GIARDIA AND *CRYPTOSPORIDIUM* INFECTIONS IN DOMESTIC LIVESTOCK: ZOONOTIC POTENTIAL, TRANSMISSION DYNAMICS, AND THREAT TO DRINKING WATER

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Abstract

The main aims of this thesis were to obtain information on the prevalence of zoonotic and host-specific *Cryptosporidium* and *Giardia* in livestock on Prince Edward Island, Canada, to determine if the presence of cattle and pigs increase the levels of *Giardia* and *Cryptosporidium* in source water. *Giardia* genotypes and *Cryptosporidium* species in humans on PEI were also analysed to determine if they were the same as those found in cattle and pigs.

The role that dairy farms may play in contaminating source water on Prince Edward Island, Canada, with *Giardia* and *Cryptosporidium* is described in Chapter 2. Thirty-two percent and 14% of 752 fecal samples, and 100 and 55% of 20 herds, were positive for *Giardia* and *Cryptosporidium*, respectively. Most of the *Cryptosporidium* isolates belonged to *C. andersoni* and *C. bovis*. *Giardia duodenalis* assemblage E was predominant among all cattle age groups, while only 6 and 4% of isolates belonged to assemblages A and B, respectively. *Giardia* and *Cryptosporidium* were detected in 46 and 85% of surface water samples, respectively, while one groundwater sample was positive for *Cryptosporidium*. All four genotyped *Cryptosporidium* isolates from water were *C. parvum*. This study indicates that dairy cattle were predominantly infected with non-zoonotic species/genotypes of *Cryptosporidium* and *Giardia*. However, some surface water sources were contaminated with the zoonotic *C. parvum* and may represent a threat to the watershed and subsequent disease transmission to humans. Cattle >6 months of age were less likely to be infected with *Giardia* while there was no significant association between either the location of the farm or the housing type and infection with *Giardia*. Cattle >6 months of age in free-stall housing were more likely to be infected with *Cryptosporidium* species. While there was no impact of age, infection with *Cryptosporidium* tended to be higher in Queens County.

Chapter 3 describes the prevalence and genotypes of *Giardia* and *Cryptosporidium* on beef farms, including the water sources associated with the farms, and the risk factors for infection of cattle with these parasites. *Giardia* and *Cryptosporidium* were detected in 42% and 17% of 739 fecal samples, and 100 and 80% of 20 farms, respectively. *G. duodenalis* livestock genotype assemblage E predominated (89%). The zoonotic assemblages A and B were found in 4 and 7% of the fecal samples that were genotyped, respectively. Overall, the most common *Cryptosporidium* species detected in this study was *C. andersoni*. *Giardia* cysts and *Cryptosporidium* oocysts were detected in 14 and 93% of surface water samples of 14 farms, respectively. *Cryptosporidium* oocysts were detected in three (15%) ground water samples of 20 farms. Age of animals and location of the farm, were not associated with the risk of infection in cattle with either *Cryptosporidium* spp. or *G. duodenalis*. The presence of zoonotic species of *Cryptosporidium* and *Giardia* in beef cattle represents a potential threat to source water and subsequent disease transmission to humans from these parasites

In Chapter 4, the prevalence of infection with *Cryptosporidium* and *Giardia,* and the genotypes and species of isolates, were determined in order to establish the zoonotic potential of pigs. Eighteen herds (86%) and 163 animals (26%; 95% CI: 22-29%) tested positive for *Cryptosporidium*, while just 3 herds (14%) and 6 animals (1%; 95% CI: 0.4-2%) tested positive for *Giardia.* The *Cryptosporidium* spp. most isolated among those successfully genotyped, belonged to pig genotype II (61%) and *C. suis* (36%). Pigs are not a major source of *Giardia* and the common occurrence of the host-specific genotypes and species of *Cryptosporidium*, indicate that domestic pigs, likely do not pose a significant health risk to humans from these parasites.

The species and genotypes of *Cryptosporidium* and *Giardia* in livestock were compared with those from humans to determine if zoonotic transmission of these species occurred, in

Chapter 5. Overall 22% and 0.5% of human fecal samples were positive for *Cryptosporidium* and *Giardia* respectively. The species of *Cryptosporidium* found in humans were *C.parvum* and *C hominis*. *C.parvum* shedding in humans was more frequent than *C. hominis*. Sub-genotyping of *C. parvum* isolates from beef cattle revealed three sub-genotypes: IIaA16G2R1, IIaA16G3R1 and IIaA15G2R1. Two of these sub-genotypes: IIaA16G2R1 and IIaA15G2R1 were also found in humans. The results indicate that prevalences of the zoonotic species and genotypes of *Cryptosporidium* and *Giradia* in the herds sampled in this study were low. However, the data suggests zoonotic transmission of *Cryptosporidium* to humans on PEI and that cattle are the possible source of the parasite.

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List of Abbreviations

AVC	Atlantic Veterinary College
bp	Base pair
CI	Confidence Interval
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein-isothiocyanate
g	Gravity
IMS	Immunomagnetic separation
mAb	Monoclonal antibody
min	Minute(s)
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEI	Prince Edward Island
rDNA	Ribosomal dexoyribonucleic acid
rRNA	Ribosomal ribonucleic acid
IFA	Immunofluorescence assay
DFA	Direct immunofluorescence assay
USEPA	United States Environmental Pretection Agency
IFAT	Indirect Fluorescence Antibody Tests
DAPI	4'-6-Diamidino-2-phenylindole

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1.0 INTRODUCTION

1.1 General Introduction

Giardia and *Cryptosporidium* are commonly identified in humans and a wide range of animals including livestock (Fayer and Ungar, 1986; Farthing, 1994; Majewska, 1994; Fayer et al., 1997; Xiao et al., 1999; Heitman et al., 2002). They are of clinical and economic importance having been recognized as a common cause of gastrointestinal disorders (Fayer, 1997). In humans, symptoms of giardiasis and cryptosporidiosis include diarrhea, abdominal pain, vomiting and fever which may last from a few days to several weeks (Fayer, 1997). While giardiasis is treatable, there is currently no effective therapeutic agent for cryptosporidiosis, which can be prolonged and even life threatening for immune suppressed individuals.

