ions
Conc.	concentration (parasites per gram feces)
dH_2O	distilled and deionized water
dNTP	deoxiribonucleoside 5` tri-phosphate
DNA	deoxyribonucleic acid
EPA	environmental protection agency
ETS	external transcribed spacer region
ft.	feet
g	gram
HIV	human immunodeficiency virus
ICR	information collection rule
IGS	intergenic spacer region
ITS1	internal transcribed spacer region 1
ITS2	internal transcribed spacer region 2
kg	kilo gram
L	litre

LLD	Legal land description
mg	milli gram
ml	milli litre
mm	milli metre
mM	milli molar
mRNA	messenger ribonucleic acid
M	molar
n	sample size
ng	nano gram
N/A	not applicable
Na^+	sodium ions
No.	number
NSR	North Saskatchewan River
PBS	phosphate buffered saline
PCR	polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid
rpm	revolutions per minute
RAPD	random amplified polymorphic DNA
RNA	ribonucleic acid
RT	reverse transcription
s	second
s. g.	specific gravity
spp.	species
UV	ultra violet
x g	times gravity

1 Introduction

The ubiquitous protozoan waterborne parasites, *Giardia* and *Cryptosporidium* are both clinically and economically important for humans and many animals including livestock and wildlife species. These parasites are found worldwide and currently contaminate Canadian water systems (Wallis *et al.*, 1996). More specifically, both parasites are routinely found in Alberta rivers and streams, and have been detected in treated drinking water supplies (Gammie & Goatcher, 1998). Contaminated water systems are a threat to our population, as one point source of contamination has the potential to infect thousands of individuals. There is a need for control measures which, in order to be effective, must be based on an understanding of both the biology and epidemiology of *Giardia* and *Cryptosporidium,* such that transmission to human and animal hosts can be greatly reduced.

The environmental distribution of both parasites is dependent upon farm animal, wildlife, companion animal, and human effluent sources. The significance of each source on the presence of waterborne parasites in the environment has yet to be evaluated. In addition, an understanding of transmission dynamics, and parasite host-specificity are required. This can be accomplished through recent advances in molecular genotyping of isolates obtained from specific animal species. One such method compares the internal transcribed spacer region of rDNA sequence amplified from various *Cryptosporidium* isolates (Morgan *et al.*, 1999b). This has demonstrated that species-specific transmission cycles exist, and that perhaps, *Cryptosporidium* is not a zoonoses as previously thought, but instead is a parasite with a clonal population structure (Awad-El-Kariem, 1996). Through the recognition of significant parasite sources, and the understanding of parasite transmission dynamics, a complete epidemiological picture of waterborne parasite presence in the environment will begin to unfold.

The primary objective of the present work was to determine the general contribution of cattle in cow-calf operations, human sewage influent, and wildlife as sources of the waterborne parasites *Cryptosporidium* and *Giardia* in the North Saskatchewan River (NSR) basin in central Alberta, Canada. The initial survey allowed for the collection of many *Cryptosporidium* positive samples isolated from human and various animal species. This led to the development of a subsequent research objective, the molecular characterization of various isolates collected. It is hoped that the information presented will contribute to the epidemiological picture of *Giardia* and *Cryptosporidium* in Canadian water systems, and the development of control measures for waterborne parasites. Ultimately, control strategies will require a coordinated and collaborative effort between physicians, epidemiologists, local and national health authorities, environmental specialists, water engineers, producers, microbiologists, and academics.

1.1 Parasite Biology

1.1.1 Taxonomy

Although *Giardia* and *Cryptosporidium* share many commonalties including transmission route, preferred environment, and site of infection, there exist innumerable differences between the two. The discovery of each parasite was independent. *Giardia* was first reported by Anton van Leeuwenhoek in 1681 (Adam, 1991), and Tyzzer (1907) first described *Cryptosporidium* as a cell-associated organism in the gastric mucosa of mice.

Giardia with its vesicular nucleus, flagella, and lack of chloroplasts belongs to the phylum Sarcomastigophora, and class Zoomastigophorea. As a member of the order Diplomonadida, *Giardia* is free living or parasitic, contains two nuclei, and possesses four pairs of flagella (Adam, 1991). Sogin *et al.* (1989) suggested that *Giardia* be one of

the most primitive eukaryotes, based on its unusually small sized ribosomal RNA subunits and rDNA phylogeny. Traditionally, the speciation of *Giardia* was based on host specificity, however it is now due largely to trophozoite morphology, intracellular structure, and genetic heterogeneity both between and within species (Wolfe, 1992; Thompson & Meloni 1993). *Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*) is the species most commonly associated with disease as it infects humans and a wide range of mammalian hosts, and is endemic throughout the world (Wolfe, 1992).

The coccidian *Cryptosporidium* belongs to the phylum Apicomplexa. All Apicomplexans are parasitic, possess an apical complex that aids in the penetration of host cells, contain a single type of nucleus, lack cilia and flagella in all non-reproductive stages, and the lifecycle includes both asexual and sexual phases (Clark, 1999). *Cryptosporidium* produces a resilient oocyst following sexual reproduction and therefore is a member of the class Sporozoea. Presently there is much confusion regarding the speciation within the genus *Cryptosporidium*, and furthermore regarding the interspecific variation within individual species. Based on the tremendous and increasing variation observed within the *Cryptosporidium* genome, a clonal population structure has been postulated (Awad-El-Kariem, 1996).

1.1.2 Morphology

Two distinct forms of *Giardia* exist. The oval cysts (8 to 12 μm long by 7 to 10 μm wide) develop in the large intestine where they form a tough hyaline cyst wall (Wolfe, 1992). These quadri-nucleate cysts are robust and can survive for extended periods of time in a favorable environment (Upcroft & Upcroft, 1998). Within the cyst, it is possible to observe the nuclei usually situated at one end, curved median bodies, and linear axonemes using staining and light microscopy techniques. Using similar techniques, it is also possible to observe the internal structures of the second form of

Giardia, the motile trophozoite. Trophozoites are binucleate, possess four pairs of flagella, and an adhesive disc which is located on the ventral surface of the cell (Meyer, 1994). Unlike the cyst, the *Giardia* trophozoite is not similar for all species of *Giardia* present. Filice (1952) identified morphological differences between trophozoites and proposed that the nomenclature be based on biological difference and not host specificity. Based on these observations, three species of *Giardia* currently exist; Giardia *duodenalis*, *Giardia muris*, and *Giardia agilis* (Figure 1.1; Table 1.1).

Unlike *Giardia*, *Cryptosporidium* has many distinct phases. The sporulated oocyst containing four uni-nucleate sporozoites within a two-layered wall is the only exogenous stage (Fayer *et al.*, 1997). Oocysts contain a residuum that consists of many small granules and a membrane-bound globule (O'Donoghue, 1995). Depending on the host species, recovered oocysts can vary in size (3 to 9 μm in length by 3 to 8 μm in width) and shape (elliptical or ovoid) (Upton & Current, 1985). Oocysts of *Cryptosporidium* are similar to *Giardia* cysts in that they are extremely resilient to various inhospitable environmental conditions. The sporozoites and merozoites are bounded by a three-layered pellicle and contain several electron-dense bodies, subpellicular microtubules, and a rudimentary apical complex (O'Donoghue, 1995). Until recently, it was thought that *Cryptosporidium* lacked any evidence suggesting the presence of a mitochondrion. Riordan *et al.* (1999) reported an acristate, ribosome-studded organelle closely resembling mitochondria of other protists. Using molecular techniques, they have provided additional support for their conclusions by confirming the presence of at least three mitochondrion-specific genes.

1.1.3 Life-Cycle

Giardia and *Cryptosporidium* develop entirely within one host and are therefore said to have monoxenous life cycles (O'Donoghue, 1995). Infection with either parasite

typically begins with the oral ingestion of the infective cyst or oocyst, and ends with the passing of cysts or oocysts in the feces. One species of *Cryptosporidium*, *C. baileyi* is unique in that infects the respiratory tract of chickens, and infective oocysts are passed with respiratory or nasal secretions (Blagburn *et al.*, 1987). In general, transmission of both *Giardia* and *Cryptosporidium* is through the fecal-oral route (Wolfe, 1992; O'Donoghue, 1995).

Following ingestion of a *Giardia* cyst, excystation occurs in the duodenum where two motile trophozoites are released (Adam, 1991). Each trophozoite adheres to the intestinal epithelium via the ventral sucking disk, and binary fission occurs resulting in further gut colonization (Meyer, 1994). This occurs in both the duodenum and the first part of the jejunum where the alkaline pH is favorable for growth (Wolfe, 1992). Trophozoites typically attach firmly to the intestinal microvillus surface or move about freely in the lumen. Invasion of the mucosa is rare and has been reported on one occasion (Saha & Ghosh, 1977). Trophozoites encyst in the ileum and large intestine where the newly formed cysts are passed with the feces into the environment (Meyer, 1994). The infection rate of *Giardia* in human and animal hosts is difficult to estimate due to the low sensitivity of current parasite detection methods in many human and veterinary health clinics, and due to the intermittent nature of cyst excretion during infection (Olson & Buret, 2000). Adam (1991) reported that the ingestion of as few as 10 cysts has led to giardiasis in humans and animals.

Excystation of the infectious *Cryptosporidium* oocyst occurs following ingestion. The mechanism by which excystation occurs is thought to be triggered by various factors including reducing conditions, carbon dioxide, temperature, pancreatic enzymes and bile salts (Fayer & Leek, 1984; Robertson *et al.*, 1993). During excystation, a small slit-like opening is created at one end of the oocyst, and the sporozoites are able to exit and subsequently attach to epithelial cells (O'Donoghue, 1995). Here, they become enclosed within parasitophorous vacuoles, which are intracellular yet extracytoplasmic (Fayer

Figure 1.1

Diagram of A) *Giardia duodenalis*, B) *G. muris*, C) *G. agilis* trophozoites showing morphological variation between species. Abbreviations: N = nuclei, M = median bodies, VF = ventral flagellae.

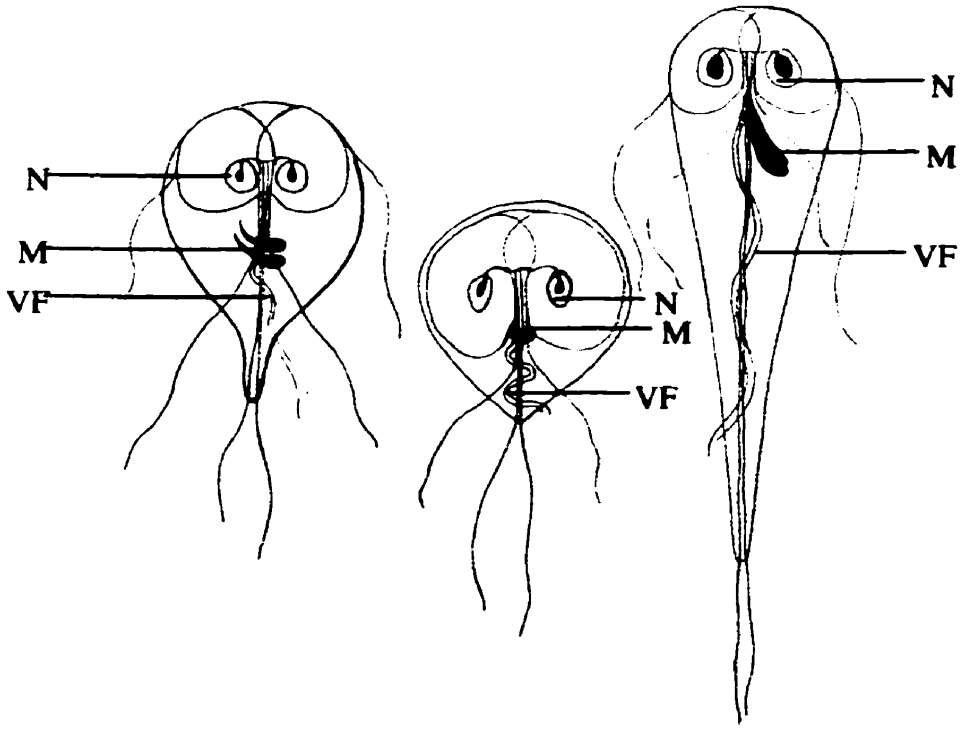

N
M
VF
N
M
VF
N
M
VF

Table 1.1 Accepted species of *Giardia* spp., their hosts, and morphological features.

Species	Host	Shape of Median Bodies	Other Morphological Features
Giardia duodenalis	approximately 40 species of vertebrates including humans	claw hammer	trophozoites are tear-drop shaped
Giardia muris	rodents	small and round	stout and rounded trophozoite
Giardia agilis	amphibians	club shaped	trophozoites have a long and narrow body
Giardia ardeae	avian species	small and round or claw hammer	tear drop nuclei, single caudal flagellum
Giardia psittaci	avian species	claw hammer	incomplete ventro-latero flange in trophozoite

et al., 1997). Compartmentalized sporozoites undergo asexual proliferation by merogony with the formation of meronts through internal budding. Meronts can be one of two types, and depending on which, will either invade new epithelial cells resulting in rapid parasite amplification, or will produce gamonts which in turn led to sexual reproduction (Göbel & Brändler, 1982). The fertilization of gametocytes results in the production of a zygote, which undergoes further asexual development to form the sporulated oocyst, which contains the four sporozoites (O'Donoghue, 1995). The majority of oocysts that are produced at this stage are usually thick-walled and are excreted with fecal matter from the host. Current (1985) observed that some oocysts are thin-walled and can excyst within the same host leading to a new developmental cycle.

The entire life-cycle of *Cryptosporidium* can be completed in a minimum of two days, however some infections may persist for several months. The prepatent period is variable depending on the host species, however in domestic animals, it ranges from 2 to 14 days (Tzipori *et al.*, 1983). Factors such as host immunocompetency, and *Cryptosporidium* species affect the duration of infection (O'Donoghue, 1995). DuPont *et al.* (1995) measured the 50% infective dose of *Cryptosporidium* to be 132 oocysts for a healthy host with no previous serological immunity to the disease.

1.1.4 Clinical Symptoms of Infection

The clinical features of giardiasis and cryptosporidiosis are similar in that they both cause enteric infection in mammalian hosts. Giardiasis differs from host to host depending on factors such as duration of infection, and inoculum size. There are three types of giardiasis; the asymptomatic, acute, and chronic infections (Farthing, 1994). Asymptomatic infection occurs in human adults and children, and other mammalian hosts. This is probably the most common form of giardiasis. In these types of infection, it is unclear whether the host had a transient diarrheal episode that went undetected, or if

an infection failed to produce any substantial symptoms. Factors such as variation in parasite virulence and host immune defense may contribute to asymptomatic giardiasis (Farthing, 1990).

Acute giardiasis is often recognized as traveler's diarrhea in humans, scours in calves, and generalized gastrointestinal illness in other animal hosts. During the acute stage, intestinal uneasiness, nausea and anorexia are the primary symptoms (Farthing, 1994). These are accompanied by low-grade fever, chills, explosive, watery diarrhea, and abdominal cramping. This stage typically lasts 3 or 4 days with most patients reporting continual diarrhea during this time (Wolfe, 1992). In agricultural animals, acute giardiasis is economically important as it causes scours in young animals, and has been associated with weight loss, reduced feed efficiency, and decreased production due to lower carcass weight (Olson *et al.*, 1995). Most cases of acute giardiasis clear spontaneously, however chronic infections can develop. This form of the disease may involve two or more years of mild intermittent symptoms with weight loss of 10% to 20% reported, chronic lassitude, headache, and anorexia persisting (Farthing 1994; Wolfe, 1992). In children, chronic giardiasis is reported to result in a failure to thrive (Craft, 1982). Other symptoms of chronic *Giardia* infection include urticaria and allergic disease (Webster, 1958), cholecystitis (Soto & Dreiling, 1977), arthritis (Shaw & Stevens, 1987), and lactose intolerance (Mantovani *et al.*, 1989).

The symptoms of cryptosporidiosis differ greatly between immunocompetent and immunocompromised hosts. In the immunocompetent patient, the disease is acute and self-limiting, producing diarrheal illness that typically occurs for one to two weeks. Symptoms are similar to acute giardiasis and include frequent, watery diarrhea, nausea, vomiting, low-grade fever, and abdominal cramping (Juranek, 1995). *Cryptosporidium* in production animals has become a major economic concern for producers, as scours caused by cryptosporidiosis leads to reduced weight gain and calf mortality (Harp & Goff, 1998). In poultry, natural infection with *Cryptosporidium* occurs in chickens

under 11 weeks of age (Goodwin, 1989). Avian cryptosporidiosis can be respiratory causing coughing, dyspnea, and sneezing, enteric resulting in intestinal and caecal distension, or renal where the kidneys become inflamed and pale (Goodwin, 1989). There is limited information on the effect of cryptosporidiosis on wildlife species.

In the immunocompromised host, such as the AIDS patient, infection frequently results in a chronic and life-threatening disease (Valdez *et al.*, 1998). *Cryptosporidium* takes advantage of the impaired or destroyed immune system, causing a persistent infection that seriously debilitates the patient and ultimately results in a higher mortality rate. Such is the case in AIDS patients, where cryptosporidiosis has been shown to significantly reduce lifespan (Valdez *et al.*, 1998). Symptoms in the immunocompromised include a debilitating cholera-like diarrhea that rapidly dehydrates the patient as up to 20 litres of excreta is lost per day (Keusch *et al.*, 1995). Severe abdominal cramping, malaise, weight loss and anorexia are also demonstrated in these patients (O'Donoghue, 1995).

1.1.5 Pathology

Diarrhea typically develops when intestinal absorption is impaired or secretion is enhanced. Both of these processes occur with *Giardia* and cryptosporidial infection. In experimental models of cryptosporidiosis, investigators have identified impaired Na^+ and water absorption and increased Cl^- secretion (Moore *et al.*, 1995). These transport problems, in addition to a breakdown in the intestinal epithelium intercellular junctions all contribute to *Cryptosporidium* related diarrhea (Clark, 1999). There are many factors, which ultimately led to cell death, thereby causing increased malabsorption throughout the duration of infection. These include the release of various enzymes and prostaglandins by the epithelial cells in response to the presence of *Cryptosporidium* in

the monolayers (Clark, 1999). In AIDS patients, cryptosporidiosis results in decreased vitamin B_{12} and D-xylose absorption (Goodgame *et al.*, 1995).

The colonization of *Giardia* trophozoites in the small intestine results in a reduction in the height of the microvilli and therefore a loss of absorptive surface area (Buret *et al.*, 1992). This loss of absorptive surface leads to the malabsorption of glucose, electrolytes and water, and reduces disaccharidase activity (Olson & Buret, 2000). This results in the small intestine filling with mucous and fluid, and ultimately maldigestion and hypermotility, all responsible for the clinical manifestation of diarrhea (Deselliers *et al.*, 1997).

1.1.6 Treatment

The effective 5-nitroimidazole family and more specifically, the drug metronidazole (Upcroft & Upcroft, 1998) easily control *Giardia* infections. Metronidazole (Flagyl) cures giardiasis in the range of 85% to 95%, which although is less effective than quinacrine, is well tolerated by the patient causing minimal side effects (Upcroft & Upcroft, 1998). The control of naturally acquired giardiasis in young ruminants can be achieved with the use of fenbendazole (O'Handley *et al.*, 1997). Drug resistance has been induced in the laboratory against metronidazole, therefore complacency and abuse must be controlled (Upcroft & Upcroft, 1993). Although resistance in *Giardia* is a problem to be taken seriously, there is another option in the treatment of giardiasis. A disrupted trophozoite-derived vaccine to prevent giardiasis in domestic animals is currently available, and hopefully soon, there will be a form of the vaccine for all animals and humans (Olson *et al.*, 1997b).

Presently there is no effective treatment for cryptosporidial infections. This is however, an area where tremendous amounts of money and research efforts are being directed. Because cryptosporidiosis is self-limiting in immunocompetent hosts, most

drug-related research is being directed towards HIV-infected patients. Bobin *et al.* (1998) tested the efficacy of the protease inhibitors indinavir and saquinavir on cryptosporidial infections in twenty HIV patients. All of the patients had resolved clinical symptoms following treatment, and a mean increase in body weight of 10.5 kg was demonstrated. Three drugs; azithromycin, Immuno-C and DAP-092 are currently in clinical trials in the USA (*Cryptosporidium* Capsule, 1998). Studies evaluating the efficacy of azithromycin have been inconclusive, however a recent study by Smith *et al.* (1998) demonstrated that azithromycin combined with paromomycin is effective. The development of anti-cryptosporidial drugs is tremendously difficult, as there are few patients with cryptosporidiosis who can be used in control trials.

A recombinant protein (rC7) colostrum-based vaccine to prevent bovine cryptosporidiosis has recently been developed by Perryman *et al.* (1999). The protein was administered three times to late gestation Holstein cows, and resulted in the production of immune bovine colostrum. This colostrum was given repeatedly to neonatal calves and provided substantial protection, as the calves did not develop diarrhea and shed a significantly lower number of oocysts than did the control group. The development of this vaccine for commercial use is underway, and it is hoped that the antigens inducing protection in bovine hosts will be the same antigens that will induce protection in other species (Perryman *et al.*, 1999).

1.1.7 Epidemiology

Human giardiasis and cryptosporidiosis occur worldwide, with higher prevalence where sanitation is poor. People of all ages are affected, however infections are more common in infants, the elderly, and the immunocompromised. Recent epidemiological investigations demonstrate that *Cryptosporidium* is one of the most commonly identified enteric pathogens both in developing and developed countries with infection rates

varying from 1% to 30% (Casemore *et al.*, 1997). Children aged 1 to 5 years are most commonly infected. The prevalence of cryptosporidiosis in adults is less, although in adults with exposure to children (familial or occupational) incidence is greater (Casemore *et al.*, 1997). A significant association between severity of infection and increasing age beyond 65 years has also been reported (Bannister & Mountford, 1989). The prevalence of giardiasis throughout temperate and tropical locations varies between 2% to 5% in the industrialized world, and up to 20% to 30% in the developing world (Farthing, 1994). As with cryptosporidiosis, giardiasis in the developing world is most common in children, and its age-specific prevalence rises through infancy and childhood, and declines after adolescence (Oyerinde *et al.*, 1970).