The mode of transmission ranges from direct fecal-oral transmission or ingestion of food or water inadvertently contaminated with oocysts or cysts from feces of an infected host (Atwill et al., 2002; Bevilacqua et al., 2005). Foodborne transmissions can occur due to the inappropriate handling of food, from food washed with contaminated water or the direct contact with fecal material. Apple cider prepared from contaminated apples, uncooked vegetables, improperly pasteurized milk, sausages, tripe, canned salmon, ice cream and contamination from infected food handlers have all been implicated in the spread of *Giardia* and *Cryptosporidium* (Millard et al., 1994; Laberge et al., 1996; Rose and Slifko, 1999; Doyle, 2003; Dawson, 2005).

Cryptosporidium oocysts and *Giardia* cysts occur commonly in the aquatic environment and there have been numerous reports of waterborne outbreaks of cryptosporidiosis and giardiasis throughout the world (Rose, 1988; Cruan, 1990; LeChevallier et al., 1991; Mackenzie et al., 1994; Cruan et al., 1998; Hoogenboezem et al., 2001). In North America, waterborne

transmission has emerged as the most important route for *Giardia* and *Cryptosporidium* infections in humans. *Giardia* cysts and *Cryptosporidium* oocysts have been identified in raw and treated drinking water in every region of Canada (Wallis et al., 1996). *Giardia* and *Cryptosporidium* are among the most frequently identified waterborne illness in North America (Lee et al., 2002) and contaminated water supply usually results in epidemic outbreaks of giardiasis and cryptosporidiosis, often on a massive scale. For example, a 1993 outbreak of cryptosporidiosis in Milwaukee resulted in an estimated 400,000 infections and 100 deaths. Also, between 5800 and 7100 individuals were infected with *Cryptosporidium* from contaminated drinking water in North Battleford, Saskatchewan, a rural community of about 15,000 inhabitants (Stirling et al., 2001). Characteristics of *Cryptosporidium* and *Giardia*, such as their low infective dose (one to ten (oo)cysts), possibility of environmental dispersal leading to the contamination of drinking water and food (Caccio et al., 2005), persistence for months in the environment, difficulty to reliably isolate from samples, (oo)cysts small enough to pass through commonly used rapid-rate granular filter media in water treatment plants and the relative resistance to disinfection processes (Sterling, 1990; Olson et al., 1999; Erickson and Ortega, 2006) make these parasites a challenging public health threat.

The potential to contaminate water sources in a watershed with *Cryptosporidium* oocysts and *Giardia* cysts from wildlife (Kulda and Nohynkova, 1978; Jakubowski, 1990; Atwill, 1996), and a variety of human practices such as sewage processing and discharge, recreational use of water and animal agricultural activities exists (Gordon and Belosevic, 2002).

Livestock, and cattle in particular, are often perceived to be the leading source of waterborne *Giardia* and *Cryptosporidium* outbreaks in humans (Fayer et al., 2000; McLauchlin et al., 2000; Goh et al., 2004). The perception that livestock is a leading cause of water pollution

with *Cryptosporidium* oocysts and *Giardia* cysts is derived from the high prevalences of these parasites in livestock (O'Handley and Olson, 2006) and the magnitude of (oo)cysts shedding from infected animals. During peak shedding, infected animals can excrete up to 10^7 (oo)cysts per g of feces (Blewett, 1989; Xiao et al., 1994a; Fayer et al., 1998; de Graaf et al., 1999; O'Handley and Olson, 2006) for a number of days. Higher rates of infection and of (oo)cyst shedding tend to occur in young animals (Kirkpatrick, 1985; Sanford, 1987; Ongerth and Sibbs, 1989; Xiao et al., 1994b; Malgorzata et al., 1998; Atwill et al., 1999; Xiao et al., 1999; Ralston et al., 2003).

The association of animal agricultural activities with increased numbers of (oo)cysts in surface water has been suggested in some studies (Hansen and Ongerth, 1991; Ong et al., 1996; Ono et al., 2001) (Table 1.1). These studies examined the relationship between the occurrence and concentrations of (oo)cysts in surface water and typical land uses within a watershed. A consistent finding was that watersheds with more animal agriculture usages had increased *Cryptosporidium* oocyst and *Giardia* cyst concentrations compared with the more protected watersheds. The impact of animal agricultural activities on the contamination of surface water was demonstrated by the significantly higher numbers of (oo)cysts in water samples collected from the downstream locations than those from the upstream locations (Hansen and Ongerth, 1991; Ong et al., 1996; Ono et al., 2001) (Table 1.1).

Practices related to the storage and the application of animal manure to agricultural lands, on-farm discharge of contaminated water to land or to water sources, livestock pasturing on land adjoining water sources, and the disposal of fecally-contaminated waste from abattoirs may result in the contamination of the environment with large numbers of (oo)cysts (Slifko et al., 2000; Schijven et al., 2004).

As livestock, particularly cattle, are known to harbor the zoonotic species of *Cryptosporidium* and *Giardia*: *Cryptosporidium parvum* and *Giardia duodenalis* assemblages A and B, there is potential for serious public health consequences.

Prince Edward Island (PEI), Canada, is a rural region where animal husbandary is a common economic activity. It is therefore important to understand the public health risks posed by *Cryptosporidium* and *Giardia* infections in domestic livestock.

Table 1.1. Selected studies where animal agriculture has been implicated in the increased concentration of *Giardia* and/or *Cryptosporidium* (oo)cysts in surface water.

Location	Type of activity	Water sample	*Giardia/Cryptosporidium* spp	Distribution and incidence of (oo)cysts/L	Reference
Watershed in central cascade mountains, western Washington State, USA	Dairy farm	Surface water in protected watershed & unprotected watershed	*Cryptosporidium* spp	18 2 oocysts in unprotected watershed, 0 2 oocysts in protected watershed	(Hansen and Ongerth, 1991)
Watershed in western USA	Cattle, sheep, chicken farms	Site of river in agricultural area & 19 km upstream	*Cryptosporidium* spp *Giardia* spp	1 09 oocysts at site of agricultural area, 0 58 oocysts 19 km upstream of agricultural area 0 22 cysts at site of agricultural area, 0 08 cysts 19 km upstream of agricultural area	(Rose et al , 1988)
Central Taiwan	Swine farm	River upstream of swine farm & river downstream of the swine farm	*Cryptosporidium* spp *Giardia* spp	0 19 oocysts in upstream river sample, 0 31 oocysts in downstream river sample 0 11 cysts in upstream river sample, 0 14 cysts in downstream river sample	(Tai-Lee, 2002)
Southern British Columbia, Canada	Cattle ranch	Upstream & downstream water from stream on farm	*Cryptosporidium* spp *G duodenalis*	0 133 oocysts downstream, 0 056 oocysts upstream 0 090 cysts downstream & 0 052 cysts upstream	(Ong et al , 1996)
Japan	Cattle farms	Water sample from area with high density of cattle & area with low density of cattle	*C parvum*	0 12 oocysts in high cattle density area & 0 07 oocysts in low cattle density area	(Ono et al , 2001)

1.2 *Cryptosporidium*

1.2.1. Taxonomy

1.2.1.1. Historical perspective

The genus *Cryptosporidium* was discovered by Ernest Edward Tyzzer who described the type species, *Cryptosporidium muris*, found in gastric glands of laboratory mice in 1907 (Tyzzer, 1907). Later in 1910, he noted that similar organisms were attached to the epithelium of the small intestines of rabbits (Tyzzer, 1910). In 1912, he discovered a second isolate in the small intestines of mice which he named *Cryptosporidium parvum* to differentiate from other species because of its smaller size and intestinal origin (Tyzzer, 1912). It was not known to be of economic importance for the next 60 years. Then it was discovered to cause diarrhea in young calves in 1971 (Panciera et al., 1971), followed by the first case of human cryptosporidiosis in 1976 which involved a 3-year old girl from rural Tennessee who suffered severe gastroenteritis for two weeks (Flanigan and Soave, 1993). As a result, interest in *Cryptosporidium* was generated and it was subsequently reported in other animals with increasing frequency, and cryptosporidiosis was recognized as a zoonosis (Levine, 1984).

1.2.1.2. Taxonomic classification

Members of the genus *Cryptosporidium* in the phylum Apicomplexa were long thought to be closely related to coccidia. However, despite strong morphological similarities to these organisms, recent taxonomic studies, such as those based on the small subunit (SSU) rRNA and β-tubulin, indicated a closer relationship to the gregarines than the coccidia (Morrison and Ellis, 1997; Carreno et al., 1999; Leander et al., 2003).

Table 1.2. List of currently acknowledged *Cryptosporidium* species

Species	Type of host
C. andersoni	*Bos taurus* (domestic cattle)
C. baileyi	*Gallus gallus* (chicken)
C. bovis	*Bos taurus* (domestic cattle)
C. canis	*Canis familiaris* (domestic dog)
C. fayeri	*Macropus rufus* (red kangaroo)
C. felis	*Felis catis* (domestic cat)
C. fragile	*Duttaphrynus melanosticus* (Black-spined toad)
C. galli	*Gallus gallus* (chicken)
C. hominis	*Homo sapiens* (human)
C. macropodium	*Macropus giganteus* (grey kangaroo)
C. meleagridis	*Meleagris gallopavo* (turkey)
C. molnari	*Sparys aurata* (gilthead seabream)
	Dicentrarchus labrax (European seabass)
C. muris	*Mus musculus* (house mouse)
C. parvum	*Bos taurus* (domestic cattle)
C. ryanae	*Bos taurus* (domestic cattle)
C. scophthalmi	*Scophthhalmi maximus* (turbot)
C. serpenti	*Elaphe guttata* (corn snake)
	Elaphe subocularis (rat snake)
	Sanzinia madagascarensus (Madagascar boa)
C. suis	*Sus scrofa* (domestic pig)
C. ubiquitum	*Bos taurus* (domestic cattle)
C. varanii	*Varanus prasinus* (Emerald monitor)
C. wrairi	*Cavia porcellus* (guinea pig)
C. xiao	*Ovis aries* (domestic sheep)

Modified from Xiao and Fayer 2008

The closeness of *Cryptosporidium* to the gregarines is supported by the occurrence of extracellular stages in the life cycle

(Hijjawi et al., 2002; Hijjawi et al., 2004; Rosales et al., 2005). Currently, there are 22 recognized species (Table 1.2) and over 40 distinct genotypes of *Cryptosporidium* (Xiao and Fayer, 2008; Fayer et al., 2010). The *Cryptosporidium* species causing human disease are *C. parvum* and *C. hominis* (Morgan-Ryan et al., 2002). Nevertheless, host adapted species including

C. canis, *C. meleagridis*, *C. felis*, *C. suis*, *C. muris*, *C. ubiquitum* (previously cervine genotype) and several other genotypes: *C. suis*-like genotype, a *C. andersoni*-like genotype, the chipmunk genotype, the skunk genotype, the *C. hominis* monkey genotype and the pig genotype II have been detected in humans (Xiao and Fayer, 2008; Kvac et al., 2009b).

1.2.2. Life cycle and morphology

Cryptosporidium has a complex life cycle; involving both sexual and asexual cycles, which are entirely completed in one host (Monis and Thompson, 2003). Infected hosts shed oocysts, the environmentally resistant transmission stage of the parasite, with their feces (Fayer and Ungar, 1986; Fayer et al., 1997). These oocysts are immediately infectious and may remain in the environment for very long periods without losing their infectivity, due to a very robust oocyst wall that protects the four sporozoites against physical and chemical damage. Following ingestion by the host, the suture in the oocyst wall opens (excystation), triggered by both host- and parasite derived components (Smith et al., 2005). Four motile sporozoites are released into the small intestine of the host and they actively probe, attach, invade and become engulfed by host epithelial cells at the luminal surfaces mainly in the jejunum and ileum, establishing an intracellular but extracytoplasmic position within a parasitopherous vacuole (PV) (Chalmers and Davies, 2010). Occasionally, intracytoplasmic invasion occurs at Peyer's patches (Marcial and Madara, 1986). The sporozoites differentiate asexually into trophozoites which undergo merogony (asexual reproduction), producing two different types of meronts (O'Donoghue, 1995). Type I meronts transform into six or eight merozoites, structurally similar to sporozoites and released from the PV. The merozoites invade neighboring cells and either develop into trophozoites, repeating the asexual cycle, or into Type II meronts (Chalmers and Davies, 2010).