Giardia and *Cryptosporidium* cause infections in wildlife, agricultural, and domestic animal species. In many cases of animal infection, it is widely accepted that animal hosts act as a reservoir for possible human infection. The possibility of transmission between animal and human hosts, and the economic importance of production animal infections have resulted in many investigations into the epidemiology of giardiasis and cryptosporidiosis in a wide variety of animals. The prevalence of both parasites in Canadian cattle has been reported up to 29% for *Giardia* and 20% for *Cryptosporidium* (Olson *et al.*, 1997a). Domestic pets, such as dogs and cats have been shown to be between 1 and 25% infected with *Giardia*, and in wild mammals, *Giardia* prevalence may vary from very low to 100% (Olson & Buret, 2000). *Cryptosporidium* has been found in 80 mammalian species including ferrets, rabbits, opossums, kangaroos, antelopes, gazelles, camels, and a variety of other ruminant, equine, cervine, and ovine species (Fayer *et al.*, 1997). Many avian species have shown infection with *Giardia* and *Cryptosporidium*, and this has led to public health concern as birds are mobile, tend to aggregate around water, and in some cases have a close association with humans (Erlandsen & Bemrick, 1987).

1.1.8 Parasite Species and Their Hosts

Both *Cryptosporidium* and *Giardia* are currently in need of taxonomic revision. Limitations with conventional diagnostic methods due to difficulties recognizing morphological features has resulted in an inability to identify species clearly. Recent advances in molecular techniques have enabled a clearer picture of the speciation of *Cryptosporidium* and *Giardia* to emerge.

A genetic comparison between various isolates of *Cryptosporidium* recovered from different hosts has revealed tremendous genetic heterogeneity among and between species. This suggests that host-adapted genotypes/strains/species of the parasite exist (Morgan *et al.*, 1999e). Recent studies demonstrate that the species-level taxonomy of the genus does not reflect molecular phylogenetic analyses or epidemiological data (Ortega *et al.*, 1991; Morgan *et al.*, 1998b; Xiao *et al.*, 1999a). A current listing of accepted *Cryptosporidium* species, and the genotypes associated with each species is summarized in Table 1.2. The taxonomy of the *Cryptosporidium* genus is now composed of ten species, whereas previously, over twenty species had been described (Fayer *et al.*, 1997).

Until recently, the causative agent of human cryptosporidiosis was thought to be solely *C. parvum*. Genotyping studies on *Cryptosporidium* isolates from AIDS patients have identified various *C. parvum* genotypes, *C. felis* (Pieniazek *et al.*, 1999) and *C. meleagridis* (Morgan *et al.*, 1999f). Of the seven recognized *C. parvum* genotypes, only the human, cattle, and dog isolates have been found in HIV infected persons (Pieniazek *et al.*, 1999), and in healthy human hosts, only the human and cattle genotypes have been identified. Further genetic study is needed such that the transmission dynamics of human cryptosporidiosis in both the immunocompromised and immunocompetent host can be fully understood. This will determine the extent of public health risk associated with different sources of the parasite.

Table 1.2 Accepted species of *Cryptosporidium*, current genotypes determined, and biological data.

Species	Genotypes Identified	Host	Site of Infection	Reference
Cryptosporidium parvum	cattle	*Bos taurus* (cattle)	small intestine	Ortega *et al.* (1991)
	human	*Homo sapiens* (human)		Ortega *et al.* (1991)
	mouse	*Mus musculus* (mouse)		Morgan *et al.* (1999d)
	pig	*Sus scrofa* (pig)		Morgan *et al.* (1999a)
	marsupial	*Phascolarctos cincereus* (koala)		Morgan *et al.* (1999b)
	dog	*Canis familiaris* (dog)		Xiao *et al.* (1999a)
	ferret	*Mustela furo* (ferret)		Morgan *et al.* (1999e)
Cryptosporidium muris	mouse	*Mus musculus* (mouse)	peptic gland	Tyzzer (1907)
	bovine	*Bos taurus* (cattle)	abomasum	Esteban & Anderson (1995)
	camel/hyrax	*Camelus bactrianus* (camel)	stomach	Anderson (1991a)
Cryptosporidium felis	N/A	*Felis catis* (domestic cat)	intestine	Iseki (1979)
Cryptosporidium wrairi	N/A	*Cava porcellus* (guinea pig)	intestine	Vetterling *et al.* (1971)
Cryptosporidium meleagridis	N/A	*Meleagris gallopavo* (domestic turkey)	caeca	Slavin (1955)
Cryptosporidium baileyi	N/A	*Gallus gallus* (domestic chicken)	respiratory tract	Current *et al.*, (1986)
Cryptosporidium serpentis	needs further study	*Elaphe guttata* (corn snake)	intestine	Levine (1980)
Cryptosporidium andersoni	N/A	*Bos taurus* (cattle)	abomasum	Lindsay *et al.* (2000)
Cryptosporidium saurophilum	N/A	*Eumeces schneideri* (Schneider's skink)	intestine	Koudela & Modry (1998)
Cryptosporidium nasorum	N/A	*Naso lituratus* (fish)	intestine	Hoover *et al.* (1981)

The population structure within the genus *Giardia* is also undetermined. The subdivisions within the *Giardia* genus are based on host specificity, cell dimensions and median body morphology, and result in five accepted species, namely *G. muris*, *G. ardeae*, *G. psittaci*, *G. agilis*, and *G. duodenalis* (Table 1.1). As with *Cryptosporidium*, *Giardia* shows tremendous genetic heterogeneity at the subspecific level. This heterogeneity strongly indicates that a complex of genotypes or subspecies exists within each species. The question of how much diversity is present within the *G. duodenalis* grouping remains to be answered. Because *Giardia* is thought to represent one of the most ancient lineages among eukaryotes, genetic variation among contemporary isolates is expected. This diversity could account for observed differences in virulence, host range, infection duration, sensitivity to drugs, and host immune response (Weiss *et al.*, 1992).

Most generally agree that *G. duodenalis* from humans and other mammals consists of at least two groups that differ in their DNA sequence at a number of genes (Homan *et al.*, 1992; Nash, 1992; Weiss *et al.*, 1992; Upcroft *et al.*, 1994; Mayrhofer *et al.*, 1995). The two groups, formerly labeled "Polish" and "Belgian" (Homan *et al.*, 1992) have been relabeled A and B by Monis *et al.* (1996). The isolates within these groups have also demonstrated a degree of heterogeneity as Hopkins *et al.* (1997) described additional genetic groups unique to A and B that were isolated from dogs. In addition, van Keulen *et al.* (1998) proposed that the *Giardia* isolated from muskrats and voles be referred to as *G. microti* due to extensive differences in the 18S rDNA sequence. In contrast, it was further suggested by Monis *et al.* (1999) that *G. microti* be viewed as a member of the *G. duodenalis* species complex based on analysis of the 18S rDNA sequence. Future isolate analysis involving polymerase chain reaction (PCR)-based DNA fingerprinting methodology should clarify the genetic division within the species, and as well, will provide much evidence regarding host range for each isolate or species of *Giardia*.

1.2 Transmission

1.2.1 Risk Factors

The incidence of giardiasis and cryptosporidiosis has dramatically increased in recent years. Some of this increase can be attributed to recent advances in diagnostic techniques, however most of this increase appears to be related to social and behavioural patterns (Casemore *et al.*, 1997). This has altered the perception of risk that was once associated with waterborne parasitic infection. Risk factors include; (1) compromised immunity – this includes AIDS and other acquired or congenital immunodepression, immunosuppression due to transplants or cancer treatment, and malnutrition (Casemore, 1997), (2) contact with infected animals – pet owners and activities such as camping, backpacking, and visiting or working on farms increase the potential for zoonotic contact (Dawson *et al.*, 1995), (3) occupational exposure – veterinary, agricultural, medical, laboratory, and child daycare (Casemore *et al.*, 1997), (4) insufficient sanitation and hygiene practices – where fecal material can contaminate drinking water and food (Crawford & Vermund, 1988), (5) exposure to improperly treated water whether it be a breakdown in normal treatment practices or improper techniques (Wallis, 1994), (6) exposure to contaminated food (Crawford & Vermund, 1988), (7) travel – risk is increased if movement is from developed to underdeveloped or from urban to rural sites (Dawson *et al.*, 1995), (8) younger age, (9) contact with a potentially infected individual demonstrating diarrhea – risk groups include daycare workers, long-term care facility workers, patients in a nosocomial setting, parents, and siblings (Craft, 1982), and (10) sexual practices involving an infected individual. The risk of cryptosporidial infection is highest when behaviours or activities favor transmission, be it zoonotic, nosocomial, waterborne, or foodborne.

1.2.2 Transmission Through Water and Food

The importance of waterborne *Cryptosporidium* and *Giardia* transmission is evaluated based on both the number of waterborne cases reported, and on the various economic, regulatory, recreational, environmental and social aspects of waterborne disease control (Wallis, 1994). Outbreaks of giardiasis and cryptosporidiosis have occurred all over the world, and five major outbreaks of cryptosporidiosis where more than 1000 people were infected have occurred as a result of contaminated drinking water (Juranek, 1995). One of these occurred in Milwaukee in 1993 where over 400 000 people were infected as a result of a breakdown in the municipal water treatment facility. Of those infected, 1000 were AIDS patients (MacKenzie *et al.*, 1994). An outbreak of 360 confirmed cases of cryptosporidiosis this past year in north west England was attributed to possible contamination from sheep living within the watershed area (*Cryptosporidium* Capsule, 1999).

Outbreaks where many people are affected usually occur where drinking water is taken from surface water sources such as lakes and rivers (Juranek, 1995). Many surveys indicate that *Cryptosporidium* and *Giardia* are found in all types of water sources, and their presence is more common in surface water than in groundwater (Smith & Rose, 1998). These surveys also suggest that many bodies of surface water have intermittently low background levels of *Cryptosporidium* and *Giardia*. These levels may be increased rapidly through contamination from agricultural and human effluents. These sources typically are a problem following heavy rainfall events, such as spring runoff. In fact, the incidence of human cryptosporidiosis and giardiasis tends to increase during the spring and summer seasons in North America (O'Donoghue, 1995). The relative importance of both human and agricultural pollution on surface water reserves has yet to be determined. Both sources have great potential for waterborne contamination, as high numbers of *Cryptosporidium* and *Giardia* have been isolated from agricultural and human effluent in many geographical locations (Smith & Rose, 1998).

The consumption of certain foods has resulted in cases of human cryptosporidiosis and giardiasis. Foodborne transmission, although not a major route of transmission, is possible if foods become fecally contaminated. Infections have been associated with inappropriate food handling and/or using contaminated water to wash food (Adam, 1991). Both *Giardia* and *C. parvum* transmission from certain foods has been documented. These include; unwashed produce (Kasprazak *et al.*, 1981), produce washed with contaminated water (Adam, 1991), undercooked sausage, raw milk, and fresh pressed apple cider (Millard *et al.*, 1994).

1.2.3 Zoonotic Transmission

Giardia and *Cryptosporidium* have been isolated from many animal species including domestic pets, livestock, and various wildlife species. Previously, both parasites were thought to be zoonoses, however recent debate has arisen concerning the potential for zoonotic transmission of these species. Genetic analysis of *Giardia* and *Cryptosporidium* isolates from different animal hosts suggests that independent cycles of species specific infection are present.

Transmission of *Cryptosporidium* between animals and humans has been documented. Konkle *et al.* (1998) reported the nosocomial transmission of *Cryptosporidium* between four species in a veterinary hospital, as *Cryptosporidium* originating from an infected calf caused the infection of veterinary students, a llama, and two horses. This report is substantiated by recent molecular data suggesting that two genotypes within the species *C. parvum* are infectious to immunocompetent humans (Morgan *et al.*, 1998b). The 'cattle' and 'human' genotypes are both infectious, however the 'cattle' genotype is also found in various ruminant hosts. This suggests that the only potential animal reservoirs of human cryptosporidiosis are the ruminant and rodent host, as the 'cattle' genotype has also been isolated from farm mice (Morgan *et al.*, 1999e).

Genetic heterogeneity and its associated host specificity (Morgan *et al.*, 1998b) suggest that the current attitude that all *Cryptosporidium* isolates be considered hazardous to human health needs to be reassessed. This study lends support for a clonal population structure of *Cryptosporidium*, where each parasite is host-species specific.

The transmission dynamics of cryptosporidiosis in AIDS patients seems to paint a different picture. *Cryptosporidium* isolated from different AIDS patients has been genetically characterized as *C. felis*, *C. meleagridis*, and *C. parvum* 'human', 'cattle', and 'dog' genotype (Morgan *et al.*, 1999f). In this analysis, no recombinant genotypes and no mixed infections were detected. Although much more study is needed, it seems that *Cryptosporidium* is much more of a zoonotic threat for the immunocompromised population.

There is much biological evidence in support of *Giardia* as zoonoses. *Giardia duodenalis* is the only species infectious to humans, and it also infects a wide range of mammals. *G. duodenalis* has been shown to lack host specificity, as *Giardia* of human origin can infect other mammals such as dogs, cats, beavers, gerbils, and rats (Majewska, 1994). Genetic differences between dog and human *G. duodenalis* isolates recovered from the same community have also been documented (Hopkins *et al.*, 1997). These results suggest that two distinct cycles of *Giardia* transmission are present within these communities and the occurrence of cross transmission is low. Research into genotyping *Giardia* isolates recovered from various species is ongoing, and with further genetic data the transmission dynamics of giardiasis will be better understood. However, the endemic nature of *Giardia* infections, the tremendous potential for cross-species transmission, and the low infectious dose needed for establishing colonization all support the theory that all *G. duodenalis* cysts are potentially infectious to humans.

Recent studies have looked into the role of animal and insect vectors in the transmission of *Giardia* and *Cryptosporidium*. These transmission routes were previously unexplored. New evidence suggests that house flies (Graczyk *et al.*, 1999)

and dung beetles (Mathison & Ditrich, 1999) can act as transport hosts of *C. parvum*. Bird species can also act as transport vectors of the parasites, as both *Giardia* and *C. parvum* were detected in the feces of migratory Canada geese (Graczyk et al., 1998). Finally, infectious *C. parvum* oocysts were detected in oysters and clams located in the Chesapeake Bay (Fayer *et al.*, 1998). These studies suggest that more effort is needed in understanding the role of various animal species that are closely associated with water resources.

1.2.4 Human-Human Transmission

The mechanisms for the transmission of *Giardia* cysts and *Cryptosporidium* oocysts among human hosts include direct person to person fecal-oral transfer, or indirect transfer through fomites. Fomite transmission is rather limited as both parasites are highly susceptible to desiccation, and therefore the capability of viable (oo)cysts to produce infection would drastically decrease with time. Transmission has been reported in day care centers (Mai Nguyen, 1987), within households (Casemore *et al.*, 1994), in urban communities (Newman *et al.*, 1994), and in nosocomial settings (Casemore *et al.*, 1994). Fecal-oral transmission is the major route for the transmission of human infection. This is exemplified in developing countries, day-care centers and public institutions where there is poor hygiene or inadequate sanitary conditions leading to a high prevalence (5% to 43%) of giardiasis (Adam, 1991). *Cryptosporidium* from sputum and vomitus has been detected, therefore these body fluids may act as other vehicles for transmission (Casemore *et al.*, 1994). Less common, but still an important means of transmission between people, include venereal spread or sexual practices involving oro-anal contact as is frequently found in homosexual men with HIV/AIDS (Flannigan & Soave, 1993; Owen, 1984).

1.3 Presence in the Environment

1.3.1 Survival

An important factor regarding waterborne parasite contamination of the environment is the extent to which the parasites can survive varying conditions. *Cryptosporidium* and *Giardia* are highly susceptible to desiccation and therefore, viable organisms are associated with water or damp environments. Water temperature is a key factor to parasite survival. Extremely high (>100°C) and freezing (*Giardia*) or extremely low (-70°C) temperatures (*Cryptosporidium*) rapidly inactivate both parasites (Fayer & Nerad, 1996; Bingham *et al.*, 1979). Survival is optimal at water temperatures near 4 °C, where *Giardia* and *Cryptosporidium* remain viable for 84 to 176 days (Jakubowski, 1990; Robertson *et al.*, 1992). Jakubowski (1990) demonstrated in the laboratory that high concentrations of bacteria in the surrounding environment led to cyst degradation. Parasites are also able to survive for long periods of time in fecal deposits, which protect the (oo)cysts from desiccation and prolong the parasite lifespan. This allows for rapid environmental contamination, if infected animals reside in areas adjacent to waterways.

Giardia is susceptible to standard chlorine disinfection methods used by water treatment facilities. The levels of chlorine required to inactivate *Cryptosporidium* oocysts is too high to be used in drinking water disinfection (Rose, 1997). *Giardia* and *Cryptosporidium* are sensitive to ultraviolet and pulsed light (Campbell *et al.*, 1995) and ozone disinfection (Rose, 1997). Ultraviolet light units that generate a maximum of 40,000 $\mu watts/cm^2$ and 253.7 nm wavelength are capable of inactivating parasites, however the cost of these units is too high for most public water agencies to afford (Cullimore & Jacobsen, 1988). The exposure of *Giardia* and *Cryptosporidium* to ozone also results in the inactivation of infective (oo)cysts (Cullimore & Jacobsen, 1988). This method is gaining popularity and although it is initially quite expensive to install, it is

predicted that most water treatment facilities will employ ozone disinfection to inactivate any waterborne parasites present.

To further demonstrate the strength of protozoan parasites in the environment, researchers at the US Food and Drug Administration evaluated three commonly used hospital disinfectants based on their ability to inactivate *Cryptosporidium* oocysts (Wilson & Margolin, 1999). Phenol (10%), povidone-iodine (10%) and glutaraldehyde (2.5%) were all unable to prevent infectivity at the concentrations and contact times used. This study raises the concern that hospital equipment may not be immersed in disinfectant for long enough periods of time to allow the complete inactivation of *Cryptosporidium* (Wilson & Margolin, 1999).

1.3.2 Water Treatment

Once waterborne parasites are positively identified in water samples, treatment must ensure their removal so that outbreaks of disease do not occur and susceptible populations are protected. Municipal water utilities provide relatively good protection against waterborne giardiasis and cryptosporidiosis. Since the Milwaukee *Cryptosporidium* outbreak of 1993, much research has been done to investigate water treatment systems. Water treatment facilities purify water using several techniques, first through chemical treatment with chlorine, and then through coagulation, sedimentation, and filtration.

Chlorination is commonly used to disinfect drinking water as it kills most viruses, bacteria, and depending on the concentration used, the temperature of the water and the contact time, may effectively inactivate *Giardia* cysts (Wallis *et al.*, 1993). *Cryptosporidium* has been shown to be 240 000 times more resistant to chlorination than *Giardia* (Smith & Rose, 1998). Korich *et al.* (1990) tested oocyst viability following various chlorine treatments and found that after 18 hours of exposure to 1.05% and 3%

chlorine, oocyst viability was not affected. Currently, other disinfection methods such as ozone treatment and UV inactivation are being evaluated, but have yet to be approved for municipal and widespread use by the US Environmental Protection Agency (EPA).

Following chlorination, *Cryptosporidium* oocysts, if detected in the raw water source have yet to be inactivated. *Cryptosporidium* and all other particulate matter are physically removed from water sources through coagulation, sedimentation, and filtration (Rose *et al.*, 1997). Effective coagulation of oocysts has been achieved using alum, ferric chloride, and polyaluminum chloride (Smith & Rose, 1998). Oocyst removal is further improved with the addition of a polymer along with the metal salt (Rose *et al.*, 1997). The efficiency of sedimentation as a method to reduce parasite numbers is extremely low. Oocysts and cysts are small and their density is low, therefore sedimentation velocities are extremely slow (0.35 μm/s and 1.4 μm/s respectively) and therefore much time is required for effective settling of the parasites to the bottom of water reservoirs (Medema *et al.*, 1998). The filtration stage of water treatment is where problems are likely to occur. Past deficiencies at this stage have led to the presence of waterborne parasites in treated drinking water, and ultimately to outbreaks of disease. Close examination of filtration deficiencies have shown that proper management can provide adequate protection against the presence of *Giardia* and *Cryptosporidium* in public water supplies (Rose *et al.*, 1997).

Filtration of incoming water through ultra-fine membranes that remove particles in the range of an electron microscope ($\geq$0.01 microns) have been developed (Flannigan & Soave, 1993). In addition, high-rate filtration, dissolved-air flotation, diatomaceous earth, and slow-sand filtration can also effectively remove oocysts at greater than 2.5 logs (Smith & Rose, 1998). Improved monitoring of turbidity and optimization of the backwash procedure can minimize the threat of waterborne parasites passing through the treatment process during high runoff periods (Smith & Rose, 1998).

1.3.3 Sewage Treatment

Surface waters can become contaminated with waterborne parasites due to the release of sewage discharge containing parasites resulting from human infection in the community. The magnitude and prevalence of parasite infection within a community determines the severity of contamination in wastewater (Meinhardt *et al.*, 1996). Unfortunately, the effect of wastewater contamination is usually observed through outbreaks of infection in downstream communities where water treatment is substandard. Many studies have demonstrated the presence of *Giardia* and *Cryptosporidium* in both raw and treated human sewage (Wallis *et al.*, 1996; Ong *et al.*, 1996, Smith & Rose, 1998). Treated human sewage containing waterborne parasites occurs when the number of parasites in sewage influent is high enough to overwhelm the capacity of the treatment system. When this occurs, water sources can become rapidly contaminated with high numbers of parasites due to the release of partially treated sewage (Meinhardt *et al.*, 1996).