Type II meronts produce four merozoites, initiating the sexual cycle (gametogamy) when they are released from the PV, forming gamonts, the microgamonts (male gametes) and macrogamonts (female gametes) (Chalmers and Davies, 2010). Nuclear division in the microgamont leads to the production of numerous microgametes that are released from the PV and each macrogamont is fertilized by a microgamete (Smith et al., 2005). A zygote is formed by the process of fertilization.Within the zygote, an oocysts is formed which is fully sporurated (four sporozoites develop (sporogony) to form sporulated oocysts) (Smith et al., 2005). Both thin-walled and thick-walled oocysts are formed. While thick-walled oocysts are shed in the feces and are immediately infective, thin-walled oocysts remain within the host and can lead into autoinfection and persistent infections (Smith et al., 2005). Autoinfection may result in heavy infection of the epithelium of the small intestine, leading to malabsorptive or secretory diarrhoea. (Smith et al., 2005).

Studies on the *in vitro* maintenance of *C. parvum*, *C. andersoni* and *C. hominis* (Hijjawi et al., 2001; Hijjawi et al., 2002) revealed additional developmental stages in the life cycle of *Cryptosporidium*, and the existence of extended extracellular phases of development. While it is not clear where these stages fit into the life cycle, they resemble stages in gregarines, which provides further credence for the close relationship between *Cryptosporidium* and the gregarines.

1.2.3. Pathogenesis and clinical signs

The small intestine is the site commonly affected, although infection can be spread throughout the gastrointestinal tract and extra-intestinal sites (Chalmers and Davies, 2010). Once the host cell is invaded, *Cryptosporidium* resides in the luminal border of the enterocytes, leading to displacement of the microvillous border and loss of the surface epithelium. This causes villous

atrophy, blunting and crypt cell hyperplasia, and mononuclear cell infiltration in lamina propia (Meisel et al., 1976; Farthing, 2000).

In humans, the major symptom is watery diarrhea which may be associated with abdominal cramps, anorexia, weight loss, nausea, vomiting, fatigue and low grade fever (Fayer and Ungar, 1986; Casemore, 2000). The causative mechanisms involved in the development of diarrhea are not fully understood but they seem to be multi-factorial, consisting of effects of the parasite and its products on the epithelial layer and the immunological and inflammatory responses of the host leading to impaired intestinal absorption and enhanced secretion (Farthing, 2000). *Cryptosporidium* infection is also known to cause apoptosis of human intestinal epithelium (Griffiths et al., 1994; Ojcius et al., 1999). Similar to human cryptosporidiosis, the common clinical sign in animals is watery diarrhea which leads to dehydration, weight loss, fever and inappetence (Ramirez et al., 2004). The severity of clinical signs depends on the age, health and genetic background of the host, the environment, genotype and infective dose of the parasite (Xiao and Fayer, 2008). *Cryptosporidium* infection is more severe in immunocompromised and young hosts (Xiao and Fayer, 2008; Chalmers and Davies, 2010).

1.2.4. Diagnosis

1.2.4.1. Diagnosis of *Cryptosporidium* in fecal samples

Conventional methods for demonstrating *Cryptosporidium* oocysts in fecal specimens from human and other animals are Ziehl-Neelsen acid fast stain, modified Kinyoun's acid fast method (Mehta, 2002), negative staining (Pohjola, 1984) and Sheather's sugar flotation (Current et al., 1983). Diagnosis of *Cryptosporidium* in fecal specimens can also be achieved by examination of fecal smears using fluorescent stains such as auramin-carbolfuchsin (Mehta,

2002), auramine-rhodamine (Garcia et al., 1983), and acridine orange (Garcia et al., 1983). When these fluorescent stains are used, other staining methods may be required for confirmation of suspected oocysts (Current and Garcia, 1991). To maximize recovery of oocysts, quantitative gradient techniques such as simple flotation or centrifugal flotation in Sheather's sugar solution, zinc sulfate (1.18 or 1.20 specific gravity), or in saturated sodium chloride (1.27 specific gravity) are employed prior to microscopy (Zajac and Conboy, 2006). These staining methods are time-consuming, tedious and require experienced microscopists to accurately identify oocysts (Garcia et al., 1987; Kehl et al., 1995). As a result, direct immunofluorescence assay (DFA) procedures with *Cryptosporidium*-specific polyclonal or monoclonal antibodies have been developed for the identification of oocysts in stool specimens (Arrowood and Sterling, 1989). This is perhaps, the most sensitive method available for the diagnosis of cryptosporidiosis (Arrowood and Sterling, 1989).

Other immunological based detection methods have been developed for use in both clinical and environmental monitoring. These include indirect fluorescence antibody tests (IFAT) (Arrowood and Sterling, 1989; Casemore, 1991), enzyme linked immunosorbent assay (ELISA) and passive agglutination (Casemore, 1991). However, antigenic variability within clinical isolates of *Cryptosporidium* can result in some infections remaining undetected, and there are also conflicting reports about the sensitivity of immunodetection methods over microscopy (Morgan et al., 1998a).

1.2.4.2. Detection of *Cryptosporidium* in water samples

A variety of methods have been developed for the detection of *Cryptosporidium* in water samples which have specific advantages and disadvantages. While there is no universally accepted procedure, the predominant usage of the United States Environmental Protection

Agency (USEPA) method 1623 (USEPA, 2003) seems to make it the current standardized procedure. All conventional methods for retrieving *Cryptosporidium* oocysts from water samples involve four major steps: filtration, elution, clarification and concentration, and microscopic examination (Hu et al., 2004; Smith and Nichols, 2010). *Cryptosporidium* spp. oocysts occur in low numbers in water samples (Smith and Grimason, 2003). As a result, a system that enables efficient recovery from large volumes of water is required. Generally, two approaches are used in taking water samples. In large-volume sampling, water is passed through a concentrating filter over a period of several minutes to a few hours at a defined flow rate where oocysts are trapped in the filter, whereas in small-volume sampling, a volume of 10-20 l, typically is taken as a grab sample (Smith and Girdwood, 1999). Source water conditions determine the volume to be collected. As filtration may be difficult with high turbidity, such water is normally collected as a defined volume grab sample. While different methods have varied approaches for recovering (oo)cysts, in the USEPA method 1623 (USEPA, 2003), *Cryptosporidium* oocysts are eluted by several washes of the sampling (capsular) filter while oocysts are concentrated by membrane disc filtration. After concentration, the filter is washed and the sediment concentrated by centrifugation. Oocysts are isolated from the concentrate by immunomagnetic bead separation (IMS). The final pellet is applied to a microscope slide well, stained with fluorescent-based anti-*Cryptosporidium* antibody and viability stain, 4'-6-Diamidino-2-phenylindole (DAPI) and examined by fluorescence microscopy.