The type and extent of sewage treatment depends largely on the size of the contributing population, and on the daily volume of wastewater received. Sewage treatment can range from simple septic tank storage with yearly discharges, to complicated tertiary treatment schemes comprised of clarification, digestion, and stabilization stages (Chauret *et al.*, 1999). Geldreich (1986) studied the effect of various sewage treatment processes on microbial populations in raw sewage. The removal range of various organisms was calculated for each phase of wastewater treatment. Primary clarification and septic tank storage removed the least percentage of microorganisms (5% to 75%) and anaerobic digestion and tertiary treatment with flocculation and sand filtration removed the most pathogens (25% to 99.99%). Following aerobic wastewater treatment at the treatment plant in Ottawa, Canada, a 2.96 $\log_{10}$ reduction of *Cryptosporidium* and a 1.40 $\log_{10}$ reduction of *Giardia* were observed (Chauret *et al.*, 1999). Parasite reduction following anaerobic sludge digestion was also calculated and a

0.30 $\log_{10}$ reduction of *Cryptosporidium* but no reduction in *Giardia* was observed (Chauret *et al.*, 1999). Further studies investigating the effect of treatment on parasite viability in wastewater systems are needed.

1.4 Detection

1.4.1 Microscopy

In order to control parasite presence in the environment, adequate surveillance and detection methods are required. The characteristics of a good detection method should include high specificity, high sensitivity such that low parasite numbers can reliably be detected, reliable quantification and methods to determine parasite viability. Current microscopic methods used to detect, quantitate, and determine viability for both *Giardia* and *Cryptosporidium* employ acid-fast staining, and immunofluorescence using parasite specific monoclonal antibodies. Protozoan parasite diagnostics have progressed tremendously from histologic staining of gut and biopsy samples to sensitive assays capable of detecting (oo)cysts in clinical and environmental samples (Arrowood, 1997).

Immunofluorescent methodologies have found widespread application in clinical, research, and environmental laboratories. This technique employing (oo)cyst reactive monoclonal antibodies is currently the accepted detection method for *Giardia* and *Cryptosporidium* by the Information Collection Rule (ICR) and is used as a standard operation in most laboratories (Smith & Rose, 1998). Oocysts and cysts are recovered and concentrated from environmental or clinical samples either by immunomagnetic separation, or by filtration, followed by the pelleted filtrate over- or underlayed with Percoll-sucrose flotation medium with a specific gravity of 1.13 (Rose *et al.*, 1997). Through a series of centrifugations and washes, the resulting pellet contains minimal debris, *Giardia* cysts and/or *Cryptosporidium* oocysts. The sample is layered onto a 25 mm filter, saturated with a monoclonal antisera mixture and labeled. Visualization and

enumeration is by epifluorescent microscopy (Rose *et al.*, 1997). Recovery using this method has been estimated between 50% and 90% (Smith & Rose, 1998).

The US EPA sponsors testing of many commercial laboratories using this method of parasite detection (Newman, 1995). Although most labs are able to detect *Giardia* and *Cryptosporidium* in spiked samples, many misidentify algae and yeast. This suggests that positive results do not necessarily indicate a public health threat, and similarly, a negative result does not necessarily signify a clean sample. Based on this evaluation, it is necessary when dealing with protozoa monitoring to test multiple samples, and to view the total context of all water quality and treatment parameters before triggering a public health alert. There is also a need for new methodology, which can generate consistent and more specific results.

Analytical methods testing parasite viability are of tremendous use to water and environmental monitoring. One technique determines viability based on the exclusion or inclusion of fluorogenic vital dyes such as propidium iodide in *Cryptosporidium* and a nucleic acid stain in *Giardia* (Campbell *et al.*, 1992). These assays also require extensive microscopy using various filters to determine dye permeability and hence, viability. In general, microscopic methods are labour-intensive, lack sensitivity, specificity and reproducibility, and require repeated washing, filtration, and gradient centrifugation prior to detection (Morgan & Thompson, 1998).

1.4.2 Molecular Techniques

Many molecular biologic techniques are being applied to the detection of *Cryptosporidium* and *Giardia* in clinical and environmental samples. The new detection techniques are all PCR- based using gene-specific DNA primers from various gene loci. The advantages of PCR for the detection of waterborne parasites in both clinical and environmental samples include, ease of use, sensitivity, ability to analyze many samples

at one time, low cost, and speciation or strain-typing ability (Morgan & Thompson, 1998). In a comparison trial between microscopy and PCR detection of *Cryptosporidium parvum* in human fecal samples, microscopy was found to exhibit 83.7% sensitivity compared to 100% sensitivity for PCR (Morgan *et al.*, 1998a). Although the benefits of PCR technology seem endless, the reoccurring problem of inhibition remains. Humic acids, bile constituents, non-target organisms and their genomic DNA, and a variety of other inhibitors all negatively affect clinical and environmental PCR reactions. This problem is close to being overcome as reliable, fast, and standardized extraction methods are being developed (Morgan & Thompson, 1998).

A multitude of PCR protocols for the detection of both *Giardia* and *Cryptosporidium* are described in the literature. Multiplex PCR reactions, where both parasites are simultaneously detected in one single reaction would be ideal. To date, a primer combination with ideal sensitivity, specificity and compatibility have yet to be developed. Rochelle *et al.* (1997) were able to amplify fragments from both the 18S rDNA of *Cryptosporidium* and the heat shock protein of *Giardia* using the same primer set. Unfortunately, the amplified product was unable to provide species/strain-typing information from either parasite (Rochelle *et al.*, 1997). Nested PCR protocols, which involve two amplification rounds, have been applied to *Cryptosporidium* detection methodology. Nested PCR increases the specificity and sensitivity of detection, however with these protocols comes an increased risk of contamination (Morgan & Thompson, 1998). Parasite viability detection is an important tool in environmental diagnostics. This has recently been shown possible through the use of reverse transcription (RT)-PCR of ß-tubulin mRNA (Widmer *et al.*, 1999). Researchers correlated the loss of oocyst infectivity in neonatal mice to a decrease in mRNA levels following prolonged incubation at room temperature (Widmer *et al.*, 1999). Ideally, the development of one protocol that could combine detection, speciation, and viability would greatly improve current parasite diagnostics. At the moment however, this is unrealistic, as extraction

procedures for RT-PCR involve the digestion of any DNA present, and speciation has only been achieved through the direct amplification of conserved genomic DNA regions.

1.4.3 Characterization and Genome Sequencing of *Cryptosporidium* Isolates

The *Cryptosporidium* genus is composed of a large number of almost indistinguishable species. This problem, compounded by the inability to culture the parasite in vitro has led to substantial progress in the development and application of molecular characterization tools (Morgan *et al.*, 2000). Many PCR-based characterization studies have revealed significant inter- and intraspecies sequence differences (Morgan *et al.*, 1999c; Xiao et al., 1998; Xiao et al., 1999a; Xiao et al., 1999b). These include conventional restriction fragment length polymorphism analysis (RFLP) and PCR-based molecular techniques which have transformed the genetic characterization of *Cryptosporidium* due to the small amounts of material required (Morgan *et al.*, 2000).

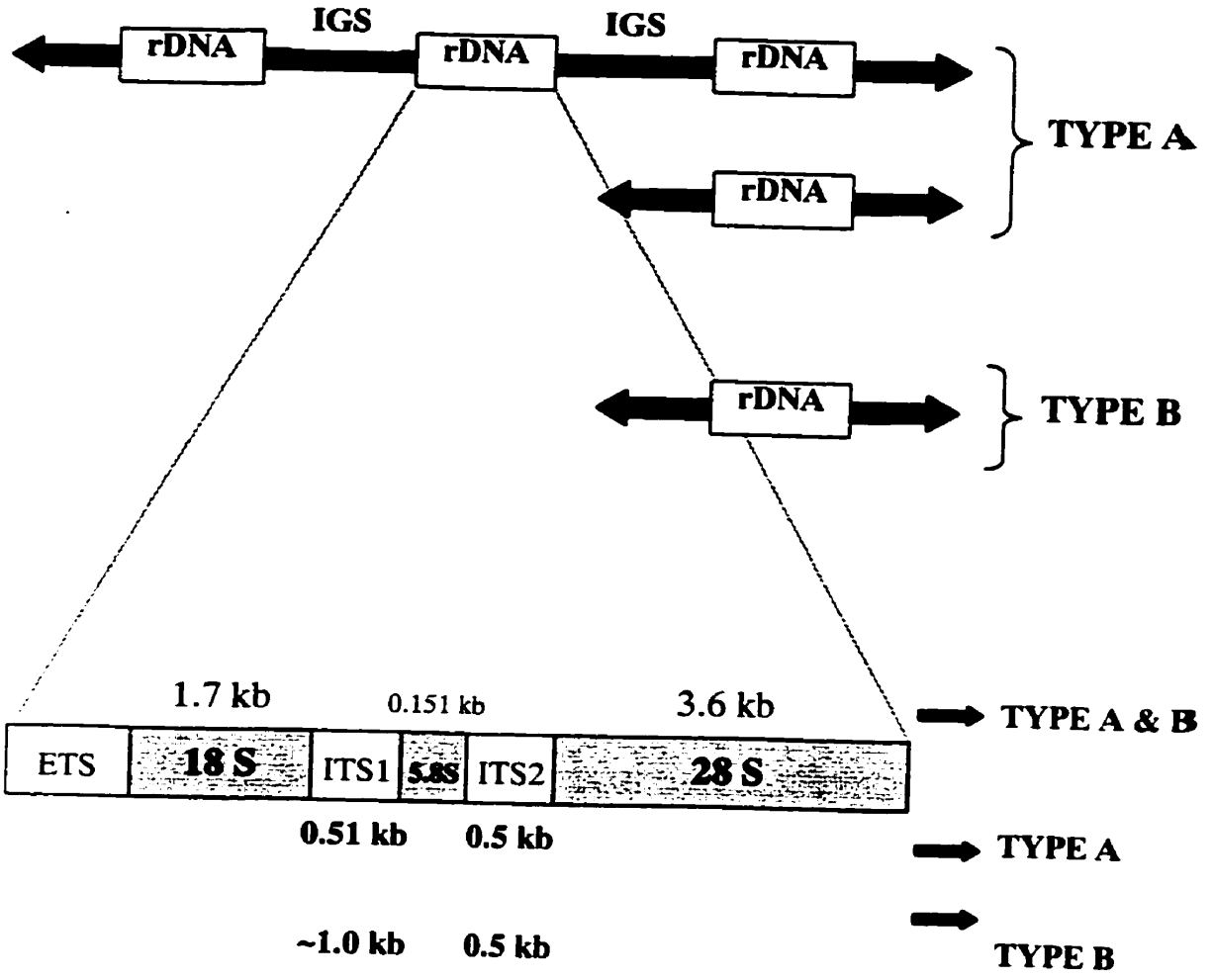
One of the first PCR-based techniques used to investigate genetic variation within *Cryptosporidium* was random amplified polymorphic DNA (RAPD). This technique, developed by Williams *et al.* (1990), detects nucleotide sequence polymorphisms without the need for previously determined sequence information. RAPD analysis of human and calf *C. parvum* isolates determined two groups, one containing the majority of the human isolates and the second, containing all of the animal isolates (Morgan *et al.*, 1995). The use of RAPD analysis is relatively restricted due to its lack of specificity and high potential for replicating contaminating DNA, and due to its lack of reproducibility (Morgan *et al.*, 2000). New methods involving PCR-RFLP and sequence analysis are typically used as they are more specific and can be repeated between laboratories.

Many genes have been targeted for comparison between *Cryptosporidium* isolates. These include AcetlyCo A Synthetase (Morgan *et al.*, 1998b), ß-tubulin

(Widmer *et al.*, 1998), oocyst wall protein (Spano *et al.*, 1997), dihydrofolatereductase-thymidylate synthase (Vasquez *et al.*, 1996), thrombospondin-related adhesion proteins (Spano *et al.*, 1998), and the heat shock protein (Di Giovanni *et al.*, 1999) genes. Perhaps the most highly utilized region in terms of characterization of the *Cryptosporidium* genome is the rDNA unit (Figure 1.2). This is due to the highly conserved nature of the coding regions, the variability in both the transcribed and non-transcribed spacer regions, and the presence of multiple copies in the haploid genome. Sequence information of the rDNA unit has allowed for the development of various diagnostic tests employing a wide range of restriction enzymes that differentiate both between and within species (Morgan *et al.*, 1999b; Xiao *et al.*, 1999a). These PCR-RFLP approaches can be used to study the molecular epidemiology of *Cryptosporidium* and allow for a wider range of isolates to be examined (Morgan *et al.*, 2000).

Figure 1.2

Ribosomal DNA unit of Cryptosporidium. Size measurements represent that of KSU-1 isolate (5-day old calf) (Le Blancq *et al.*, 1997). Size variation among different isolates exists to a small degree. Five copies of the rDNA unit per 1 haploid genome exist, with 2 types of unit, type A and B. It is thought that the 5 copies are dispersed within 3 separate chromosomes.


rDNA
IGS
rDNA
IGS
rDNA
TYPE A
rDNA
rDNA
TYPE B
1.7 kb
0.151 kb
3.6 kb
TYPE A & B
ETS
18 S
ITS1
5.8S
ITS2
28 S
0.51 kb
0.5 kb
TYPE A
~1.0 kb
0.5 kb
TYPE B

1.5 Summary and Objectives

An understanding of the epidemiology of *Giardia* and *Cryptosporidium* in central Alberta is of particular interest, as both parasites currently contaminate Canadian watersheds. Both *Giardia* and *Cryptosporidium* are medically, and economically important parasites, and their distribution in the environment is ubiquitous. The comparative significance of agriculture with emphasis on the cow-calf industry, wildlife and human sources of *Giardia* and *Cryptosporidium* would be of tremendous importance in terms of management strategies that could reduce the impact of problematic sources on parasite presence in the environment. Source prevalence coupled with an understanding of parasite genetic differences and thus transmission limitations will shed light on the molecular epidemiology of waterborne parasites. This may directly lead to the reduction of waterborne parasites in the nearby water systems. As such, preliminary work on the generalized prevalence of *Giardia* and *Cryptosporidium* in cow-calf, sewage influent, and wildife sources within the NSR basin in central Alberta was the first objective in what follows.

With the parasite prevalence of each source established within the general NSR basin area, a second objective was to assess the impact of the various sources on specific subwatersheds. By focusing efforts onto localized areas, additional agricultural sources such as hog and dairy operations were assessed. This allowed for an in depth appraisal of agriculture as a potentially significant source of *Giardia* and *Cryptosporidium* in the NSR basin. Finally, the collection of *Cryptosporidium* positive samples from human and a wide range of animal species allowed for the molecular characterization of various isolates. This has enabled the testing of various PCR-based genotyping tools, which has resulted in the differentiation of various *Cryptosporidium* isolates.

The specific objectives of the present work can be summarized as follows:

(1) To determine and compare the general parasite prevalence in the cow-calf industry, human sewage influent, and wildlife scat within the NSR basin.

(2) To determine and compare the localized parasite prevalence in specified subwatersheds with emphasis on agricultural sources such as hog, dairy, cow-calf operations.

(3) To use and improve current PCR-based molecular tools for the identification and differentiation of various *Cryptosporidium* isolates found in samples collected from the Alberta region.

2 Materials and Methods

2.1 Sample Collection and Questionnaire Completion

All samples were collected during a two-year period that began during the spring of 1998 and continued through until the winter of 2000. Fecal (cow-calf, dairy, hog, and wildlife) and sewage influent samples were obtained from various areas within the North Saskatchewan River (NSR) basin watershed (Figure 2.1) and all producers cooperated. Survey information was collected during interviews with each respective producer.

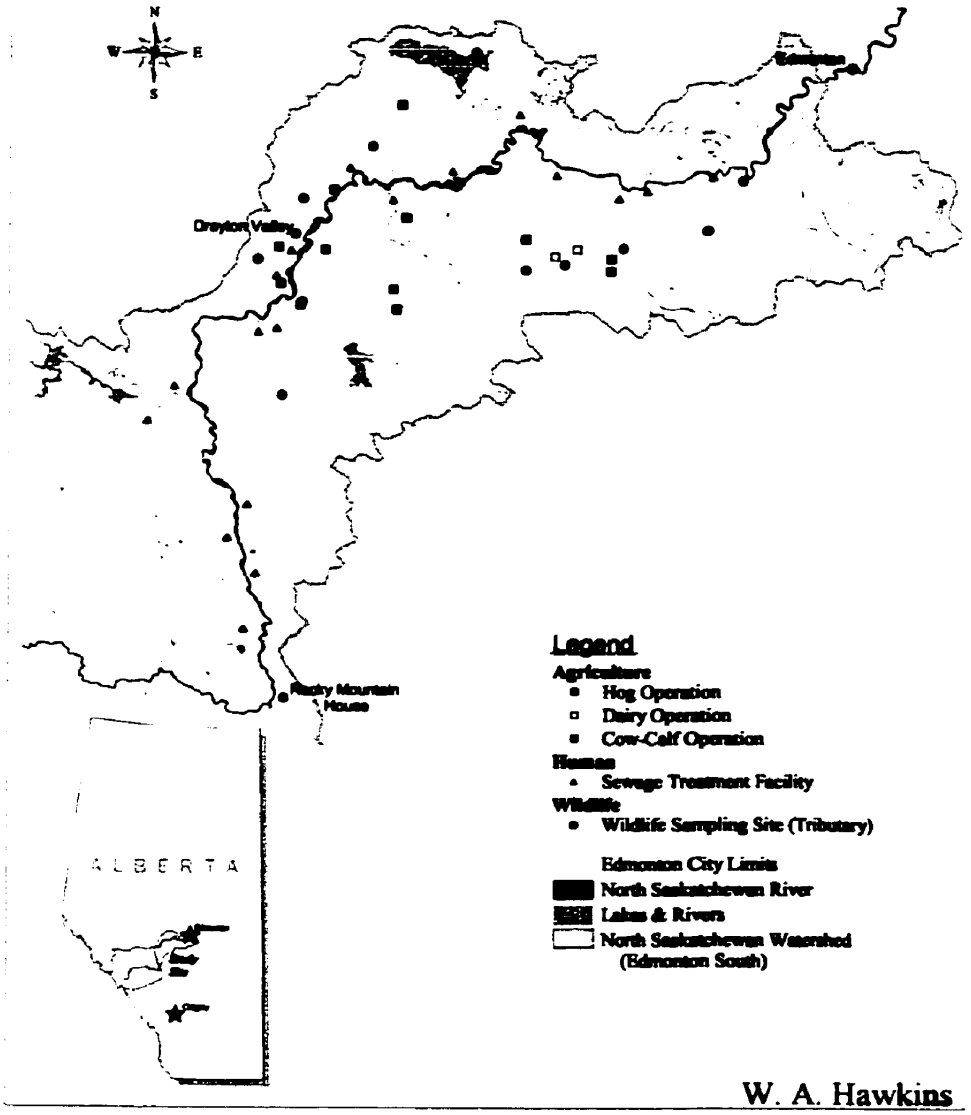
2.1.1 Cow-calf

Seven cow-calf operations located within the Brazeau Municipality were sampled during the spring of 1998, and four additional cow-calf operations located within close proximity to NSR tributaries, but in various Municipalities, were sampled during the spring of 1999. Sampling times were coordinated with the average age of the calf herd such that the majority of samples could be collected from animals aged three to six weeks. Approximately 25 to 50 fecal samples from adult animals and 75 to 125 fecal samples from calves within the age bracket were collected. Samples were collected in small, one to five gram volumes from individual fecal deposits found on the ground in pastures at each location. Care was taken to ensure that only one sample from each fecal deposit was collected. Collection preference was given to fresh verses old, and thawed verses frozen deposits. Feces were identified as cow or calf based on size, colour, and texture. All samples were weighed and suspended in 2.5% potassium dichromate (Sigma Chemical Co., St. Louis, Missouri, USA) for preservation upon laboratory arrival. Information regarding management practice, water accessibility, herd and feed characteristics, medical history, and water treatment was collected through the on-sight completion of a questionnaire given to each producer (Figure 2.2).

Figure 2.1

The North Saskatchewan River (NSR) basin watershed area and associated agriculture, wildlife, and sewage influent sampling sites.

North Saskatchewan River Basin
Sampling Sites



W. A. Hawkins

2.1.2 Dairy

Two dairy operations located within close proximity to Strawberry Creek, a major tributary of the NSR, were sampled in early January 2000. A small volume sample (1 to 5 g) was collected from each fecal deposit found within calf pens in order to maximize the number of calf samples obtained. Small volume samples from all age groups at each dairy were also collected, such that a total of 25 to 50 samples from each location were obtained. For all pens sampled, approximate animal age was determined as the mean date of birth of all animals present in each pen. Each dairy producer completed a questionnaire, similar to that previously described, during the farm visit (Figure 2.2). Specific dairy-related questions pertaining to manure management were added to the standard cow-calf questionnaire. Samples were weighed upon arrival at the laboratory.

2.1.3 Hog

One hog operation located directly adjacent to a major tributary of the North Saskatchewan River was sampled and tested for the presence of both *Giardia* and *Cryptosporidium* in early January, 2000. Samples were collected and analyzed according to Guselle *et al.* (unpubl.). The farm operator received a cooler containing collection tubes (Becton-Dickinson Labware, Franklin Lakes, New Jersey, USA), a questionnaire (Figure 2.2), and detailed instructions. Small samples (1 to 5 g) were collected from the pens of boars, dry sows, nursing sows, weaners, growers, and finishers, and also from the pits of the above. Following sample collection, the cooler containing all samples and the completed questionnaire was picked up and driven directly to the lab for processing.

Figure 2.2

Questionnaire used during agricultural sampling.

A. Cow-calf and Dairy operators.

B. Hog operators.

A) Cow-calf and Dairy Survey

Name:	Phone:
Address (mailing):	Fax:
Location of Farm (LLD):	

Type of Farm: mixed / beef / dairy	Total number acres:
Total acres of pasture:	
Type of forage: grass / alfalfa / clover / hay / silage / grainfed / other	

Total number of cattle:	
Number of cattle in each age group:	1. Calf (<1 year)
	2. Heifer (1-2 years)
	3. Steers / bulls (1-2 years)
	4. >2 year cows
	5. breeding bulls
Change in herd size last 1-5 years:	
Number of other animals on farm:	1. Sheep / goats
	2. Pigs
	3. Horses
	4. Dogs / cats
	5. Poultry

Do you supplement with any of the following (if yes, give name of brand used)	
1. trace mineral salt	Y / N name:
2. 1:1 mineral	Y / N name:
3. Vitamin ADE	Y / N name:
4. Protein	Y / N name:
5. Selenium	Y / N name:

Months of calving:	Number of calves born:	Calf mortality:
Mortality in last 5 years: 5% / 5-10% / >10%		

History of scours in last 5 years (describe):

Treatment of scours: no treatment / cut back on feed / electrolytes / antibiotics

Brand name of antibiotics:

Do cattle have ready access to running water:

Water source for cattle : well / stream / cistern / spring / pond / municipal

Water source for house: well / stream / cistern / spring / pond / municipal

Water treatment – cattle: Y / N / municipal supply / unknown

Water treatment –house: Y / N / municipal supply / unknown

Distance of barn water source from septic system (ft.):

Distance of pasture water source from septic system (ft.):

Type of bedding material used for calves:

Is there any effort to clean calf bedding areas (if yes, describe):

When are cows brought to calving area:

How long are cows in calving area:

When are pairs released to pasture:

Where does calving take place:

Is there any pen rotation during calving:

Does location of pairs change between seasons (if so, describe movement):

¶ How do you dispose of manure?

¶ When do you dispose of manure?

¶ question asked only to dairy operators

B) Hog Survey

Farm name:		Sample date:
Farm address, phone, fax:		
Farm size:		Type of operation:
Number of swine:	Breed:	Source of Stock:
Other animal species on farm:		

Medication:	
How frequently do you worm your pigs?	Product:
Disease problems in herd?:	

Type of water source for swine:	
Any standing water?:	

How do you dispose of swine fecal material?:	Size of Lagoon:
How often is fecal material disposed of? times of year? # acres covered:	
If used as fertilizer, is it sprayed or injected?	
If injected, to what depth:	
Land it is spread on (crop/pasture)	
What is the rate of manure application?:	
Do you own the manure equipment or have it custom applied?	

2.1.4 Wildlife

Immediately following the spring 1998 snowmelt, wildlife scat samples were collected from a total of 39 forested or riparian-band locations chosen within 18 NSR-tributary sub-watershed areas (Table 2.1). Areas within each tributary sub-watershed were ranked according to their proximity to the NSR. Areas designated "one" were closest to the NSR and areas designated "three" were furthest away. The legal land description and date of sampling for each location was recorded. Up to fifteen fecal samples from each type of wildlife scat encountered and identified was collected in a manner to minimize collecting from the same animal. Fresh samples were selected over older samples, and distant samples were selected over adjacent samples. Small volume samples (1 to 5 g) were collected, and scat was identified to genus based on shape, colour, size, texture, and location. All samples were weighed and preserved in 2.5% potassium dichromate until processed.

Fecal samples from culled beaver and muskrat present in the NSR watershed were also collected in the fall of 1998 and 1999. Local trappers and Municipality workers collected fresh samples directly from the intestine immediately following death of the animals. The location of animals before death was recorded, and the samples were weighed, and stored in 2.5% potassium dichromate before being shipped to the laboratory on ice for analysis.

Additional samples from migratory or resident Canada and Snow Geese were collected from various regions of central Alberta. Feces were collected from frozen lakes and riverbanks, during the late fall of 1998. The location of sample collection and the type of bird predominating in the nearby area were recorded. Samples were weighed, preserved, and shipped to the laboratory as described above.

2.1.5 Sewage influent

Sixteen sewage treatment facilities located upstream of Edmonton were sampled bimonthly throughout the two-year study period (Table 2.2). Alberta Environment employees collected sewage influent samples as 500 ml of a ten-point composite collection from each storage lagoon or holding facility. Tween 20 (BDH Inc., Toronto, Ontario, Canada) was added to the composite collection as a surfactant prior to mixing and sub-sampling. Collected samples were immediately shipped on ice to the laboratory for processing.

2.2 Parasite Isolation and Concentration

2.2.1 Fecal analysis (cow-calf, dairy, hog, and wildlife)

Fecal sample preparation was performed according to methods previously described by Olson *et al.* (1997a). A small volume of phosphate buffered saline (PBS) was added to all unpreserved fecal samples. Samples were vortexed to ensure adequate mixing before being filtered through a 4-ply surgical gauze sponge (NuGauze, Johnson & Johnson, Montreal, Quebec, Canada). Additional PBS (Table 2.3) was added during the filtering process to further liquefy feces and allow greater parasite recovery. The resultant 7 to 10 ml of filtrate was carefully layered over 5 ml of a 1M sucrose solution with a specific gravity of 1.13 (Table 2.3). The layered samples were centrifuged (model HN-S IEC, International Equipment Co., Needham Heights, Massachusetts, USA) at 800 x g for 7 minutes. Following centrifugation, a crescent shaped pellet was apparent at the interface between the sample and the sucrose. This pellet and the upper layer of liquid were removed and centrifuged again at 800 x g for 7 minutes. Following this last centrifugation, the supernatant was removed, the final pellet suspended in 1 ml 2.5% potassium dichromate, and refrigerated for storage.

Table 2.1 Sites within the NSR basin where wildlife sampling occurred spring of 1998.

Tributary Sampled (number of sites)	Distance to NSR	Legal Land Description	Date Sampled
Weed Creek (2)	1	NW 13-50-R28-W4	April 6
	2	NW 24-50-R28-W4	April 6
Strawberry Creek (2)	1	SW 5-50-R1-W5	April 7
	2	NE 28-50-R1-W5	April 7
Wabamum Creek (2)	1	NE 34-52-R3-W5	April 7
	2	NE 9-52-R3-W5	April 7
Tomahawk Creek (3)	1	NW 14-50-R5-W5	April 8
	2	NE 33-50-R5-W5	April 8
	3	SW 29-50-R5-W5	April 8
Buck Lake Creek (2)	1	SW 19-49-R5-W5	April 8
	2	SW 4-49-R5-W5	April 9
Drayton Valley Tributary (2)	1	SW 34-48-R7-W5	April 14
	2	NE 14-48-R7-W5	April 14
Mishow Creek (3)	1	NE 21-50-R6-W5	April 9
	2	SW 30-50-R6-W5	April 14
	3	SW 13-50-R6-W5	April 14
Tributary of Violet Grove (2)	1	SE 18-48-R7-W5	April 15
	2	NW 26-48-R7-W5	April 15
Sand Creek (2)	1	NE 33-46-R10-W5	April 15
	2	NW 12-46-R10-W5	April 15
Washout Creek (2)	1	NE 10-45-R10-W5	April 15
	2	SW 16-45-R10-W5	April 15
Rose Creek (2)	1	NE 35-47-R8-W5	April 15
	2	NE 25-47-R8-W5	April 15
Nordegg River (2)	1	SW 26-45-R10-W5	April 16
	2	SW 23-45-R10-W5	April 16
Baptiste River (2)	1	NW 34-42-R8-W5	April 16
	2	SW 26-42-R8-W5	April 16
Canyon Creek (2)	1	NE 8-41-R7-W5	April 16
	2	SW 10-41-R7-W5	April 17
Big Beaver Creek (3)	1	NE 11-41-R7-W5	April 16
	2	SE 31-41-R7-W5	April 16
	3	SW 27-41-R7-W5	April 16
Prentice Creek (2)	1	NE 18-40-R7-W5	April 17
	2	NE 12-40-R7-W5	April 17
Conjuring Creek (2)	1	NE25-50-R26-W4	April 22
	2	SW18-50-R26-W4	April 22
Shoal Lake Creek (2)	1	NW 19-50-R4-W5	April 24
	2	NE 24-50-R4-W5	April 24

Table 2.2 Sixteen sewage treatment facilities located upstream of Edmonton, Alberta that were sampled bimonthly from June 1998 to January 2000.

Municipal Sewage Facility	Storage Type	Treatment/ Disinfection	Discharge frequency
Alder Flats	lagoon	N/A	1 per year
Birchwood Village	lagoon	N/A	1 per year
Breton	lagoon	N/A	2 per year
Buck Creek	lagoon	N/A	1 per year
Calmar	lagoon	N/A	1 per year
Devon	mechanical	chlorination	continuous
Drayton Valley	mechanical	chlorination	continuous
Edmonton-Goldbar	mechanical	chlorination and UV	continuous
Rocky Mountain House	mechanical	N/A	continuous
Rocky Rapids	lagoon	N/A	1 per year
Sunnybrook	lagoon	N/A	1 per year
Thorsby	lagoon	N/A	1 per year
Tomahawk	lagoon	N/A	2 per year
Tomahawk School	lagoon	N/A	1 per year
Violet Grove	lagoon	N/A	1 per year
Warburg	lagoon	N/A	1 per year

2.2.2 Sewage influent

Of the 500 ml volume obtained for each sewage influent sample, only a 350 ml aliquot was processed. Each 350 ml sub-sample was centrifuged at 800 x g for 10 minutes in a refrigerated super-speed centrifuge (Sorvall model MN RC-5B, Dupont Instruments, New Town, Connecticut, USA). The supernatant was vacuum aspirated and the pellet retained. PBS was added to each pellet to equal a volume of 10 ml. This represented the "wash" step. Finally, the sample was centrifuged again at 800 x g for 10 minutes, the supernatant discarded, and the refrigerated pellet retained in 1 ml of PBS.

2.3 Immunofluorescent Visualization

Microscopic investigation was used to visualize all samples. For all fecal samples and sewage influent, 0.015 ml of the 1 ml suspended pellet was dried onto each ring of an immunofluorescent slide (Erie Scientific Co., Portsmouth, New Hampshire, USA). A small volume (1 to 2 ml) of acetone (Sigma Chemical Co., St. Louis, Missouri, USA) was dried onto the slide to fix any parasites present to the glass. Once dry, 0.02 ml of a 1x fluorescein isothiocyanate (FITC)-labeled monoclonal antibody solution specific for either *Giardia* (Giardi-a-Glo™, Waterborne Inc., New Orleans, Louisiana, USA) or *Cryptosporidium* (Crypt-o-Glo™, Waterborne Inc., New Orleans, Louisiana, USA) was used to completely cover one of the two rings. Slides were then incubated in a humidity chamber (sealed plastic container containing wet cloths) at 37°C for 45 minutes. After incubation, the slides were allowed to air-dry and a 22 x 22 mm coverslip was mounted to each half using glycerol (Aqua-polymount, Polysciences, Warrington, Pennsylvania, USA). Once dry, all slides were stored in low-light areas until which time they were viewed under the microscope.

Table 2.3 Formulations for Phosphate Buffered Saline (PBS), Sucrose Solution, TAE buffer, and Tsg polymerase 10x buffer.

Phosphate Buffered Saline (PBS)
10.0 g NaCl
0.25 g KCl
1.44 g $Na_2HPO_4 \bullet 12H_2O$
0.25 g KH_2PO_4
dH_2O to 1.0 litre; pH 7.2

Sucrose Solution (1.13 s. g.)
1000 ml PBS
342.2 g sucrose
5 ml Methylene Blue (for colour)

TAE buffer (50 X)
242 g tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA pH 8.0
dH_2O to 1.0 litre; pH 8.5

Tsg DNA Polymerase 10x buffer (Biobasic Inc., Scarborough, Ontario, Canada)
500 mM KCl
100 mM Tris HCl (pH 9.0) at 25°C
1% Triton X-100 Buffer

Microscopic analysis was performed using an epifluorescence microscope (Nikon Labophot, Nikon Canada Inc., Mississauga, Ontario, Canada). The *Giardia* half of the slide was viewed under 100X, and the *Cryptosporidium* half was viewed under 400X. *C. parvum*-like oocysts were those that were between 3 and 5 μm in length, and *C. muris*-like oocysts were those that were between 5 and 9 μm in length. Parasites if present, were enumerated by counting the total number of cysts (*Giardia*) or oocysts (*Cryptosporidium*) over the spotted area. The parasite concentration per gram or milliliter of sample was calculated by dividing the total number observed by 0.015 ml and by the weight or volume of the original sample. This value was recorded for each sample analyzed. The sensitivity of this method for the analysis of one gram or one milliliter of sample has been shown to be approximately 67 parasites (Olson *et al.*, 1997c).

2.4 Viability Testing of Positive Sewage Influent

Parasite viability testing was performed only on sewage samples found to contain either *Giardia* or *Cryptosporidium*. Analysis was started immediately following positive identification as described above.

2.4.1 *Giardia* positive samples

Viability analysis of *Giardia* positive sewage influent samples was done according to previously described methods (Taghi-Kilani *et al.*, 1996). This method involved the use of a LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes, Eugene, Oregon, USA). The manufacturer's instructions of mixing equal volumes of component A with component B were followed, and subsequently 0.003 ml of this mixture was added to 0.097 of each positive sample. Following incubation in the dark at 37°C for one hour, 0.01 ml of the sample was trapped between a standard glass

slide (VWR Scientific Inc., Media, Pennsylvania, USA) and an 18 x 18 mm glass coverslip (VWR Scientific Inc., Media, Pennsylvania, USA). The slide was observed with an epifluorescence microscope. Cysts were deemed viable if they stained dark green, and were considered dead or nonviable if they stained light green or orange/yellow. The number of viable versus the number of nonviable cysts observed over the spotted area were counted, and the percentage of viable *Giardia* was determined, and recorded for each sample.

2.4.2 *Cryptosporidium* positive samples

To differentiate between viable and nonviable *Cryptosporidium* oocysts, 0.01 ml of 0.5 mg propidium iodide (Sigma Chemical Co., St. Louis, Missouri, USA) in 50 ml PBS was added to 0.1 ml of the suspended sample pellet (Campbell *et al.*, 1992). This preparation was allowed to incubate at 37°C for 10 minutes. Following incubation, 0.015 ml was dried and fixed onto an immunofluorescent slide, and stained with monoclonal antibody as described above in section 2.3. The slide was visualized using the fluorescein filter of the epifluorescence microscope. Once *Cryptosporidium* oocysts were identified, the fluorescein filter was removed and the rhodamine filter was used. Oocysts appearing red in colour were deemed nonviable, and those that became invisible or black in appearance were viable. The number of viable versus the number of nonviable oocysts observed over the spotted area were counted, and the percentage of viable *Cryptosporidium* was determined, and recorded for each sample.

2.5 Molecular Characterization of *Cryptosporidium*

2.5.1 Sources of Cryptosporidium isolates

The sources of *Cryptosporidium* isolates used for the proceeding molecular characterization study are listed in Table 2.4. All isolates were derived from fecal samples collected directly from their host of origin.

2.5.2 Ficoll purification

Following identification, *Cryptosporidium* positive samples were further purified using a ficoll (Sigma Chemical Co., St. Louis, Missouri, USA) and sodium diatrizoate (Sigma Chemical Co., St. Louis, Missouri, USA) gradient separation and purification technique (Lumb *et al.*, 1988). A discontinuous gradient consisting of 1 ml volumes of 0.5, 1.0, 2.0, and 4.0% ficoll in PBS containing 16% sodium diatrizoate was prepared by layering each volume into a 15 ml polypropylene centrifuge tube (Becton-Dickinson Labware, Franklin Lakes, New Jersey, USA). Layering began with the high percentage (4.0%) gradient and ended with the lowest percentage (0.5%) layer on top. This gradient was spun at 1500 x g for 10 minutes in a refrigerated swinging-bucket centrifuge (Centra model 7-R, Damon International Equipment Co., Needham Heights, Massachusetts, USA). The 1 ml sample suspension was layered on top of the complete gradient and centrifuged at 1500 x g for one hour. Oocysts were harvested from between the 0.5% and 1.0% ficoll layers, washed three times in PBS, and sterilized with 10% Javex bleach (Colgate-Palmolive Canada Inc., Toronto, Ontario, Canada) for 10 minutes at 4°C. The resulting, clean, 1 ml sample was maintained in PBS at 4°C until time for DNA extraction.

Table 2.4 Sources of *Cryptosporidium* isolates used for molecular characterization.

Code	Host	Species	Genotype	Origin	Source
Ca-1	calf	*C. parvum*	calf	Drayton Valley	University of Calgary
St-1	steer 356	*C. andersoni*	N/A	Calgary, AB	University of Calgary
Hu-1	human	*C. parvum*	human	Calgary, AB	Provincial lab
Hu-2	human	*C. parvum*	human	Calgary, AB	Provincial lab
Cat-1	kitten	*C. felis*	N/A	Calgary, AB	University of Calgary
Pig-1	pig	*C. parvum*	pig	Central Alberta	University of Calgary
Pig-2	pig	*C. parvum*	pig	Central Alberta	University of Calgary

2.5.3 DNA extraction

The extraction of any *Cryptosporidium* DNA present in the sample preparation was done using a modified version of the technique described by Morgan *et al.* (1995). The entire 1 ml sample was resuspended in 0.08 ml ATL Tissue Lysis Buffer (Qiagen Inc., Mississauga, Ontario, Canada) and boiled for 40 minutes. This aided in the degradation of the oocyst and released any DNA present. The sample was allowed to cool before 0.285 ml AL Binding Buffer (Qiagen Inc., Mississauga, Ontario, Canada) was added, and the sample was gently agitated. A subsequent addition of 0.01 ml Prep-A-Gene Matrix (BioRad Laboratories, Hercules, California, USA) to bind DNA, followed by 20 minutes of mixing occurred before the sample was spun at 15 000 rpm in a desktop microcentrifuge (Biofuge 15, Heraeus Instruments, Germany) for two minutes. Two wash steps followed whereby the sample was mixed with 0.25 ml AW Wash Buffer (Qiagen Inc., Mississauga, Ontario, Canada) and spun at 15 000 rpm for one minute. Washing was necessary to remove any protein or debris present, and to yield a clean and pure DNA sample. The supernatant was removed, the sample vacuum dried (SpeedVac Concentrator, Savant Instruments Inc., Hicksville, New York, USA), and incubated at 60°C for 10 minutes with 0.05 ml AE Elution buffer (Qiagen Inc., Mississauga, Ontario, Canada). Following incubation, the sample was spun once more at 15 000 rpm, and the supernatant containing the eluted DNA was retained.

2.5.4 ITS rDNA PCR amplification

All primers were designed from available rDNA sequence data of the KSU-1 isolate (Genebank accession numbers: AF040725). Two sets of primers were developed and used in a nested PCR reaction. Both forward primers were located in the conserved 18S region of the rDNA and both reverse primers were located in the conserved 5.8S region. The outer primer set (primary reaction), designated F1-18S (5' GTCTGTGATGC

CCTTAGATTCC 3') and R1-5.8S (5' CTGCTATTTGCGTTGAGAGATCTG 3') amplified a region approximately 992 base pairs in length. The internal primer set (secondary reaction), designated F2-18S (5' TTGAATATGCATCGTGATGGG 3') and R2-5.8S amplified a region ranging in size from 958 to 714 base pairs in length depending on the isolate present. The resulting amplicon from both the primary and secondary PCR reaction amplified a fragment encompassing the end of the 18S, the entire ITS1 and the beginning of the 5.8S rDNA regions.

Both the primary and secondary PCR reaction mixtures contained the same ingredients. Each 0.025 reaction mixture consisted of 200 M of each dNTP (Gibco BRL®, Frederick, Maryland, USA), 1 x PCR buffer (BioBasic Inc., Scarborough, Ontario, Canada) (Table 2.3), 1 unit of Tsg DNA Polymerase (BioBasic Inc., Scarborough, Ontario, Canada), 1.5 mM $MgCl_2$, and 80 ng of each forward and reverse primer. The volume of template DNA added to the primary reaction equaled 0.002 ml such that the total reaction volume was 0.025 ml. A 0.005 ml volume of the completed primary reaction was added as template to the secondary, and the total reaction volume again was 0.025 ml. Primary and secondary DNA amplification was carried out for 35 cycles, each consisting of denaturing (94°C, 30 seconds), annealing (58°C, 60 seconds), and elongating (72°C, 90 seconds), with an initial hot start of 94°C for 2 minutes, and a final extension of 7 minutes at 72°C. Reactions occurred in a Perkin Elmer Gene Amp PCR 2400 thermocycler (Perkin Elmer Applied BioSystems Division, Foster City, California, USA). Each experiment contained one negative control (reaction mixture without template DNA).

2.5.5 18S rDNA PCR amplification

A nested set of primers were designed (Morgan, unpublished) to amplify a 798 base pair region within the 18S rDNA that through sequencing would characterize

specific isolates of *Cryptosporidium*. The outer set of primers consisted of the forward primer, 18S BEG (5′ ACCTGGTTGATCCTGCCAGTAGTC 3′), and the reverse primer, $18SiR_3B$ (5′ TTAACAAATCTAAGAATTTCACC 3′), and the secondary reaction consisted of the internal forward primer, 18S STA (5′ AGATTAAGCCATGCATG TCTAAG 3′) and the internal reverse primer, 18SiR3A (5′ CCAACTGTCCCTATTAA TCAT 3′) (Morgan, unpublished). The reaction conditions for both the primary and secondary reaction are the same as what was previously described in section 2.5.4.