1.2.4.3. Molecular detection methods

In addition to staining and immunodiagnostic methods, *Cryptosporidium* detection has been enhanced with the application of molecular diagnostics. The development of sensitive and

specific molecular detection methods based on nucleic acid hybridization (Johnson et al., 1993), polymerase chain reaction (PCR) amplification techniques (Laxer et al., 1991) and PCR-restriction fragment length polymorphism (RFLP) has greatly increased our knowledge about the taxonomy and epidemiology of *Cryptosporidium* (Quintero-Betancourt et al., 2002).

Genetic loci generally used to identify *Cryptosporidium* species/genotypes include, the small subunit (SSU) rRNA (18S rRNA) (Fayer et al., 2006), the heat shock protein (HSP70 gene) (Guselle et al., 2003), the *Cryptosporidium* outer wall protein (COWP) gene (Spano et al., 1997), the gene for the thrombospondin-related adhesive protein of *Cryptosporidium*- 1 (TRAP-C1) (Spano et al., 1998), and the actin gene (Sulaiman et al., 2002). Approaches using multiple genetic loci increase the sensitivity of detection and the the accuracy of identification of *Cryptosporidium* (Smith et al., 2006).

1.2.4.4. Sub-genotyping of *Cryptosporidium*

To accurately understand transmission patterns, track outbreaks, and elucidate the population genetic structure of *Cryptosporidium*, the detection of genetic variation within a species is central. Researchers have used highly discriminatory sub-genotyping/fingerprinting techniques to examine the population structure and transmission dynamics of *C. parvum* and *C. hominis*. The 60 kDa glycoprotein (GP60, also known as gp15/40) –based PCR sequencing tool has proven to be useful (Xiao and Ryan, 2004; Alves et al., 2006; Thompson et al., 2007). Not only is it the most polymorphic marker identified so far in the *Cryptosporidium* genome (Gatei et al., 2006; Leoni et al., 2007; Wielinger et al., 2008), but also it is biologically pertinent. It encodes surface glycoproteins (gp45 and gp15), both of which are implicated in zoite attachment to and invasion of enterocytes (Strong et al., 2000).

The GP60 gene is similar to a microsatellite sequence by having tandem repeats of the serine-coding trinucleotide TCA, TCG, or TCT at the 5' end of the gene (Strong et al., 2000). In addition to the variations in the number of trinucleotide repeats, there are extensive sequence differences in the non-repeat regions, which categorize *C. parvum* and *C. hominis* each to several subtype families. Within each family, subtypes differ from each other mostly in the number of trinucleotide repeats (TCA, TCG, or TCT microsatellite).

The name of GP60 subtypes starts with the subtype family designation (Ia, Ib, Ie, If, etc. for *C. hominis* and IIa, IIb, IIc, IId, etc. for *C. parvum*) followed by the number of TCA (represented by the letter A), TCG (represented by the letter G) or TCT (represented by the letter T) repeats (Sulaiman et al., 2005). For example, the designation IIaA12G2R1 indicates the parasite belongs to *C. parvum* subtype IIa and has 12 copies of the TCA repeats and 2 copies of the TCG repeat in the trinucleotide repeat region of the GP60 gene. R1 and R2 are used to indicate the number of ACATCA repeats immediately after the trinucleotide repeats.

Using the GP60 gene locus in subtype analysis of *C. parvum*, two zoonotic (IIa, IId) and 10 non-zoonotic (IIb, IIc, IIe-III) subtype groups have been identified (Sulaiman et al., 2001; Alves et al., 2003; Peng et al., 2003a; Peng et al., 2003b; Sulaiman et al., 2005; Abe et al., 2006; Akiyoshi and Tzipori, 2006; Alves et al., 2006; Trotz-Williams et al., 2006). Only *C. parvum* subtype families (IIa and IId) have to date, been found in both humans and animals (Alves et al., 2003; Sulaiman et al., 2005; Trotz-Williams et al., 2006).

Different subtypes may be clustered in different geographic locations. For example, the *C. parvum* subtype IIaA15G2R1 is the most commonly identified zoonotic infection in calves in the US and Canada and in humans from around the world, including the US, UK, Portugal, Slovenia, Australia, Japan, and Kuwait (Xiao et al., 2007). The IIa *C. parvum* subtypes

A17G2R1, A18G3R1, A19G3R1 and A20G3R1 are common among humans in Australia, Canada and Northern Ireland (Trotz-Williams et al., 2006; Ng et al., 2008; Zintl et al., 2009). Subtype IIaA17G1R1 was the most common subtype in the UK while subtypes IIaA16G2R1 and IIaA15G2R2 are mostly responsible for zoonotic *Cryptosporidium* infections in other parts of Europe (Portugal, Ireland, Slovenia) (Glaberman et al., 2002; Alves et al., 2003; Alves et al., 2006).

The zoonotic *C. parvum* belonging to the subtype family IId has been found only in a few specimens from humans, cattle, sheep and water samples in Kuwait, Portugal, Spain, Serbia and Hungary (Quilez et al., 2008). The detection of this allele has not been reported in animals or humans in North America, suggesting that this group is not a major zoonotic pathogen in North America (Xiao et al., 2007).

1.2.5. Epidemiology

1.2.5.1. Prevalence of *Cryptosporidium* in cattle and pigs

1.2.5.1.1. Cattle

Cryptosporidium parvum is widely distributed throughout North American cattle operations, with the majority of studies being completed in dairy cattle (Mann et al., 1986). In the United States, a study of 1103 dairy farms from 28 states reported the presence of *Cryptosporidium* on 59.1% of the farms and in 22.4% of calves (1648/7369).. Fecal samples collected from one to three week old calves were positive 48% of the time (Garber et al., 1994). Also, in a well-defined watershed in the northeastern United States, *Cryptosporidium* was found on 91% percent of 11 dairy farms (Sischo et al., 2000). The difference in the rate of infection in the two studies might be accounted for by the difference in the design of the two studies. While

the former was a straight survey, the latter involved sampling of farms over a 6-month period, which resulted in the increased likelihood of getting more positives.