2.5.6 Gel electrophoresis

Following PCR amplification, all reactions were fractionated on a 2.0% agarose gel containing 1.5 g agarose (Gibco BRL, Frederick, Maryland, USA), and 0.75 L 1 x TAE buffer (Table 2.3). Gel electrophoresis at 85 Volts for 1.5 hours was performed using a SubCell tank (BioRad Laboratories, Hercules, California, USA) and immersing the gel in 1 x TAE buffer. The gel was visualized by 10 μg/ml ethidium bromide (Sigma Chemical Co., St. Louis, Missouri, USA) staining as fluorescent bands under ultra-violet light illumination (FotoPrep®, Fotodyne Inc., New Berlin, Wisconsin, USA) at the anticipated molecular size.

2.5.7 RFLP analysis of ITS amplicon

To differentiate between isolates amplified with the ITS reaction, restriction fragment length polymorphism (RFLP) was performed using the restriction enzyme, *Dra I* (New England Biolabs Inc., Beverly, Massachusetts, USA). The digestion reaction contained 40 units of *Dra I*, 0.0004 ml bovine serum albumin (BSA) (New England Biolabs Inc., Beverly, Massachusetts, USA), 0.004 ml 10 x buffer (New England Biolabs Inc., Beverly, Massachusetts, USA), and 0.015 ml of the PCR product as template. This

reaction mixture was incubated at 37°C overnight. The digested product was fractionated on a 3.5% MetaPhor agarose (FMC® BioProducts, Rockland, Maine, USA) gel, electrophoresed at 60 Volts for 2.5 hours. Following electrophoresis, the gel was stained with ethidium bromide, and visualized using an ultra-violet transilluminator. Expected restriction product sizes are described in Table 2.5.

2.5.8 Sequencing of 18S amplicon

Following gel electrophoresis and visualization, all bands present were cut out of the gel and purified using the QIAquick Gel Extraction Kit (Qiagen Inc., Mississauga, Ontario, Canada). This purified product was used as template in the dRhodomine Terminator Cycle Sequencing Kit reaction (Perkin-Elmer Applied BioSystems Division, Foster City, California, USA). For each isolate, both a forward and reverse sequencing reaction was set up. The following reagents were added to each reaction; 0.004 ml Terminator Ready Reaction Mix, 0.005 ml template, and 0.001 ml 20 ng of either the forward or reverse primer. A Perkin-Elmer Gene Amp PCR 2400 thermocycler was used for the reaction, where 25 cycles of denaturing (96°C for 10 seconds), annealing (50°C for 5 seconds), and elongating (60°C for 4 minutes) occurred. Following the PCR reaction, all extension products were purified. A 1.5 ml microcentrifuge tube containing 0.002 ml 3 M sodium acetate (pH 4.6) and 0.05 ml 95% ethanol was prepared for each reaction. The entire reaction was then added to this tube, and placed on ice for 10 minutes. Following this precipitation step, the tubes were centrifuged at 15 000 rpm for 30 minutes, and the supernatant was aspirated and discarded. The tubes were spun once more to remove any excess supernatant, and the final pellet was vacuum dried for 15 minutes. This preparation was then sent to UC*DNA* services (University of Calgary, Canada) where sequencing was carried out using an ABI 377 Automated Sequencer.

Table 2.5 Expected amplicon sizes and restriction products for ITS region of all *Cryptosporidium* isolates tested.

Host	Genotype or *species*	Size of PCR product (bp)	Expected number and size of fragments (bp)
calf	calf	847	598, 249
human	human	884	348, 174, 132, 126, 104
steer	*C. andersoni*	~ 650	~360, ~160, ~130
cat	*C. felis*	714	617, 97
pig	pig	958	670, 288

2.6 Statistical Analysis

Statistical analysis of the parasite prevalence data was completed using the statistical software package STATA (Stata Press, College Station, Texas, USA). Based on assumptions made with regards to the data collected, it was determined that an analysis of variance (ANOVA) model could not be used for interpretation. This was due to many factors including the non-normally distributed dependent variable, the unequal number of cases in the design, and the underlying factor that the data collected were not experimental. Likewise, ordinary least squares regression could not be used because it was assumed that the error was not normal in distribution. It was assumed that the data collected more closely resembled a Poisson distribution (Figure 2.3), however with the Poisson regression model the mean must equal the variance. Based on the calculated alpha value (used to measure the extent of variation in the distribution of the dependent variable) it became clear that this assumption was not reasonable for these data due to a greater variation in distribution than expected. This indicated the presence of a negative binomial distribution. The negative binomial regression model does not require this underlying assumption. Finally, the distribution of data most closely represented that shown in Figure 2.3. This was due to an excessive number of cases without parasites, indicating that the model must be adjusted for these inflated zero values (Long, 1997). Therefore, interactions between the various factors present in farm animal, wildlife, and sewage data were compared using negative binomial regression for inflated zeros. In this manner, it was possible to determine those interactions more significant with respect to parasite prevalence, and sources most important with respect to parasite presence in the environment.

Sequence comparison and alignment was conducted using Clustal X Multiple Sequence Alignment Program, version 1.63b (EMBL Data Library, Heidelberg, Germany).

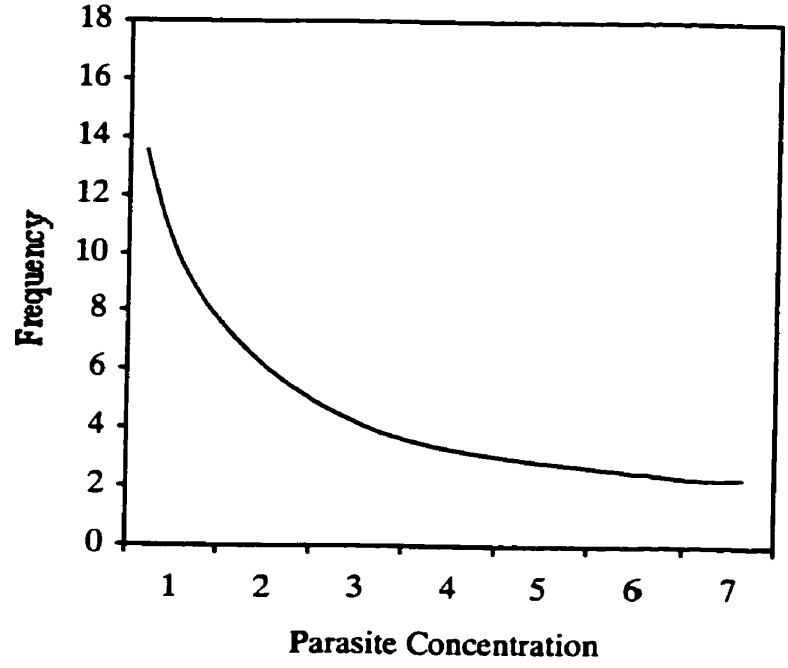
Figure 2.3

Data distribution models.

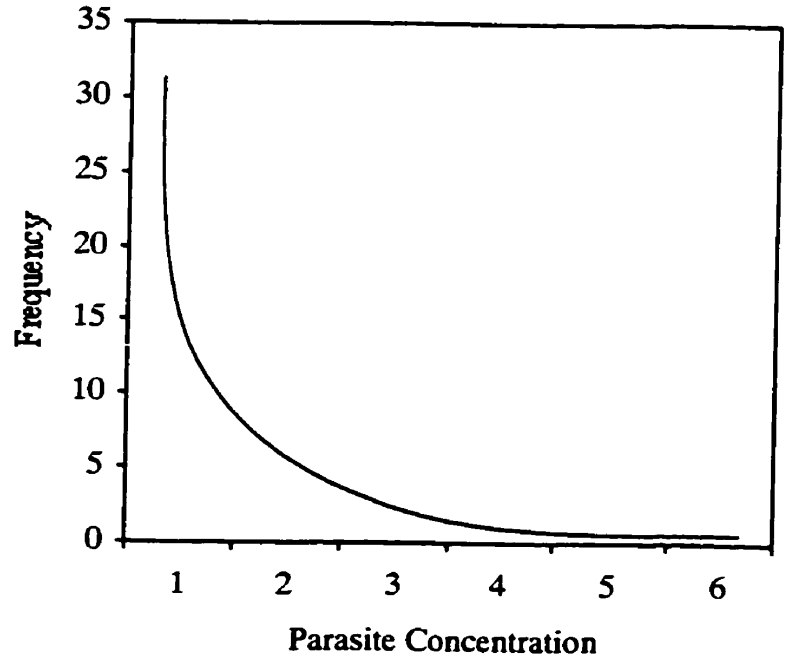
A. Typical Poisson regression distribution.

B. Skewed distribution with inflated zeros.

A


18
16
14
12
10
8
6
4
2
0
Frequency
1
2
3
4
5
6
7
Parasite Concentration

B


35
30
25
20
15
10
5
0
Frequency
1
2
3
4
5
6
Parasite Concentration

3 Results

3.1 Parasite Prevalence

Giardia and *Cryptosporidium* were isolated from all sources tested. The microscopic analysis for all agriculture, wildlife, and sewage influent samples for *Giardia* cysts, *C. parvum*-like oocysts, and *C. muris*-like oocysts are summarized in Table 3.1. Sewage influent had the highest prevalence of *G. duodenalis* (48.80%), *C. parvum*-like oocysts (5.42%), and *C. muris*-like oocysts (4.82%). *Giardia* and *Cryptosporidium* were least prevalent in wildlife scat samples, where 2.25% contained *G. duodenalis* and 0.36% contained *C. parvum*-like oocysts. *C. muris*-like oocysts were not detected in any wildlife samples collected. Parasites in pooled agriculture samples were less prevalent than in sewage influent and were more prevalent than in wildlife samples. *G. duodenalis* was detected in 29.07%, while *C. parvum*-like oocyts (4.25%) and *C. muris*-like oocysts (0.20%) were detected less frequently.

3.1.1 Sewage Influent

The prevalence of both *Giardia* and *Cryptosporidium* in sewage influent was found to vary from facility to facility from June 1998 to January 2000 (Table 3.2). *Giardia* was detected at all facilities except Tomahawk School. The Calmar, Edmonton-Goldbar, Warburg, and Devon facilities were most prevalent for *Giardia*. *C. parvum*-like oocysts were not detected at many facilities. These included the Rocky Rapids, Buckcreek, Alderflats, Tomahawk, Tomahawk School, Calmar, Devon, Rocky Mountain House, Thorsby, and Edmonton-Goldbar facilities. Oocysts resembling the size of *C. muris* were found in sewage influent samples from rocky Rapids, Breton, Warburg, Rocky Mountain House, and Thorsby. The mean concentration of parasites detected for

Table 3.1 Summary of agriculture, wildlife, and sewage influent microscopic analysis for *G..duodenalis*, *C. parvum*-like oocysts, and *C. muris*-like oocysts.

Sample Type	Total no.	*G. duodenalis*-positive samples		*C. parvum*-like-positive samples		*C. muris*-like-positive samples	
		No.	%	No.	%	No.	%
Agriculture	1531	445	29.07	65	4.25	3	0.20
Wildlife	1686	38	2.25	6	0.36	0	0
Sewage Influent	166	81	48.80	9	5.42	8	4.82
Total no.	**3383**						

Table 3.2 The prevalence and mean parasite concentration of *G. duodenalis*, *C. parvum*-like oocysts, and *C. muris*-like oocysts in sewage influent samples collected from each of 16 wastewater facilities in the NSR basin.

Facility	sample size	*G. duodenalis*		*C. parvum-like*		*C. muris-like*	
		%	conc. (cysts/ml)	%	conc. (oocysts/ml)	%	conc. (oocysts/ml)
Rocky Rapids	11	18.18	0.22	0	0	18.18	62.3
Violet Grove	11	27.27	0.16	9.09	0.03	0	0
Buck Creek	10	30	0.42	0	0	0	0
Birchwood Village	10	50	0.70	10	0.13	0	0
Breton	11	54.55	0.39	18.18	0.07	9.09	0.12
Sunnybrook	10	10	0.17	20	1.78	0	0
Warburg	14	78.57	3.98	7.14	0.04	14.29	0.03
Alderflats	10	60	1.47	0	0	0	0
Tomahawk	12	33.33	5.27	0	0	0	0
Tomahawk School	10	0	0	0	0	0	0
Calmar	9	88.89	2.21	0	0	0	0
Devon	9	77.78	0.88	0	0	0	0
Drayton Valley	11	63.64	0.93	18.18	0.22	0	0
Rocky Mountain House	9	66.67	0.70	0	0	11.11	2.07
Thorsby	11	45.45	0.40	0	0	18.18	0.17
Edmonton-Goldbar	8	87.5	1.26	0	0	0	0

each facility remained less than 10 parasites per 1 ml of sewage influent. This however, was not the case for *C. muris* -like oocysts detected at the Rocky Rapids facility, where 62.3 oocysts per ml were calculated.

Monthly variation in parasite prevalence was also recorded during the sampling period from June 1998 to January 2000. *Giardia* was detected most often in the late fall, and *Cryptosporidium* was mostly detected in the spring and summer months (Figure 3.1). Months with high prevalence did not correspond to months with high parasite concentrations. The highest concentration of *Giardia* was found in March 1999 (3.36 cysts/ml) and the lowest concentration was found in June 1998 (0.43 cysts/ml). Samples collected during May 1999 contained the highest concentration of both *C. parvum*-like oocysts (0.11 oocysts/ml) and *C. muris*-like oocysts (41.1 oocysts/ml).

3.1.2 Agriculture

The prevalence of *G. duodenalis*, *C. parvum*-like oocysts, and *C. muris*-like oocysts varied between cow-calf operation and between year of sampling (Figure 3.2). *Giardia* prevalence ranged from less than 10% (operation 4) to 60% (operation 10). *Cryptosporidium* was found less often. *C. parvum*-like oocysts were detected at 6 of the 11 operations with prevalence values as high as 12%. *C. muris*-like oocysts were only detected at two operations, both in low prevalence. The concentration of *Giardia* found in cow-calf samples ranged from 39 cysts/g to greater than 17 000 cysts/g. In contrast, *Cryptosporidium* found in cow-calf samples was observed in much lower concentrations. For *C. parvum*- and *C. muris*-like oocysts, the maximum concentration calculated was 906 oocysts/g and 180 oocysts/g respectively. Yearly variation in both parasite prevalence and concentration was also observed. Prevalence and parasite concentrations were higher in 1999 than they were in 1998. The average *Giardia* prevalence for the total 1998 operations (n=893) was 20%, as compared to 45% which was observed in the

Figure 3.1

Prevalence of *G. duodenalis* cysts (■), *C. parvum*-like oocysts (■), and *C. muris*-like oocysts (□) in sewage influent collected each month from June 1998 to January 2000. Numbers in parentheses above prevalence bars represent the mean parasite concentration per ml of sewage influent.

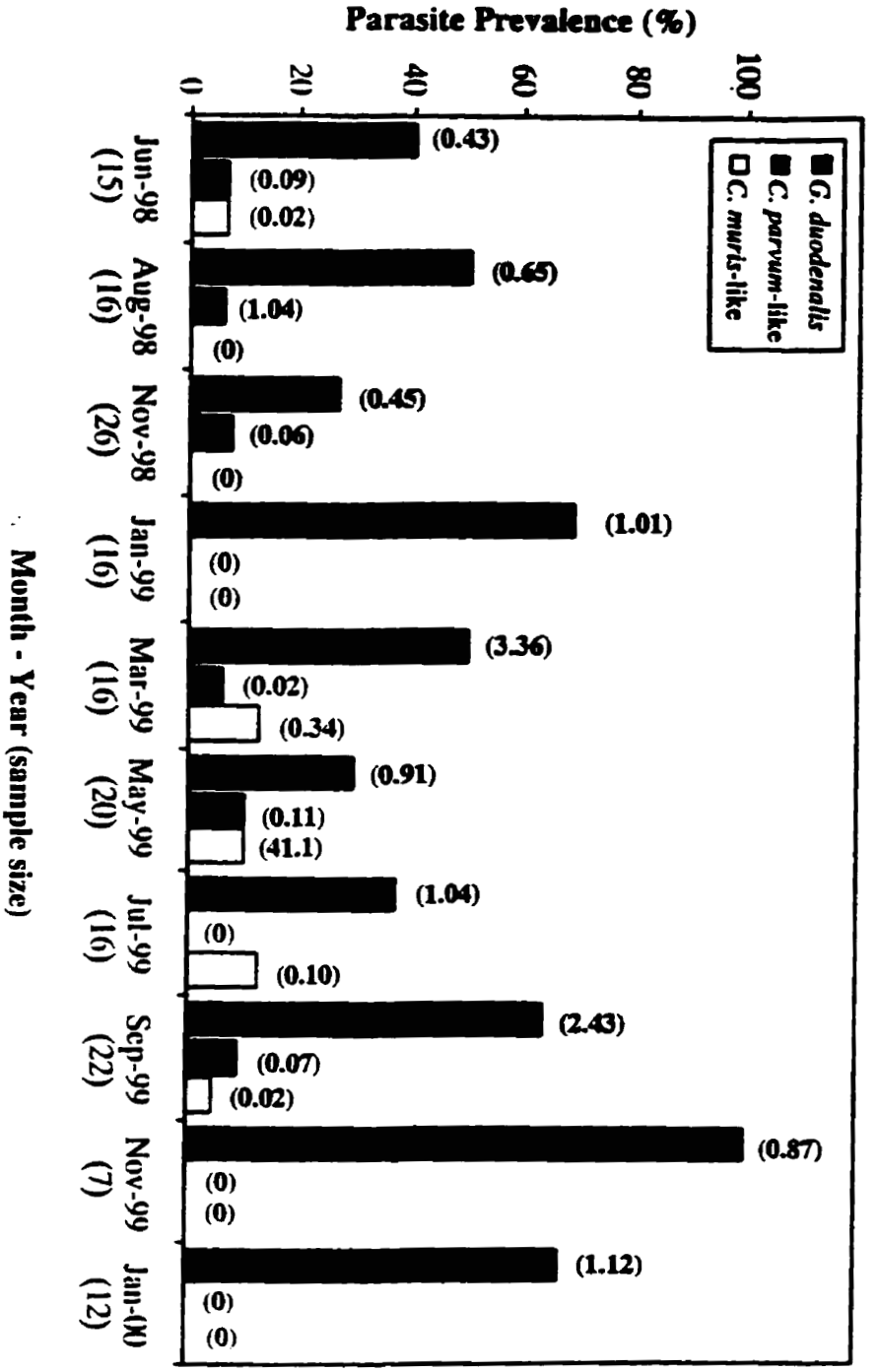
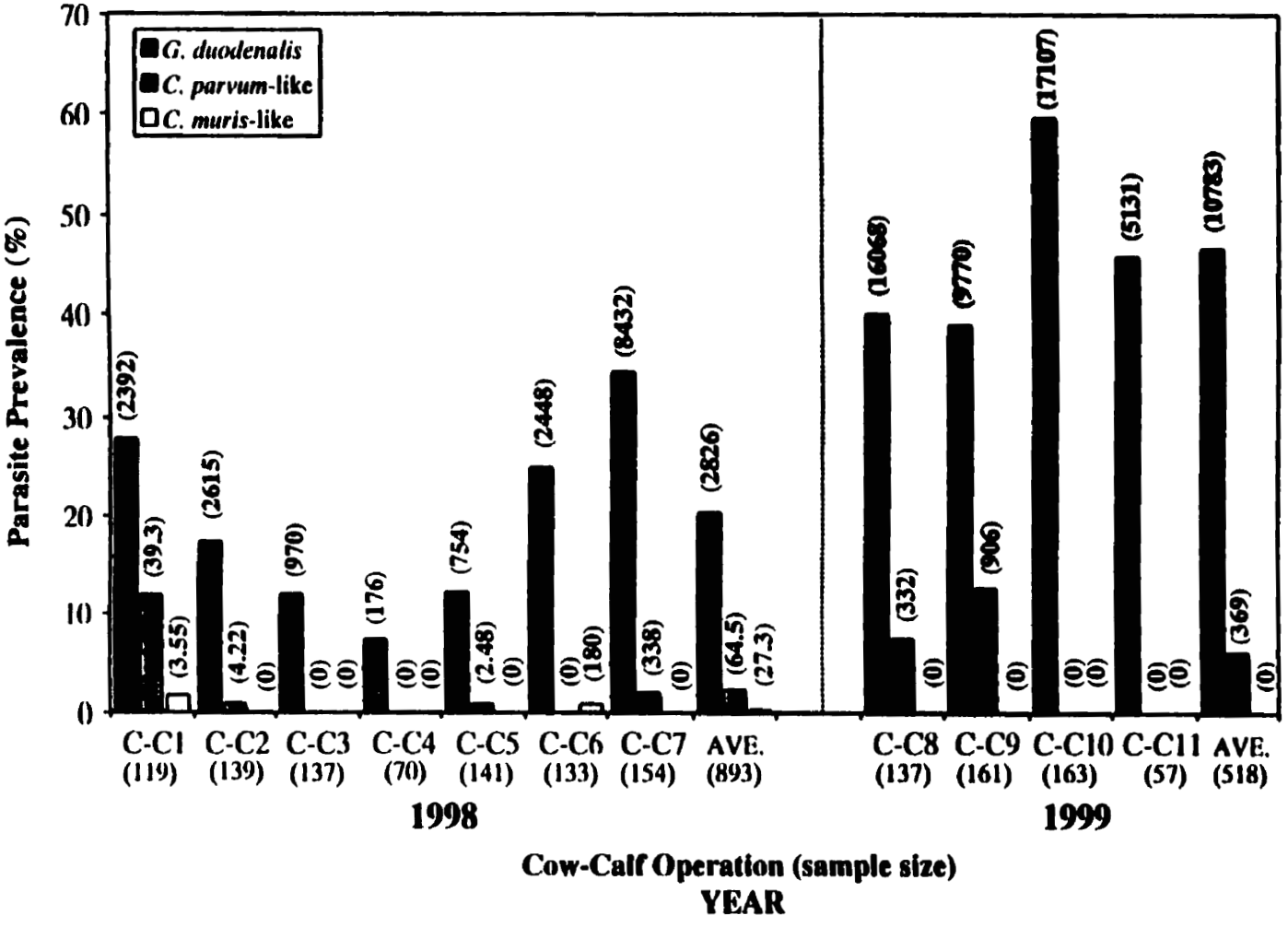

Parasite Prevalence (%)
0
20
40
60
80
100
G. duodenalis
C. parvum-like
C. muris-like
Jun-98 (15)
(0.43)
(0.09)
(0.02)
Aug-98 (16)
(0.65)
(1.04)
(0)
Nov-98 (26)
(0.45)
(0.06)
(0)
Jan-99 (16)
(1.01)
(0)
(0)
Mar-99 (16)
(3.36)
(0.02)
(0.34)
May-99 (20)
(0.91)
(0.11)
(41.1)
Jul-99 (16)
(1.04)
(0)
(0.10)
Sep-99 (22)
(2.43)
(0.07)
(0.02)
Nov-99 (7)
(0.87)
(0)
(0)
Jan-00 (12)
(1.12)
(0)
(0)
Month - Year (sample size)

Figure 3.2

Prevalence of *G. duodenalis* cysts (■), *C. parvum*-like oocysts (■), and *C. muris*-like oocysts (□) for individual cow-calf operations sampled during the spring of 1998 (n=7) and during the spring of 1999 (n=4). The total prevalence for each year is also reported. Numbers in parentheses above each prevalence bar represent the mean parasite concentration detected per gram of feces. Cow-calf operations (C-C) are numbered 1 through 11.