In a Canadian study, *Cryptosporidium* was found in 20% of fecal samples from cattle (Olson et al., 1997a). As well in the Fraser river valley of British Columbia, 80% of dairy farms tested were positive for *C. parvum* (Olson et al., 1997). Other Canadian studies have found higher herd prevalences of *Cryptosporidium* on dairy farms in Alberta and Quebec, at 100% and 88%, respectively (O'Handley et al., 1999; Ruest et al., 1998). Lower prevalences were found in beef cows (1.1%) and beef calves (3.1%) for *Cryptosporidium* in Canada (Gow and Waldner, 2006).

In other parts of the world, *C. parvum* oocysts were detected in 62.4% of fecal samples from two herds of apparently healthy adult beef cattle in the UK (Pettoello-Mantovani et al., 1995). In Australia 48% of calves <3 months of age, were positive for *C. parvum* (Becher et al., 2004) on two dairy farms while *C. parvum* was reported in 63.6% and 36.6% of dairy calves from two sale-yards respectively in southern Western Australia (Thompson, 2003).

Cryptosporidium spps. was detected in 70.3 and 31.4% of cows and calves, respectively, in Turkey (Degerli et al., 2005). The rather low prevalence for calves as compared to adult cattle in this study contravenes the results generally observed in other studies but may be due to the small number of adult cattle sampled (70 adult cattle as opposed to 387 calves).

In Zambia, herd prevalences in dairy, beef and traditional husbandry systems was 42.8, 8.0 and 6.3%, respectively. Moreover, 76% of the dairy farms, 44.0% of the beef farms and 15.2% of the traditional husbandry farms had at least one positive calf at the time of visit (Geurden et al., 2006). Possible reasons for the low incidence of oocysts on the traditional husbandry farms and animals may be related to mitigation of oocyst transmission on open range

in traditional husbandry as opposed to confinement in the other husbandry practices. In Korea, *Cryptosporidium* was detected in 22.2% adult dairy cattle (Rhee et al., 1991).

1.2.5.1.2. Pigs

A handful of studies have been conducted to determine the prevalence of *Cryptosporidium* in pigs worldwide. In a survey of 200 market swine offered for sale at a southern California livestock auction, 5% tested positive for *Cryptosporidium* oocysts (Tacal et al., 1987). In a retrospective study in Canada, a prevalence of 5.3% in pigs aged from 1 to 30 weeks and 60% in pigs from 6 to 12 weeks was reported (Sanford, 1987). *Cryptosporidium* was also identified in 3 of 4 sampling sites with an overall animal prevalence of 11% in another Canadian study (Olson et al., 1997a). In Alberta, 32% of 50 farms tested positive for *Cryptosporidium* with an overall prevalence of 2.8%, and most of the infections (10.4%) were reported in weaners (Guselle and Olson, 1999). In a longitudinal study, also in Alberta a cumulative rate of infection for *C. parvum* of 100% in pigs with a mean time of initial oocyst detection of 45.2 ± 16 days was reported (Guselle et al., 2003b). The authors suggested that the much lower prevalence reported in other studies may be due to the effect of intermittent oocysts shedding in point prevalence studies.

In other studies, *Cryptosporidium* was detected in 1.4% of piglets in Germany (Wieler et al., 2001) and 22% of 1 to 6 months old pigs but not in suckling piglets or adults in northeastern Spain (Quilez et al., 1996b). *Cryptosporidium* on Korean pig farms was 20% in the Chollabuk-do area (Rhee et. al 1991) and 11% in the rural areas of Chungcheong-do (Jae-Ran and Seo, 2004) while in Japan 33% of 1- to 3-month old weaned piglets tested positive for the parasite from 4 of 8 stock-raising farms (Izumiyama et al., 2001).

Unlike ruminants, most *Cryptosporidium* infections in pigs are delayed until after weaning at ~4-10 weeks of age and may be associated with weaning stress (Angus, 1990; Quilez et al., 1996c; Guselle et al., 2003; Zintl et al., 2007b). The low level of *Cryptosporidium* infections in pigs before weaning may relate to farm hygiene practices which reduce environmental contamination (Xiao et al., 1994a; Mohammed et al., 1999).

For example, in a study where *Cryptosporidium* infection on a swine farm described to have good hygiene practices, occurred only in weaning piglets while on another with poor hygiene *Cryptosporidium* infection was found in both nursing piglets and weanlings (Xiao et al., 1994a). It may also relate to early protection afforded by innate and acquired immune factors in the mother's milk (Guselle et al., 2003). In older pigs (fattening and sows) *Cryptosporidium* infections are rare (Vitovec et al., 2006; Suarez-Luengas et al., 2007). This is important when considering on-farm mechanisms for preventing watershed contamination with this parasite.

1.2.6. Distribution of *Cryptosporidium* species/genotypes in cattle and pigs and implications for zoonosis

The common use of molecular methods for determining species and genotypes of *Cryptosporidium* in the last decade has revealed an association of specific species/genotypes with different livestock hosts and also the existence of an age structure distribution (Armson et al., 2009; Yang et al., 2009) (Table 1.4). The fact that younger animals are more susceptible to infection and are the predominant carriers of zoonotic species/genotypes could only have been revealed through fine scale molecular analysis. With molecular methods, we finally have a means of assessing the contribution of different livestock hosts and age demographics with their

potential to contaminate the environment, foods and the watershed with zoonotic species of *Cryptosporidium*.

Cattle are infected with four species of *Cryptosporidium*: *C. parvum*, *C. andersoni*, *C. bovis* and *C. ryanae*. The prevalence of infection with *Cryptosporidium* species in cattle decreases with age (Table 1.4).

Several studies have demonstrated that in cattle, *C. parvum* appears first in young calves ~1-3 weeks of age, followed by *C. bovis* and *C. ryanae* (previously, the deer-like genotype) in older calves, 3-8 months of age. *C. andersoni* appears later and predominates in cattle >8 months of age (Feltus et al., 2008). Being more prevalent for the zoonotic *Cryptosporidium, C. parvum*, the capacity for calves to increase the environmental burden of *Cryptosporidium* oocysts with subsequent public health implications is high, and therefore contamination intervention strategies for *Cryptosporidium* from cattle should concentrate on calves.