G. duodenalis
C. parvum-like
C. muris-like
Parasite Prevalence (%)
70
60
50
40
30
20
10
0
(2392)
(39.3)
(3.55)
(2615)
(4.22)
(0)
(970)
(0)
(0)
(176)
(0)
(0)
(754)
(2.48)
(0)
(2448)
(0)
(180)
(8432)
(338)
(0)
(2826)
(64.5)
(27.3)
(16068)
(332)
(0)
(9770)
(906)
(0)
(17107)
(0)
(0)
(5131)
(0)
(0)
(10783)
(369)
(0)
C-C1 (119)
C-C2 (139)
C-C3 (137)
C-C4 (70)
C-C5 (141)
C-C6 (133)
C-C7 (154)
AVE. (893)
C-C8 (137)
C-C9 (161)
C-C10 (163)
C-C11 (57)
AVE. (518)
1998
1999
Cow-Calf Operation (sample size)
YEAR

total 1999 operations (n=518). The concentration of *Giardia* found in 1999 (10783 cysts/g) was almost 4 times greater than the concentration observed in 1998 (2826 cysts/g). *C. parvum* like oocysts were also more prevalent in the 1999 samples (5%) as compared to the 1998 samples (2%). The yearly difference in *C. parvum* concentration was greater than for *Giardia*, as 369 oocysts/g were detected on average in 1999 and only 64.5 oocysts/g were detected on average in 1998. *C. muris*-like oocysts were not detected in any 1999 cow-calf samples. The average concentration for those samples found to be positive in 1998 was 27.3 oocysts/g feces.

G. duodenalis and *C. parvum*-like oocysts were both detected in dairy operations, however only *Giardia* was found in the hog operation. No oocysts resembling *C. muris* were observed in samples collected from either dairy. Figure 3.3 summarizes the prevalence of those parasites found in samples collected from both dairies, and the one hog operation tested. *Giardia* was less than 20% prevalent in the 40 hog samples collected. A higher *G. duodenalis* prevalence (>20%) was observed at dairy 1 as compared to the prevalence observed at dairy 2 (<10%). Likewise, *C. parvum*-like oocysts were observed in a higher percentage of samples from diary 1 (>25%) than from dairy 2 (5%). The concentration of both *Giardia* and *C. parvum*-like oocysts corresponded to the prevalence observed. Dairy 1 had higher *Giardia* and *Cryptosporidium* concentrations than dairy 2.

3.1.3 Wildlife

Of 1686 wildlife scat samples collected, *Giardia* was found in 2.25% and *C. parvum*-like oocysts were detected in only 0.36% (Table 3.3). *C. muris*-like oocysts were not observed in any sample. *Giardia* cysts were present in 5 of the 18 species sampled. These included deer, moose, coyote, beaver and muskrat. *Cryptosporidium* was only observed in deer and beaver samples. Of the species tested, beaver and muskrat had the highest prevalence and concentration of *Giardia*. Beaver samples were 15% positive for

Figure 3.3

Prevalence of *G. duodenalis* cysts (■), *C. parvum*-like oocysts (■), and *C. muris*-like oocysts (□) in one hog and two dairy operations sampled winter 2000. Numbers in parentheses above each prevalence bar represent the mean parasite concentration per gram feces processed.

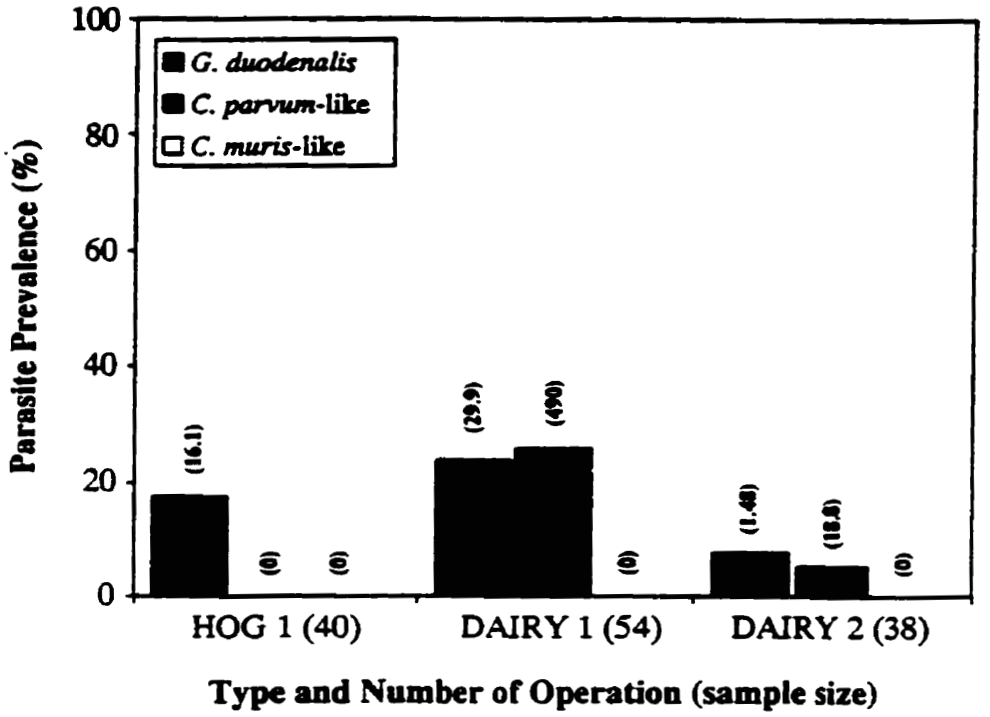

100
80
60
40
20
0
Parasite Prevalence (%)
G. duodenalis
C. parvum-like
C. muris-like
(16.1)
(0)
(0)
(29.9)
(49.0)
(0)
(1.4)
(18.8)
(0)
HOG 1 (40)
DAIRY 1 (54)
DAIRY 2 (38)
Type and Number of Operation (sample size)

Table 3.3 Prevalence and mean concentration of *G. duodenalis* cysts and *C. parvum*-like oocysts in wildlife samples collected during the early spring of 1998 and fall of 1999.

Animal	Sample Size	*Giardia*-positive samples			*C. parvum*-positive samples		
		No.	%	Conc.* (cysts/ml)	No.	%	Conc.* (oocysts/ml)
Lynx	1	0	0	0	0	0	0
Marten	4	0	0	0	0	0	0
Fisher	3	0	0	0	0	0	0
Porcupine	8	0	0	0	0	0	0
Horse	1	0	0	0	0	0	0
Deer	649	1	0.15	22963	1	0.15	238
Moose	177	1	0.56	903	0	0	0
Elk	34	0	0	0	0	0	0
Coyote	99	5	5.05	946	0	0	0
Wolf	2	0	0	0	0	0	0
Hare	453	0	0	0	0	0	0
Snow Goose	20	0	0	0	0	0	0
Canada Goose	57	0	0	0	0	0	0
Grouse	50	0	0	0	0	0	0
Squirrel	15	0	0	0	0	0	0
Mouse	2	0	0	0	0	0	0
Beaver	87	13	14.94	3800	5	5.75	10
Muskrat	23	18	78.26	12233	0	0	0
Total Prevalence (average)	**1686**	**38**	**2.25**	**7847**	**6**	**0.36**	**48**

* mean concentration of positive samples only

Giardia while muskrat samples were 78% positive. The concentration of *Giardia* was highest in positive muskrat (9574 cysts/g) samples. *C. parvum*-like oocysts were rarely observed, and the concentration in positive samples was minimal.

3.2 Sewage Viability

Viability analysis of sewage influent samples revealed large percentages of viable parasites at each facility (Figure 3.4). The viability of *Giardia* ranged from 60% at the Warburg facility to less than 5% at Rocky Rapids. Viable *C. parvum*-like oocysts were only documented at the Breton and Sunnybrook facilities, both less than 10%. *C. muris*-like oocysts were found to be viable at the Rocky Rapids, Rocky Mountain House, and Thorsby facilities. Viability analysis per month of sample collection demonstrated high percentages of viable *Giardia* cysts during the fall of 1999 extending into the winter of 2000 (Figure 3.5). This data is incomplete, as viability analysis was not done on samples collected during November 1998 nor on those collected during July 1999. This was due to samples arriving frozen or to samples arriving more than 4 weeks after their collection.

3.3 Source Statistical Analysis

Comparison of mean parasite concentration between agriculture (cow-calf, dairy, and hog), wildlife, and sewage resulted in agriculture with a significantly high mean parasite concentration for both *G. duodenalis* (β=8.322131; $p \leq 0.01$) and *C. parvum*-like oocysts (β=7.161878; $p \leq 0.01$). The mean *Giardia* concentration in wildlife was highly significant (β=4.922342; $p \leq 0.05$). The mean parasite concentration of sewage influent was highly non-significant for all parasites. No source was found to have a significant *C. muris*-like oocyst concentration.

Figure 3.4

The percent of viable *G. duodenalis* cysts (■), *C. parvum*-like oocysts (■), and *C. muris*-like oocysts (□) observed in sewage influent samples collected from sixteen sewage treatment facilities in the NSR basin. Abbreviations: RR, Rocky Rapids; VG, Violet Grove; BC, Buck Creek; BV, Birchwood Village; BR, Breton; SB, Sunnybrook; WA, Warburg; AF, Alderflats; TK, Tomahawk; TKS, Tomahawk School; CA, Calmar; DE, Devon; DV, Drayton Valley; RMH, Rocky Mountain House; TB, Thorsby; EG, Edmonton-Goldbar. Note: no parasites were detected in any of the 10 samples collected from the Tomahawk School Facility, therefore viability = 0%.

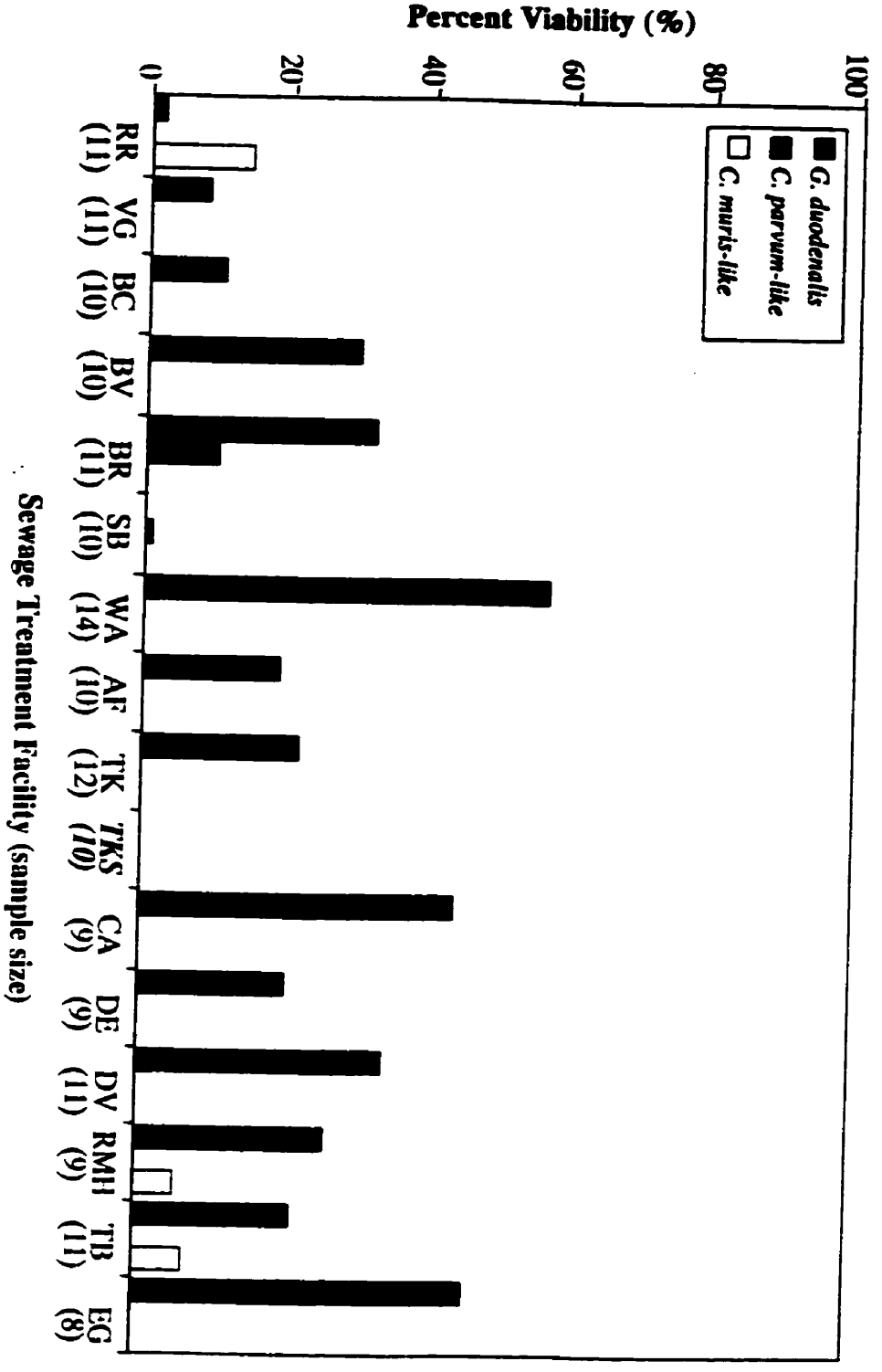
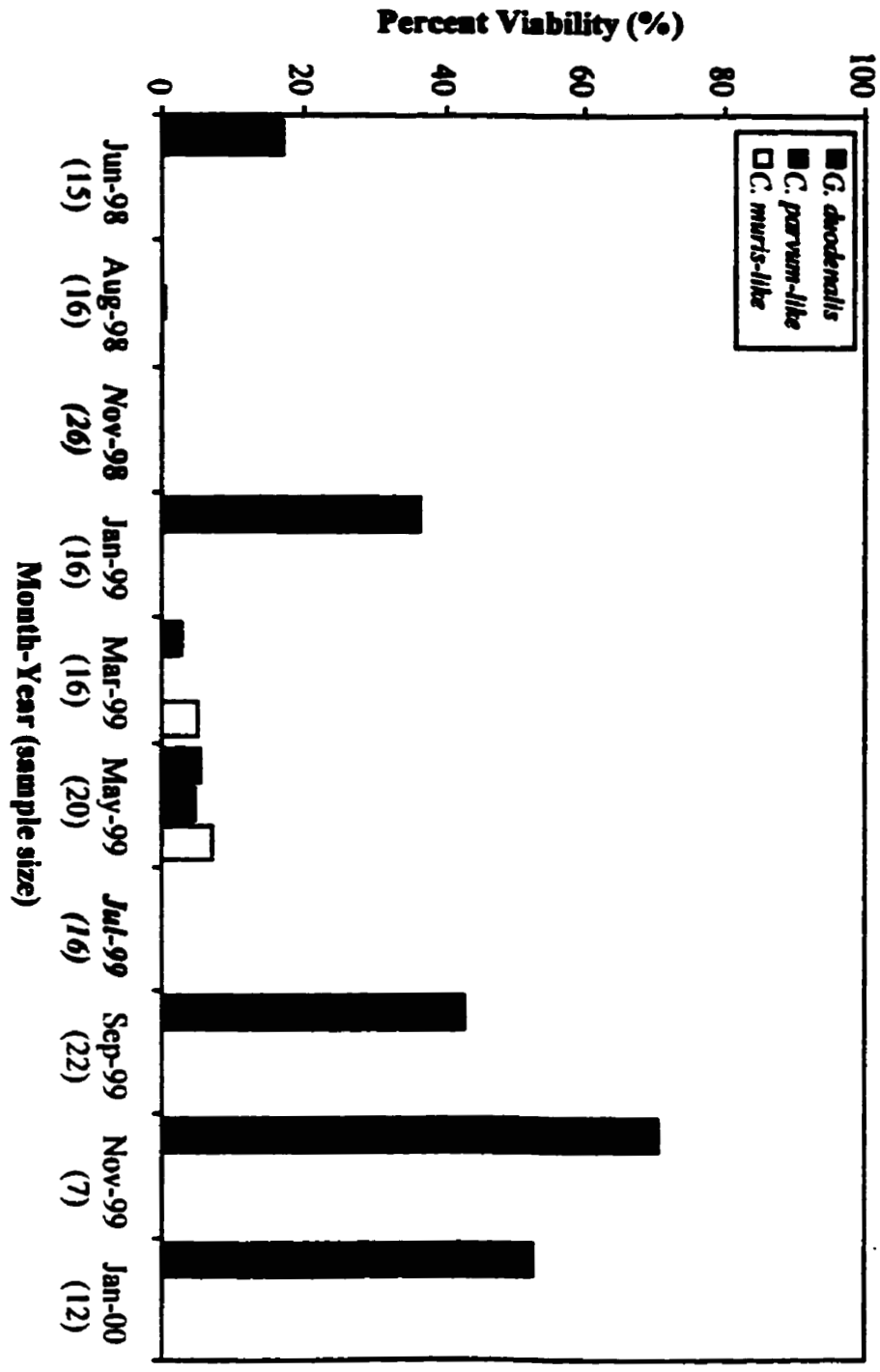

Percent Viability (%)
0
20
40
60
80
100
G. duodenalis
C. parvum-like
C. muris-like
RR (11)
VG (11)
BC (10)
BV (10)
BR (11)
SB (10)
WA (14)
AF (10)
TK (12)
TKS (10)
CA (9)
DE (9)
DV (11)
RMH (9)
TB (11)
EG (8)
Sewage Treatment Facility (sample size)

Figure 3.5

The percent of viable *G. duodenalis* cysts (■), *C. parvum*-like oocysts (■), and *C. muris*-like oocysts (□) observed in sewage influent samples collected bimonthy from June 1998 to January 2000. Note: viability analysis was not performed on samples collected during November 1998 nor on samples collected during July 1999.


Percent Viability (%)
0
20
40
60
80
100
G. duodenalis
C. parvum-like
C. muris-like
Jun-98 (15)
Aug-98 (16)
Nov-98 (26)
Jan-99 (16)
Mar-99 (16)
May-99 (20)
Jul-99 (16)
Sep-99 (22)
Nov-99 (7)
Jan-00 (12)
Month-Year (sample size)

3.3.1 Agriculture

Further analysis into agricultural sources revealed that cow-calf operations had the highest concentration of *G. duodenalis* (β=5.910057; $p \leq 0.01$) and dairy operations had the highest concentration of *C. parvum*-like oocysts (β=5.319539; $p \leq 0.01$). The mean *Giardia* concentration calculated for the one hog operation tested was non-significant. An in depth analysis of farm characteristics and management practices demonstrated the importance of herd water source, animal age, health, mortality, and proximity to NSR tributary as factors affecting the presence of *G. duodenalis* and/or *C. parvum*-like oocysts in cow-calf samples (Table 3.4). Calf samples were statistically more likely to contain both *Giardia* cysts and *C. parvum*-like oocyts than cow samples. Samples from scouring calves collected during the spring of 1998 were likely to contain both *Giardia* and *C. parvum*. In contrast, scour samples from 1999 calves were nonsignificant. Herd access to dugout or runoff water significantly contributed to the presence of *G. duodenalis* and *C. parvum*-like oocysts in 1998 and to *G. duodenalis* in 1999 cow-calf samples. Additionally, herd access to multiple water sources contributed to *Giardia* in 1998. The presence of a NSR tributary within 10 km from the 1998 cow-calf operations contributed to the likelihood of finding *C. parvum*-like oocysts. Finally, mortality was shown to be a significant factor for *G. duodenalis* infection in 1999 cow-calf samples.

Analyses of the factors affecting the presence of parasites in dairy samples was undertaken, the results of which are summarized in Table 3.5. This was limited as only two farms were sampled. Age however, was a significant factor for *Giardia* and *Cryptosporidium*. Samples from calves aged 5-10 weeks were likely to contain both *Giardia* and *Cryptosporidium*. In addition, samples from older calves, aged 10 to 15 weeks, were also more likely to contain *C. parvum*. Finally, herd size was shown to significantly affect the presence of *C. parvum*-like oocysts in dairy samples.

Table 3.4 Effect of individual farm characteristics and management practice on the concentration of *G. duodenalis* cysts and *C. parvum*-like oocysts in samples collected from cow-calf operations during the spring of 1998 (n=7) and 1999 (n=4).