Cryptosporidium infections in pigs are common and found in all age groups from nursing piglets to adults, with weaners having the highest prevalence (Zintl et al., 2007b; Armson et al., 2009). Similar to calves, piglets and weaners are the major source of environmental contamination on hog farms.

In studies where isolates have been genotyped (Table 1.4), two distinct swine-specific *Cryptosporidium* species/genotypes, *C. suis* and *Cryptosporidium* Pig genotype II have been revealed. However, occasionally, *C. parvum* and *C. muris* have been reported (Morgan et al., 1999; Langkjaer et al., 2007; Zintl et al., 2007b; Johnson et al., 2008a; Kvac et al., 2009a).

An age-related infection pattern with different species and genotypes has been suggested, with *C. suis* predominating in pigs <7 weeks old (Ryan et al., 2003; Vitovec et al., 2006; Kvac

et al., 2009a) and pig genotype II in pigs > 7 weeks old (Langkjaer et al., 2007; Zintl et al., 2007b; Johnson et al., 2008).

Although uncommon, human clinical cases of *C. muris* or *C. suis* infections have been documented (Xiao et al., 2002; Cama et al., 2003; Muthasamy et al., 2006). Recently, pig genotype II was isolated from an immunocompetent patient with diarrhea but the clinical signs could have been caused by co-infection with *G. duodenalis* assemblage A (Kvac et al., 2009b). However, the identification of *Cryptosporidium parvum* "Pig" genotype in a HIV positive patient who was not severely immunosuppressed may alter the dynamics of pigs as reservoirs of *Cryptosporidium* infectious to humans (Xiao et al., 2002).

1.3 *Giardia*

1.3.1. Taxonomy

1.3.1.1. Historical perspectives

Giardia belongs to the class *Zoomastigophorea* and the order *Diplomonadida* (Thompson and Monis, 2004). Although it was likely first described by Antony van Leeuwenhoek over 300 years ago as he was examining his own diarrheal stool under a microscope (Dobell, 1920), Vilem Lambl (1824 to 1859), a Czech physician, was credited with the discovery in 1859 of the flagellate *Giardia*. In 1888, Blanchard suggested the name *Lamblia intestinalis* (Blanchard, 1888). Thereafter, Stiles changed the name to *G. duodenalis* in 1902 (Stiles, 1902). Controversy shrouded the phylogenetic affinities of *Giardia* for many years, where some investigators suggested species names on the basis of host of origin while others resorted to morphology (Adam, 2001). Over 50 species were described based on host specificity (Thompson, 2004). In 1952, Filice proposed that three species names be used: *G. duodenalis* (also known as *G. intestinalis* and *G. lamblia*), *G. muris* and *G. agilis* on the basis of median body morphology

(Filice, 1952). *Giardia* which were previously grouped with *G. lamblia* based on morphology through light microscopy have been reclassified as different species, *G. psittaci* from parakeets (Erlandsen et al., 1989) and *G. ardeae* from herons (Erlandsen et al., 1990) using electron microscopy.

Table 1.3. List of well defined species and assemblages of *Giardia*

Species*	Hosts
G. agilis	Amphibians
G. ardeae	Birds
G. microti	Muskrats and voles
G. muris	Rodents
G. psttaci	Birds
G. duodenalis	
Assemblage A	Humans, , primates, dogs, cats, cattle, rodents, wild mammals
Assemblage B	Humans, primates, dogs, horses, cattle
Assemblage C	Dogs
Assemblage D	Dogs
Assemblage E	Artiodactyls
Assemblage F	Cats
Assemblage G	Rodents
Assemblage H	grey seal, gull

*Xiao and Fayer 2008

Another species, *Giardia microti*, has been described based on cyst morphology using electron micrography (Feely, 1988) and the 18S rRNA gene (van Keulen et al., 1998). To date six species of *Giardia* are recognized (Table 1.3).

Giardia duodenalis cysts are infective and environmentally resistant, and are ovoid, smooth-walled, and measure 8 to 15 μm in length and 7 to 10 μm in width (Dryden et al., 2005). *Giardia* cysts may appear somewhat transparent under the microscope. They contain 2 to 4 nuclei, medium bodies, and structural elements of the adhesive disc and flagella (Thompson et al., 1993). Trophozoites, the reproductive and feeding stages are environmentally fragile. They

measure between 12 to 17 μm in length and 7 to 10 μm in width and have a bilaterally symmetrical pear-shape. Eight flagella enable motility of the trophozoite, which also has 2 nuclei and 2 median bodies inside.

1.3.1.2. Taxonomic classification

The morphology of *Giardia* trophozoites has been used to differentiate six species, but only *Giardia duodenalis* is found in humans and most other mammals (Xiao and Fayer, 2008). *G. duodenalis* from humans and animals are morphologically identical but genotyping has defined eight assemblages A-H (Feng and Xiao, 2011) (Table 1.3). Only *G. duodenalis* assemblages A and B are associated with human infections.

Allozyme analysis originally divided isolates within these two assemblages into four subgroups, with the subgroup AI and AII within assemblage A and the subgroup III and IV within assemblage B (Monis et al., 1999). However, genetic characterization studies at the β-giardin, triosephosphate isomerase (*tpi*), 16S rRNA, and glutamate dehydrogenase gene (*gdh*) loci have indicated greater genetic variability exists among a number of assemblages of *G. duodenalis* than was first thought (Sulaiman et al., 2003; Lalle et al., 2005), which could lead to the further subdivision of currently recognized assemblages.

1.3.2. Life cycle and morphology

Compared to *Cryptosporidium*, *Giardia* has a relatively simple biphasic life cycle consisting of the infectious cysts, which are resistant to many environmental factors, and the trophozoite, which colonizes the intestinal lumen and causes disease (Chavez-Munguia et al., 2007). Cysts are ingested through contaminated drinking water or occasionally food, or are acquired by person-to-person contact (Slifko et al., 2000). This is followed by excystation, in

which two trophozoites are released from each cyst in the small intestine (Lauwaet et al., 2007). Excystation is a complex process, involving physiologic, structural and molecular changes on the cyst wall which are triggered by luminal host signals encountered in the stomach and duodenum, i.e. low pH followed by elevated pH and proteases (Gillin et al., 1996; Adam, 2001).