Tested Effect	Regression Model Coefficient (β) (standard error)			
	1998		1999	
	G. duodenalis	*C. parvum*-like	*G. duodenalis*	*C. parvum*-like
Age of Animal	-6.948 ††	-9.350 ††	-8.704 ††	-7.669 ††
	(0.609)	(2.553)	(0.384)	(1.247)
Health	-1.394 †	-7.196 †	-1.145	-26.473
	(0.699)	(2.854)	(0.990)	(5677.285)
Herdsize	-0.388	2.639	-0.580 †	2.639
	(2.843)	(1.968)	(0.279)	(1.968)
Type of Water	-0.3670 *	-2.117 ††	0.787 †	26.28266
for Herd -*pooled*	(0.190)	(0.927)	(0.455)	(1540.920)
Well	0.510	-19.129	0.431	6.465
	(0.721)	(948.457)	(0.373)	(0.587)
Dugout/Runoff	1.021 †	4.397 †	9.016 ††	-24.627
	(0.515)	(1.353)	(0.283)	(592.569)
Many Sources	7.293 †	0.529		
	(0.380)	(0.999)	N/A	N/A
Distance to NSR	0.842	-14.351	-0.378	-0.290
	(0.799)	(747.386)	(1.726)	(1.336)
Within 10 km				
from NSR	0.280	-2.305 †	N/A	N/A
Tributary	(1.609))	(3.470))		
Stream				
Availability for	-0.692	-3.017	N/A	N/A
Herd	(0.726)	(1.917)		
Mortality in Past	-0.890	2.288	0.014 †	0.361
5 years	(5.331)	(7.933)	(0.440)	(1.317)
Constant	**19.942**	**14.381**	**18.660**	**-35.658**
	(21.028)	**(8.063)**	**(1.013)**	**(2465.658)**
Pseudo R^2	**0.015**	**0.040**	**0.023**	**0.060**

* $0.051 \leq p \leq 0.055$
† $p \leq 0.05$
†† $p \leq 0.01$

Table 3.5 Effect of individual farm characteristics and management practice on the concentration of *G. duodenalis* cysts and *C. parvum*-like oocysts in samples collected from dairy operations (n=2) during the winter of 2000.

Tested Effect	Regression Model Coefficient (β) (standard error)	
	G. duodenalis	***C. parvum*-like**
Age of Animal -*pooled*	-2.023643 †	-4.317674 ††
	(0.8685959)	(1.581809)
3-5 weeks	1.19835	-20.26295
	(2.054866)	(6864.465)
5 - 10 weeks	3.52175 †	3.748191 †
	(1.660404)	(1.80416)
10 – 15 weeks	2.634516	6.2958 ††
	(1.544556)	(1.677325)
Health	N/A	N/A
Herdsize	-1.012232	-3.561905 ††
	(0.7159048)	(1.068765)
Type of Water for Herd	N/A	N/A
Distance to NSR	N/A	N/A
Within 10 km From NSR Tributary	N/A	N/A
Stream Availability for Herd	N/A	N/A
Mortality in Past 5 Years	N/A	N/A
Constant	**11.41645 (3.082213)**	**37.78667 (10.22256)**
Pseudo R^2	**0.0231**	**0.0291**

† $p \leq 0.05$

†† $p \leq 0.01$

3.3.2 Sewage

The effect of collection time and sewage treatment facility characteristics on the concentration of *G. duodenalis* and *Cryptosporidium* spp. in sewage influent was tested. The results are summarized in Table 3.6. Factors found to significantly affect the presence of parasites in sewage samples include year, season, and facility sampled. Samples collected in 1999 were more likely to contain *G. duodenalis*, and samples collected in the spring were more likely to contain *C. muris*-like oocysts. Samples collected from the Tomahawk sewage treatment facility contained more *Giardia* cysts, and those collected from the Rocky Rapids facility contained more *C. muris*-like oocysts. The presence of *C. parvum*-like oocysts in sewage influent was shown to be unaffected by all factors tested. Similar analysis of the above factors on parasite viability in sewage influent samples was also conducted (Table 3.7). Viable *G. duodenalis* was most likely found in samples collected during the year 2000. Additionally, samples collected during the spring and summer, and those collected from the Rocky Rapids facility contained more viable *Giardia*. This analysis did not provide those factors affecting the presence of viable *C. parvum*- and *C. muris*-like oocysts.

3.3.3 Wildlife

Factors affecting the presence of *G. duodenalis and C. parvum* in wildlife scat samples were tested (Table 3.8). Type of animal (one of the five found positive), location, and distance to river all significantly contributed to the presence of *Giardia* in these samples. Animals found within 5 km or greater than 10 km away from the river were more likely to be positive than animals within 5 to 10 km. This was likely an artifact of sampling, as few beaver and muskrat samples were collected from areas 5 to 10 km from a tributary. Beaver samples were the only sample type that significantly contributed to the presence of *C. parvum* in wildlife samples ($\beta = 13.17218$; $p \leq 0.05$).

Table 3.6 The effect of collection time and sewage treatment facility characteristics on the concentration of *Giardia* and *Cryptosporidium* spp. in sewage influent samples collected from 16 facilities located in the NSR basin.

Tested Effect		Regression Model Coefficient (β) (standard error)		
		G. duodenalis	***C. parvum*-like**	***C. muris*-like**
Year *-pooled*		0.8211531 †	-2.198519 †	3.997438
		(0.3506144)	(0.9947284)	(2.790419)
	1998	-0.7877864	19.45216	15.99264
		(0.6069826)	(8283.351)	(12457.29)
	1999	0.4657002 *	17.2892	23.33174
		(0.5665)	(8283.351)	(12457.29)
	2000	0.1051105	-20.52891	-21.34506
		(0.5373236)	(8283.351)	(12457.29)
Month		0.101655	0.0048365	-0.3807897
		(0.0555416)	(0.233515)	(0.5282353)
Season *-pooled*		-0.2431614	-0.7561756	-3.571257 ††
		(0.1440627)	(0.6240835)	(1.015506)
	Spring	0.7375728	17.75247	6.241464 ††
		(0.4675283)	(5317.569)	(1.515973)
Facility *-pooled*		0.0788776	-0.1743413	-0.3466515 †
		(0.0553396)	(0.217166)	(0.1473856)
	Rocky Rapids	-1.725979	0.0000056	26.71108 †
		(0.8951299)	(171027.7)	(28280.47)
	Tomahawk	1.428778 †	0.0000056	0.000000448
		(0.633873)	(168000.5)	(36509.94)
Treatment Type		-0.5182465	1.548321	-38.03559
		(0.45094652)	(2.22301)	(0.00000026)
Discharge Frequency		0.0983654	0.024475	1.271297
		(0.3131506)	(1.294093)	(1.31369)
Constant		**0.0128496**	**11.60165**	**-1.326712**
		(1.385829)	**(25.19925)**	**(13.71636)**
Pseudo R^2		**0.0071**	**0.1191**	**0.0387**

* $0.051 \geq p \leq 0.055$
† $p \leq 0.05$
†† $p \leq 0.01$

Table 3.7 The effect of collection time and sewage treatment facility characteristics on the viability of *Giardia* and *Cryptosporidium* spp. in sewage influent samples collected from 16 facilities located in the NSR basin.

Tested Effect		Regression Model Coefficient (β) (standard error)		
		G. duodenalis	***C. parvum*-like**	***C. muris*-like**
Year *-pooled*		1.0669 †	1.06606	4.068027
		(0.5933306)	(7.241073)	(5.182589)
	1998	-1.858444	21.00138	0.00000000817
		(1.140454)	(22076.71)	(182189.7)
	1999	-0.6701822	22.47704	-27.65959
		(1.036357)	(22076.71)	(1.171345)
	2000	3.954124 ††	-22.48946	-0.0000000015
		(0.9670853)	(22076.71)	(292828.9)
Month		0.0632843	0.5687531	-0.8355859
		(0.0883587)	(2.048408)	(0.9059139)
Season *-pooled*		0.8873121 ††	-11.51709	-11.51065
		(0.3128083)	(9090.08)	(2504.122)
	Spring	-2.371534 ††	26.31884	25.7696
		(0.794315)	(65779.6)	(30430.89)
	Summer	-1.666375 †	24.03226	-0.000000067
		(0.8197339)	(65779.6)	(41981.69)
Facility *-pooled*		0.0435659	-1.017781	-0.0058315
		(0.0666202)	(1.098491)	(0.1552987)
	Rocky Rapids	-3.254665 †	0.00000000485	25.02254
		(1.634814)	(135575.4)	(29435.49)
Treatment Type		-0.1420451	-51.69245	-27.27434
		(0.8908826)	(0.0000000021)	(41659.42)
Discharge Frequency		0.2469335	9.219836	0.9462266
		(0.5673787)	(8.155824)	(1.979033)
Constant		**0.0128496**	**11.60165**	**-1.326712**
		(1.385829)	**(25.19925)**	**(13.71636)**
Pseudo R^2		**0.0071**	**0.1191**	**0.0387**

† $p \leq 0.05$

†† $p \leq 0.01$

Table 3.8 The effect of animal type, location, and distance to NSR on the concentration of *Giardia* and *Cryptosporidium* sp. in wildlife scat samples collected from tributaries located within the NSR basin.

Tested Effect		Regression Model Coefficient (β) (standard error)	
		G. duodenalis	***Cryptosporidium* spp.**
Animal -*pooled*		1.071776 †	1.530833
		(0.5464644)	(0.9282739)
	Deer	48.39464 ††	24.29713
		(3.289898)	(220594.3)
	Moose	46.45834 ††	0.000031
		(3.436908)	(221835.2)
	Coyote	48.69453 ††	0.000031
		(3.589096)	(226980.4)
	Beaver	51.17014 ††	13.17218 †
		(3.635434)	(7.128198)
	Muskrat	53.99818 ††	0.000031
		(3.635334)	(229972)
Location		-0.4459551 ††	-0.8364849
		(0.1497984)	(0.6060796)
Distance to River -*pooled*		-1.017434 †	-5.046456
		(0.4999216)	(3.989208)
	< 5 km	3.918999 ††	24.22057
		(1.063055)	(11092.74)
	5 to 10 km	0.8147788	-25.19336
		(0.7593751)	(11092.74)
	> 10 km	5.984039 ††	21.52257
		(1.517335)	(11092.74)
Constant		**-0.0220769**	**-0.9572696**
		(3.179354)	**(1.928863)**
Pseudo R^2		**0.0266**	**0.0628**

† $p \leq 0.05$

†† $p \leq 0.01$

3.4 Molecular Analysis of *Cryptosporidium* isolates

3.4.1 ITS amplification and Dra I digestion

A total of 7 *Cryptosporidium* isolates representing those collected were analyzed using RFLP analysis (Table 2.4). The secondary reaction products are shown in Figure 3.6. Product sizes ranged from approximately 580 bp to 958 bp. Amplicon size differences can clearly be observed following the secondary reaction. The final products resulting from *Dra I* digestion of the ITS region for each isolate are shown in Figure 3.7. The isolate obtained from an infected calf displayed the 'cattle' fingerprint, while the isolate obtained from the infected steer showed a unique fingerprint consisting of 3 products, sized ~380, ~165, and ~135 bp. The pig isolate displayed the 'pig' fingerprint, and the cat isolate displayed the 'cat' fingerprint. Both human isolates digested into 5 products giving 4 observable amplicons, and thus displayed the 'human' fingerprint.

3.4.2 18S Sequence Analysis

A genomic DNA region of approximately 800 bp of the 18S ribosomal unit sequence was amplified with the primers 18S STA and 18SiR3A from 7 *Cryptosporidium* isolates of various host origin. Sequence comparison of a 318 bp region of that amplicon revealed a substantial degree of variation between the different isolates (Figure 3.8). There were some differences in length, with the cat isolate (Cat-1) exhibiting the longest region (332 bp).

Figure 3.6

Products from the secondary PCR reaction of the rDNA ITS1 region of *Cryptosporidium* isolates. Lane 1, 100 bp ladder with bright band representing 600 bp; 2, negative control containing all reaction products and no DNA; 3, Ca-1; 4, St-1; 5, Hu-1; 6, Hu-2; 7, Cat-1; 8, Pig-1; 9, Pig-2.

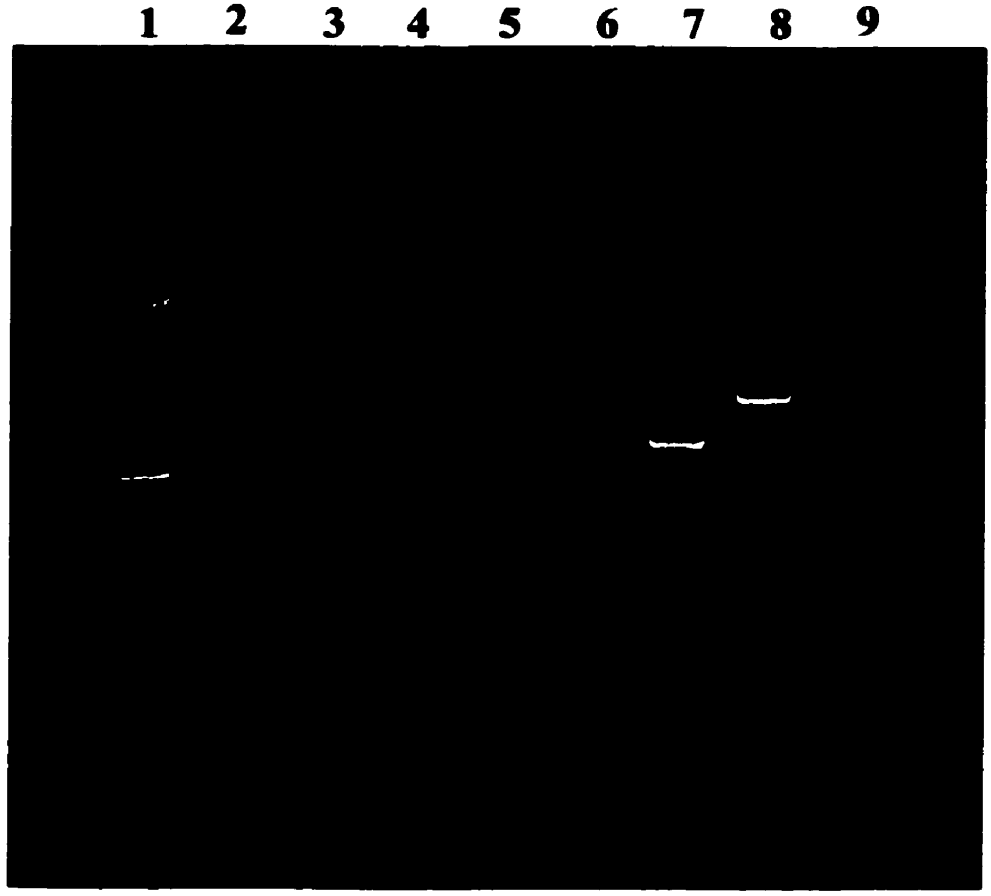

1
2
3
4
5
6
7
8
9

Figure 3.7

Electrophoretic profiles of the rDNA secondary PCR products digested with the restriction endonuclease *Dra I*. Lane 1, 100 bp ladder; 2, Ca-1; 3, St-1; 4, Hu-1; 5, Hu-2; 6, Cat-1; 7, Pig-1; 8, Pig-2.

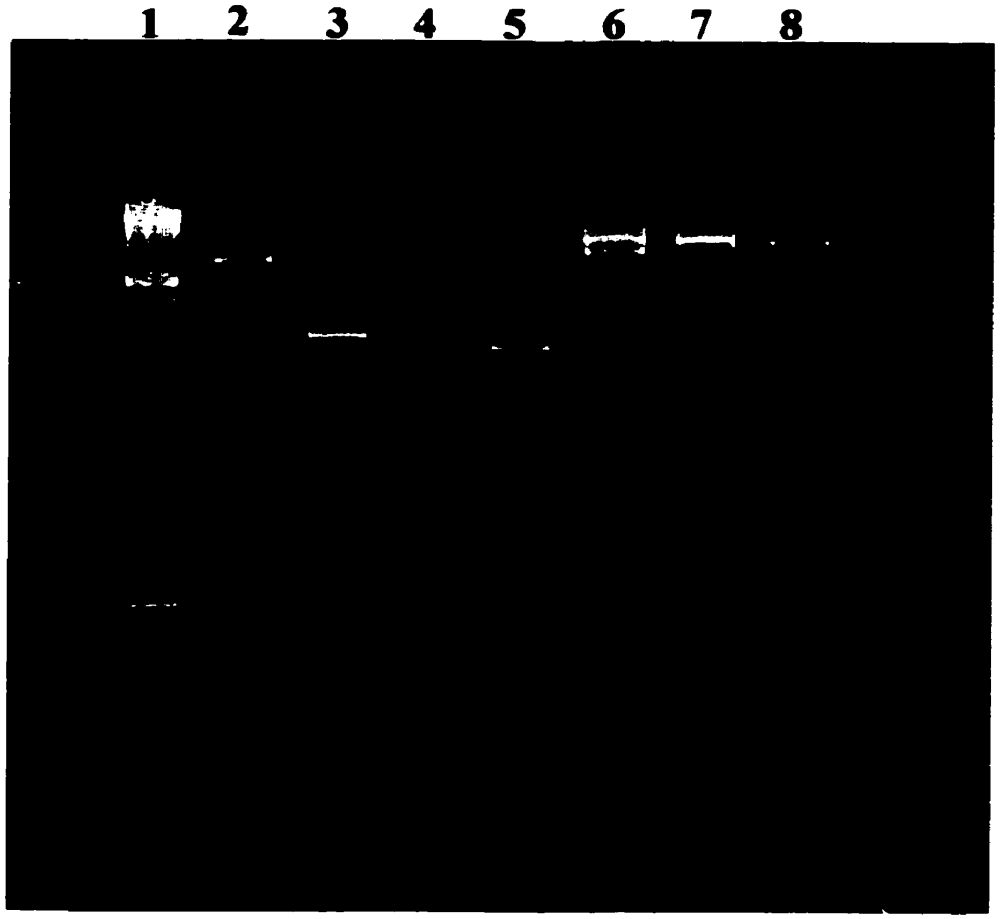

1
2
3
4
5
6
7
8

Figure 3.8

Sequence alignments of a 318 bp unit of the *Cryptosporidium* rDNA 18S region from various isolates. Primer sequence is not included. The region begins at 85 bases upstream to the start of the 18S and ends at position 403 of the 18S gene. Complete Hu-1 (human) sequence is given as a reference. Dashes represent insertions, and dots represent homologous regions.

```
                  85
Hu-1              CCTGCGAATGGCTCATTATAACAGTTA-TAGTTTACTTGATAAT--CTTTTACTTA
Pig-1             ........................GCC.................--....-.....
Ca-1              ..C.....................-.................--....-.....
Cat-1             ........GC........A........-.C.............CC....-TACA.
St-1              ..............A..A........-.................----CAA.AC..
                  ** *****  ***** ** ******   * **************          *

Hu-1              CATGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAAACTCGACTTTATG
Pig-1             ..........................................CTA........
Ca-1              ..........................................................
Cat-1             ......................A.A.............TG...G..C.A...C.GG.
St-1              ..............T.......C.A.............C.......C.A....CCC.
                  ** **********  ******* * **************  *** **   ***    *

Hu-1              GAAGGGTTGTATTTATTAGATAAAGAACCAATATAATT----GGTGACTCATAATA
Pig-1             ...A.....................................T---.....T........
Ca-1              ..........................................----..............
Cat-1             ..........T.......T.A.....C...G.C.TT..TTTC......G...C...
St-1              .........T.T.......A.A...A.......GAGC..-----..G..T...A..A.
                  *** **** * ******* * *** * *** *    **    ** **  **  * *

Hu-1              ACTTTA---CGGATCACAATT--AAT---GTGACATATCATTCAAGTTTCTGACCT
Pig-1             ......---.........T..TT...---..............................
Ca-1              ......---.........T.A--...---..............................
Cat-1             ......TTG..C.C....TG.--T..TTT.C.GG.....................G
St-1              ......---...G..C..TCCCCG.A---.CC.A.A.A.............C....C
                  ******   **   * **       *    *    * * ************ ****

Hu-1              ATCAGCTTTAAACGGTAGGGTATTGGCCTACCGTGG-CAATGACGGGTAACGGGGA
Pig-1             ..........G..............................-...................
Ca-1              ..........G..............................-...................
Cat-1             .C..A.....TT.....................T..CA.T...................
St-1              .A..A........................C...C...-.T...................
                  * ** *****  ***************** *** **  * ******************

Hu-1              ATTAGGGTTCGATTCCGGA-GAGGGAGCCTGAAAAACGGCTACCACATCTAAGGAA
Pig-1             ....................-............G.........................
Ca-1              ....................-............G.........................
Cat-1             ....................C.........A..C.............A...C..C...
St-1              ..........C.........-A.....A..C.........G.....A...C......
                  **********'********  ***** ** ** ****** ***** *** ** ***
                                                                        403
```

4 Discussion

In the preceding results, we have demonstrated the significance of agriculture, human sewage influent, and wildlife as sources of the waterborne parasites *Giardia duodenalis* and *Cryptosporidium* spp. in the NSR watershed. Furthermore, we have shown that the isolates of *Cryptosporidium* spp. collected from central Alberta are genetically similar to isolates collected from other geographical regions. These results are discussed in detail below.

4.1 Source Contribution

The results of this study confirm that agriculture, sewage influent, and wildlife are all sources of the waterborne parasites *Giardia* and *Cryptosporidium* in Alberta. Based on the comparison of mean parasite concentration between each of the sources, agriculture was found to be a significant source of both *Giardia* and *Cryptosporidium*, while sewage influent was found to be nonsignificant as a source. The role of wildlife as a source of *Cryptosporidium* was minimal to none, however beaver and muskrat samples contained significantly high concentrations of *Giardia*.

4.1.1 Sewage Influent

The concentration of both *Giardia* and *Cryptosporidium* in sewage influent was extremely low, however the presence of both parasites in consistent sampling events remained high. This resulted in high parasite prevalence for human sewage. This observation was expected as the majority of treatment facilities tested were closed cell with one or two discharges per year. Therefore, with constant input and minimal output, the background levels of *Giardia* and *Cryptosporidium* remained relatively constant. The presence of C. *muris*-like oocysts in sewage influent was unexpected. C. *muris* is a

genetically and morphologically distinct species of *Cryptosporidium* that is infectious to ruminant and rodent hosts (Morgan *et al.*, 1998b). Its presence raises questions concerning specific contributions to affected facilities, parasite transmission, and most importantly, hygiene within individual communities. For example, concentrations of *C. muris*-like oocysts were detected in the Warburg facility where a large truck-wash exists that caters to agricultural equipment. It is possible that the oocysts detected were a result of infected bovine manure and/or rodent feces associated with the machinery.

Seasonal fluctuations in parasite prevalence of sewage influent were also apparent. *Cryptosporidium* was detected in samples collected over the spring and summer months. This trend corresponds to the observation made by Fayer & Ungar (1986) that the incidence of *C. parvum* infection increases during the spring and summer seasons in North America. Bodies of surface water have intermittently low background levels of *Cryptosporidium*. These levels may be increased rapidly through contamination. Contamination typically increases following heavy rainfall events and spring runoff. Contamination of drinking water can result due to compromised filtration during periods of high particle load, such as during spring runoff (Smith & Rose, 1998). Other factors that could be attributed to this trend include increased contact with infected animals during calving season in rural areas or increased contact with potentially contaminated recreational waters (Deneen *et al.*, 1998). Human cryptosporidiosis is emerging as an important disease, as individuals with compromised immunity are extremely susceptible to severe, chronic and life-threatening infections.

The consistency with which *Giardia* was detected in sewage influent samples should be of concern for public health authorities. Estimates of giardiasis and cryptosporidiosis in the community can be gained through raw sewage monitoring. This suggests that in particular, giardiasis is present in many NSR communities. Regardless of our findings, the incidence of giardiasis in NSR communities has remained unchanged and no outbreaks of disease have occurred. This was expected as *Giardia* is a common

enteric pathogen, cysts are excreted intermittently from the infected host, and the duration and severity of infection tends to be minimal (Farthing, 1994). The end result is that many cases of giardiasis go undiagnosed.

Viability analyses suggest that parasites, mainly *Giardia*, isolated from sewage influent are infectious. We report up to 60% *Giardia* viability in sewage treatment facilities within the NSR basin. This is contrary to previous findings of less than 10% *Giardia* viability in raw sewage (Smith *et al.*, 1994). The presence of viable *G. duodenalis* is reason for concern because 12 of the 16 facilities tested do not disinfect prior to discharge. These facilities rely on time and therefore sedimentation as the sole method used to reduce parasite numbers. Sedimentation is an inefficient treatment process due to the small size and low density of cysts and oocysts (Medema *et al.*, 1998). Despite this, Wallis (1994) reports that the parasite removal efficiency of various sewage treatment systems is high. However, he further states that the proportion of viable parasites present in raw sewage may increase due to the increasing concentration of (oo)cysts that pass through the treatment system. Our reported parasite prevalence and viability in wastewater should warn against the discharge of untreated sewage, particularily because parasite survival is enhanced by cold temperature. Viable parasites released into the NSR could therefore survive and remain infectious for prolonged periods of time.

The mean concentration values calculated for human sewage influent were low. This was likely due in part to some sampling bias. Samples were collected not from various depths of the individual lagoons but from various areas around the perimeter of each facility. This could have resulted in an underestimation of parasite concentration. Oocysts and cysts could attach to various particulate matter and would therefore, sediment at the rate of the particle and not the parasite. An analysis of both prevalence and parasite concentration at various depths in each lagoon would have greatly

contributed to the understanding of sedimentation and circulation dynamics in the individual sewage treatment facilities.

4.1.2 Wildlife

Of the three sources tested, *Giardia* and *Cryptosporidium* in wildlife were the least prevalent. The overall prevalence of each parasite was extremely low, however the prevalence of *Giardia* in aquatic mammals (beaver and muskrat) was quite high. The levels detected in beaver and muskrat were expected and remain consistent with previous findings (Bajer et al., 1997). The concentration of *Giardia* in muskrat samples was extremely high. In the all source comparison, this combined with the concentration in beaver samples resulted in a significantly high mean *Giardia* concentration for wildlife when compared with the other sources. Beaver and muskrat are continually exposed to waterborne parasites in contaminated waterways. This suggests the potential for aquatic mammals to become infected by human and agricultural contamination, thus explaining the high values detected in aquatic mammal sources. As reservoirs, infected beaver and muskrat could amplify background levels of *Giardia* and *Cryptosporidium* in these waterways (Wallis *et al.*, 1996). As such, beavers have been implicated in outbreaks of giardiasis (Wilson *et al.*, 1982). The ability of a colony of beavers to contaminate downstream surface waters has been previously demonstrated (Monzingo & Hibler, 1987). It was also shown that beavers shed cysts year round, and cysts output levels are highest in the late summer and fall (Wallis, 1994). Finally, Erlandsen *et al.* (1988) demonstrated that human source *Giardia* cysts are infectious to both beavers and muskrats. Therefore, the discharge of untreated sewage and agricultural runoff may impact waterborne parasitic infections in water mammals. These infections could lead to an increase in downstream effects, such as outbreaks of giardiasis or cryptosporidiosis.

We were unable to detect *Giardia* or *Cryptosporidium* in the feces of Canada and snow geese. This was unexpected, as Graczyk *et al.* (1998) found high levels of both parasites in migratory Canada geese from Chesapeake Bay, Maryland. The lack of parasites in Alberta samples suggests that Western migratory geese either become infected in other, more southerly locations, or that they fail to become infected in contrast to their Eastern counterparts. Many of the waterfowl samples collected during this study were from resident geese. Since these samples were also negative, this further supports the conclusion that geese do not become infected in the Alberta region.

The large majority of wildlife scat samples collected were negative for both *Giardia* and *Cryptosporidium*. This may have been due to the collection methods used. Scat samples were collected in the early spring, directly from the environment, immediately after the melting of all snow-cover. In many instances, older scat would have undergone multiple freeze-thaw cycles before being sampled. Freezing causes cyst and oocyst inactivation which may result in physical degradation. It is therefore possible that some samples found to be negative for *Giardia* and *Cryptosporidium* were in fact positive when they were in a fresher state. Similarly, since beaver and muskrat samples were collected fresh from the colon of culled animals, the prevalence and concentration of parasites detected was likely representative of these populations. This created bias, particularly when comparing between wildlife species, as sample collection likely caused an under estimation for some and an actual estimation for others.

4.1.3 Agriculture

Of the sources tested in this study, agriculture had the highest mean parasite concentration for both *Giardia* and *Cryptosporidium* in the NSR watershed. Cow-calf samples contained the highest concentration of *Giardia*, and the highest concentrations of *C. parvum*-like oocysts were found in dairy samples. These findings imply that

agricultural effluent, if not contained adequately, could act as a significant contaminant to the NSR environment. The understanding of *Giardia* and *Cryptosporidium* in agricultural sources is important not only for watershed protection, but also to maintain the economical integrity of agricultural production in Alberta.

At each of the 11 cow-calf operations tested, all samples containing *C. parvum*-like ocysts, and the large majority of those containing *G. duodenalis* were from calves. Only a few cow fecal samples contained marginal levels of *Giardia*. This result corresponds to that previously published by Atwill *et al.* (1999) concerning *C. parvum* infection in California cow-calf herds. Here, calves aged 1 to 4 months were most likely to shed oocysts, and few shedding cattle were greater than 4 months in age. A watershed analysis in British Columbia linked calving season to high concentrations of oocysts in downstream surface water (Ong *et al.*, 1996). In this study, high oocyst concentrations in downstream water disappeared within a few months after calving. Giardiasis in calves has been shown to occur within 4 days of birth with infections persisting over 8 weeks (Xiao, 1994). Due to our results, and the results of the above-mentioned studies, it is likely that the greatest risk of waterborne contamination from cow-calf sources occurs during calving season.

Large variations in parasite concentration and prevalence between year and individual cow-calf operations tested were detected. Differences between operations were due to differences in management practices used. Cow-calf herds with access to dugout and runoff water as principal water source were found to have the highest concentrations of waterborne parasites, whereas herds where well water was the principal source had the lowest parasite concentrations. This was an expected risk factor, as animals tend to congregate in and around dugout water sources. By doing this, one infected animal is able to easily contaminate the water source and cause subsequent infections in the susceptible members of the herd. Well water is much more difficult to contaminate and as a result is a cleaner water source.

A tendency to detect *C. parvum*-like oocysts in cow-calf herds located within 10 km from a NSR tributary was also demonstrated. This finding suggests a possible relationship between contaminated waterways and infected calves. Animals with direct access to river water could be at greater risk for infection. Likewise, infected animals living within close proximity to or on tributaries could be a major cause of waterborne contamination, as animals will defecate directly into their water source. Runoff from unprotected areas such as cow-calf pastures may allow for the transport of *Giardia* cysts and *Cryptosporidium* oocysts in the environment to waterways. If infected calves are present in the watershed area, and snowmelt and rainfall exceed the capacity of the soil, overland flow conditions could result (Atwill *et al.*, 1999). This would cause large volumes of potentially infected manure to rapidly reach rivers and streams.

Yearly variation in parasite presence for cow-calf samples was observed. This was most likely due to differences in weather patterns during 1998 and 1999. The winter of 1998 was dry with little precipitation. During this time, the observed parasite prevalence was lower than previous reports of 29% and 20% for *Giardia* and *Cryptosporidium* respectively (Olson *et al.*, 1997c). Drier weather during this time may have contributed to an increase in susceptibility of *Giardia* cysts and *Cryptosporidium* oocysts to desiccation. Dried fecal material would become non-infectious thereby limiting transmission within the cow-calf herds. The lower than usual precipitation could also have led to a reduced spring runoff and a subsequent reduction in surface water contamination. Environment Canada (http://www.mb.ec.gc.ca) reported considerably higher precipitation levels during the winter of 1999. This led to higher recorded precipitation levels in the spring, and a high runoff potential. Subsequently, the prevalence of *Giardia* was higher than expected (Olson *et al.*, 1997c), and *Cryptosporidium* was found in similar prevalence to studies previously published (Atwill *et al.*, 1999). Increased precipitation would have raised the relative humidity and the

tendency for (oo)cysts to desiccate would have decreased. Parasite survival times could have been increased, thereby facilitating animal to animal transmission on the pasture.

Of the agricultural sources tested, dairy operations were found to be the significant source of *C. parvum*-like oocyts. Samples collected from two dairies contained high concentrations of *C. parvum*-like oocysts. There was considerable variation between the concentrations detected at each of the two dairies. However, due to minimal sample size, these variations could not be accounted for. Previous studies have shown the risk factors for dairy calf cryptosporidiosis to be contact between calves and frequent bedding changes (Sischo *et al.*, 2000). An increased frequency of bedding changes demonstrates that a more conscientious effort is being made by the farmer to be hygienic. Unfortunately, through the process of removing and adding fresh bedding, equipment can become contaminated and helps spread infection (Maldonado-Camargo *et al.*, 1998). As with the cow-calf data, age was also shown to be a significant factor for the presence of *Cryptosporidium* and *Giardia* in dairy farms. In contrast to preceding studies where the youngest animals were mostly infected (Garber *et al.*, 1994), we found the majority of infected samples to come from animals aged 5 to 15 weeks. This was an artifact due largely to a lack of samples collected from animals less than 5 weeks of age. The management of manure presents a challenging problem for dairy producers. Manure is typically applied to pastures either through spraying or spreading. Surface runoff moving from these pastures to rivers and streams could harbour many infectious parasites. However, in a recent study, water samples downstream of many dairies found to be positive for parasites were not positive for cryptosporidia. Regardless, dairy operations represent an important source of parasites, as young calves are present throughout the year and managing manure presents a challenging problem (Sischo *et al.*, 2000).

The levels of *Giardia* detected in hog samples collected from one operation within the NSR basin were highly non-significant. Our observed *Giardia* prevalence of

18% was within the 0 to 20% range of previously reported prevalencies (Olson *et al.*, 1997c). No *Cryptosporidium* was detected in hog samples, however other studies report up to 22% infection in 78% of hog operations (Quilez *et al.*, 1996). Our results suggest that hog farms do not produce substantial amounts of waterborne parasites when compared to other agricultural sources such as cow-calf and dairy operations. However, our sample size of 40 collected entirely from one operation is far too minimal to adequately assess hog production as a source. More operations would have to be tested for comparisons to be made, although in actuality, few operations exist within the watershed.

4.2 Molecular Characterization

Genetic analysis of the *Cryptosporidium* isolates collected during this study revealed the presence of distinct calf, steer, cat, human, and pig genotypes. The 'calf', 'cat', 'human', and 'pig' genotypes displayed the same fingerprinting patterns reported by Morgan *et al.* (1999b). Isolates used in their study came from various geographical locations ranging from North America, Europe, to Australia. Other studies using many of the same isolates have reported that only the 'human' and 'calf' genotypes are infectious to immunocompetent humans (Awad-El-Kariem *et al.*, 1998; Carraway *et al.*, 1997; Morgan *et al.*, 1998b). In view of the genetic similarity between our isolates and those isolates used by others, we propose that our 'calf' and 'human' isolates are also infectious to the human population. The susceptibility of humans to infection from other genotypes such as the 'cat' and 'pig' requires further molecular epidemiological study. However, to date, molecular analyses have revealed cryptosporidiosis in AIDS patients caused by 'calf', 'human', 'cat', and 'dog' isolates (Pieniazek *et al.*, 1999). Recombinant genotypes and mixed infections have yet to be reported.

The fingerprinting pattern displayed by the 'steer' genotype is unknown. A recent study by Lindsay *et al.* (2000) describes a new species of cattle infective *Cryptosporidium* labeled *C. andersoni*. This species of *Cryptosporidium* largely resembles *C. muris*, both morphologically and genetically, is not infective for mice, and is commonly found to infect the abomasum of juvenile and adult cattle (Lindsay *et al.*, 2000). *C. andersoni* is thought to interfere with milk production in dairy cows and has been found in 1.4% of United States cattle (Anderson, 1991b). The genetic similarity between *C. andersoni* and *C. muris* of 99.14% is less than the similarity between the 'human' and 'calf' *C. parvum* genotypes at the same locus (99.7%) (Lindsay *et al.*, 2000). We propose that the 'steer' fingerprinting pattern observed through the digestion of the ITS1 rDNA region with the restriction endonuclease *Dra I* represents *C. andersoni*, as described by Lindsay *et al.* (2000). Our St-1 isolate was collected from an adult bovine host, chronically infected with oocysts resembling *C. muris* in size.

Genetic analyses revealed the presence of a distinct 'pig' fingerprint. Previous studies have indicated that porcine cryptosporidiosis is caused by either the 'pig' or 'calf' genotypes (Morgan *et al.*, 1999a). The presence of the 'calf' genotype in pigs suggests that porcine hosts can act as reservoirs of human infectious *Cryptosporidium*. Both of the pig isolates tested in our study displayed the 'pig'-adapted genotype. The role of this genotype in human infections requires further epidemiological investigation. At present, the 'pig' genotype has not been isolated from human hosts.

In this study, only 7 isolates representing 5 genotypes were tested. We were unable to amplify the beaver and deer isolates using the methodology reported. Morgan *et al.* (1999b) amplified the ITS1 region of a deer isolate from Scotland and found it to resemble the 'calf' genotype. No molecular studies to date report the genotype of *Cryptosporidium* isolated from beavers. Beaver cryptosporidiosis is likely caused by infection with rodent-infective species; *C. parvum* 'mouse' genotype or *C. muris*.

Additionally, beavers could be susceptible to *C. parvum* 'human' and/or 'calf' strains, as they are likely exposed to these genotypes continually in their aquatic environment.

Our results in combination with the results from others, add strength to the proposed theory that *Cryptosporidium* has a clonal population structure. Clonal population systems occur when distinctive genotypes persist through generations and sexual recombination does not occur (Morgan *et al.*, 1999b). To further test this theory, more epidemiological investigation of different genetic loci in a greater variety of host-adapted genotypes is required.

Fingerprinting and molecular characterization of *Giardia* isolates from various hosts is also necessary so that transmission dynamics for this parasite can also be understood. Recent studies have provided further evidence suggesting that genetic divisions between populations of *G. duodenalis* isolates occur worldwide (Hopkins *et al.*, 1999). These studies demonstrate genetic diversity between different isolates, however increased resolution is needed for transmission analysis. With the continued development of new molecular procedures, this will become possible. Continued molecular epidemiology studies of *Cryptosporidium* isolates found will also contribute to our understanding of human-infectious or zoonotic genotypes. Finally, the use of these molecular tools will become of tremendous use for environmental monitoring of *Cryptosporidium* and *Giardia* genotypes present in water supplies. The use of genotyping technology in the environmental protection industry will become crucial as more information regarding *Cryptosporidium* and *Giardia* genotypes becomes available.

4.3 Perspectives on Source Management

The sources of *Giardia* and human-infectious *Cryptosporidium* in the NSR watershed must be controlled. Control efforts must incorporate ways in which to protect the watershed, such that waterways do not become overly contaminated. The results of

this study demonstrate that of the three sources of waterborne parasites tested, cow-calf and dairy sources of *Giardia* and *Cryptosporidium* contained the highest, therefore most significant concentrations. Additional genetic analysis of *Cryptosporidium* isolates confirmed the presence of 'human' and 'calf' genotypes, both of which are infectious to humans. This suggests a need for the implementation of management practices that would serve to protect waterways from all sources of waterborne parasites.

Contaminated sewage influent is one potential source of waterborne contamination. Efforts to reduce the release of untreated sewage into the NSR must be undertaken. Additional efforts are needed to reduce the loading of *Cryptosporidium* and particularly *Giardia* into these wastewater treatment facilities. The reduction of *Giardia* and *Cryptosporidium* in wastewater will ultimately led to decreased parasite loading in the watershed, a reduced potential to infect water mammals living in the watershed, and a reduced threat to public health.

Management practices targeting the reduction of *Giardia* and *Cryptosporidium* in the agricultural industry must be developed and practiced. These strategies will serve to not only protect the watershed, but ultimately will also contribute to better herd health and an increase in production at the economical level. Possible management strategies include restricting cattle access to streams during the first 4 months of calving season when the prevalence of parasitic infections are typically the highest. Additional management such as spreading manure on fields during the driest months of the year to avoid runoff and promote desiccation, composting manure for extended periods of time before spreading to reduce parasite viability, and sterilizing farm equipment between pen changes would also decrease the prevalence of *Giardia* and *Cryptosporidium* in farms. Finally, advances in the pharmaceutical industry have shown much promise in the area of *Giardia* and *Cryptosporidium* vaccines (Olson *et al.*, 1997b; Perryman *et al.*, 1999), and preventive strategies (O'Handley *et al.*, 1997).

Further study assessing parasite presence in fresh wildlife samples should be undertaken. This will allow for further clarification of the impact from species other than beaver and muskrat. Reductions in parasite loading from sources such as sewage and agriculture will decrease the exposure of water mammals living in the waterways to waterborne parasites. Overall, the prevalence of both *Giardia* and *Cryptosporidium* in wildlife was extremely low. This suggests that the development and maintenance of riparian bands along waterways could act as a filter, serving to protect rivers and streams from other sources of contamination.

The NSR basin is a good Canadian watershed model. This watershed includes areas of mountains, foothills, farmland, forest, and urban centers. Many rivers feed directly into the main NSR, which winds through the watershed and supplies resources to surrounding communities. The 1986 agricultural census estimated the presence of 260000 cattle in the watershed. The average daily manure output of one beef and one dairy cow is 36.95 kg/day (Code of Practice, 1995). This, multiplied by the average concentration of *Giardia* and *C. parvum*-like oocysts found in our agricultural analysis and by the number of cattle in the watershed, suggests that potentially 2.87×10^{12} cysts and 1.73×10^{12} oocysts per day could be released into the watershed. Calculated values for sewage influent assuming maximum daily collection volumes are 2.09×10^{12} *Giardia* cysts and 2.27×10^{11} *Cryptosporidium* oocysts per day. These values suggest that the potential for waterborne contamination in the NSR basin is extremely high. The NSR basin is no different from many other Canadian watersheds, therefore, contamination in the NSR basin should be representative of other large watersheds in Canada.

4.4 Summary of Major Findings.

The comparative significance of agricultural, wildlife, and human sewage influent sources of the waterborne parasites *Giardia duodenalis* and *Cryptosporidium* spp. was

determined. Of the three sources tested, agriculture was found to have the highest mean parasite concentration. The cow-calf operations tested demonstrated the highest concentration of *G. duodenalis*, while dairy operations had the highest concentration of *C. parvum*-like oocysts. However, the prevalence of both parasites in agricultural sources (cow-calf, dairy, and hog) was lower than that found in human sewage influent. This demonstrated that specific animals in the herds were infected, while others were not. Further statistical analysis revealed that calves were most likely infected. Additional statistical analysis linked the presence of parasites in cow-calf operations to the type of water given to the herd. Specifically, dugout and runoff water sources were significant factors contributing to *Giardia* and *Cryptosporidium* infection in calves. Wildlife, as a source of waterborne parasites was found to have minimal *Giardia* and *Cryptosporidium* prevalence. Water mammal samples had significantly high mean *Giardia* concentrations in comparison to the other species tested. This was likley due to sample bias and requires further investigation.

PCR analysis of the ITS1 region of rDNA in *Cryptosporidium* isolates collected throughout the study revealed genetic differences between isolates. The presence of specific 'human', 'calf', 'steer', 'cat', and 'pig' genotypes was documented. This analysis suggested the presence of distinct *Cryptosporidium* transmission cycles with conserved isolate heterogeneity occurring within the NSR basin.

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