Trophozoites multiply by longitudinal binary fission and colonize the mid-jejunum where they can be free or attached to the mucosa by a ventral sucking disk (Adam, 2001; Lauwaet et al., 2007). As they travel into the lower regions of the small intestine, encystation is activated by exposure to bile (Adam, 2001). During encystation, the trophozoites undergo nuclear cell division resulting in the formation of a cyst containing four tetraploid nuclei (Adam, 2001), and cysts are voided in the feces.

1.3.3. Pathogenesis and clinical signs

Studies in several animals as well as in human epithelial cell lines point to the fact that infections with *Giardia* are remarkably devoid of typical histological features (Eckmann, 2003). In humans with dramatic symptoms of infection, most (> 95%) with non-specific gastrointestinal complaints and *Giardia*-positive duodenal biopsies, there was no inflammation or gross alteration of the epithelium and the duodenal mucosa was considered 'normal' (Oberhuber et al., 1997). Despite the absence of gross histological signs, giardiasis is often accompanied by subtle changes in ultrastructure and function of the intestinal epithelium, primarily microvillus alterations including a decreased crypt to villus ratio and brush border enzyme deficiencies (Buret et al., 1990b; Buret et al., 1990c; O'Handley et al., 2001; Scott et al., 2002). Given the absence of these alterations in response to a *Giardia* infection in T- and B-cell deficient mice (Scott et al., 2000), the alterations are not only a direct consequence of the interaction between

trophozoites and the intestinal epithelium but also are mediated by the host's immune response (Geurden et al., 2010). Pathogenesis of giardiasis is therefore a multifactorial process involving both parasite characteristics and the host immune response (Geurden et al., 2010). In giardiasis, an increase in epithelial permeability has also been observed and appears to result from enterocyte apoptosis (Chin et al., 2002) and cytoskeletal reorganization induced by trophozoite toxic products (Buret et al., 2002; Scott et al., 2002), leading to local disruption of tight-junctional-proteins (Geurden et al., 2010).

Generally, *G. duodenalis* infection is more severe in immunocompromised or young hosts (Kirkpatrick, 1987; St Jean et al., 1987; Xiao et al., 1993; Faubert, 2000). In humans, clinical signs are characterized by malabsorptive diarrhea, dehydration, abdominal pain, nausea, vomiting and weight loss (Thompson and Monis, 2004). In humans, infections with assemblage A are more likely to result in clinical signs than infections with assemblage B (Read et al., 2002). However, once established, assemblage B appears to result in more persistent diarrhea (Homan and Mank, 2001). In animals, infection by *Giardia* typically occurs in calves between 5 and 10 weeks of age; however, infections can occur in calves as young as 4 days (Xiao et al., 1993; Xiao and Herd, 1994; O'Handley et al., 2000). The predominant clinical sign in animal hosts is malabsorptive diarrhea which may be accompanied by vomiting, steatorrhea, abdominal pain and weight loss (O'Handley et al., 1999; Olson et al., 2001). It is possible for infections to persist in calves for several months resulting in numerous episodes of diarrhea (Xiao et al., 1993; Xiao and Herd, 1994). The severity of infection depends on the host, environmental factors and the genotype of the parasite (Thompson, 2004).

1.3.4. Diagnosis

1.3.4.1. Diagnosis from fecal samples

Detection of *Giardia* in fecal samples follows similar isolation procedures as described for *Cryptosporidium*. Detection of cysts and trophozoites usually rely on microscopic methods (Zajac and Conboy, 2006). To increase the sensitivity of detection, simple flotation and centrifugal flotation techniques, using various salts (zinc sulphate or sodium nitrate) or sugar solutions, followed by light microscopy are commonly applied in parasitological fecal examinations (Zajac et al., 2002; Dryden et al., 2005).

Direct fluorescent-antibody (DFA) staining and enzyme immunoassays are sensitive, cost effective and widely used in detecting *Giardia* in fecal samples (Appelbee et al., 2003; Trout et al., 2004). Their use is limited by the need for numerous reagents additions and specialized equipment. Other methods of detecting and characterization of *Giardia* have included surface antigen analysis, isoenzyme analysis, RFLP analysis and pulse-field gel electrophoresis (PFGE) of chromosomal patterns (Adam, 2001).

The use of flow cytometry, an automated process has also been successfully used in the diagnosis of *Giardia* and other parasites such as *Cyclospora*, and *Cryptosporidium* (Arrowood et al., 1995; Dixon et al., 2005).

1.3.4.2. Diagnosis from water samples

The major steps in the isolation of *Giardia* cysts from water samples are similar to those described for *Cryptosporidium* above. The anti–*Cryptosporidium* and anti-*Giardia* immunomagnetic separation (IMS) kit (Dynabeads GC Combo IMS Kit; Dynal) is used for concentrating both *Giardia* cysts and *Cryptosporidium* oocysts, and FITC-labelled anti-*Giardia*

and anti-*Cryptosporidium* antibodies (AquaGlo G/C complete kit; Waterborne) are used for detecting both cysts and oocysts.

1.3.5. Molecular detection method

Molecular tools, particularly PCR and sequencing have been used to elucidate the epidemiology and zoonotic significance of *Giardia* species. Most molecular epidemiological studies were undertaken using single markers (O'Handley et al., 2000; Becher et al., 2004; Coklin et al., 2007). The majority of studies have used the 16S ribosomal RNA (16S rRNA), beta giardin (β-giardin), glutamate dehydrogenase (*gdh*), triose phosphate isomerase (*tpi*), elongation factor 1 α (EF-1α) and *G. duodenalis* open reading frame C4 (GLORF-C4) (Caccio et al., 2005a). It has been shown that using two or more markers in genotyping is more accurate and reliable (Sprong et al., 2009). However the occurrence of heterogeneous templates and difficulties in assigning some isolates to a specific assemblage is a common problem in *Giardia* genotyping (Caccio and Ryan, 2008).

1.3.6. Epidemiology

1.3.6.1. Prevalence of *Giardia* in cattle and pigs

1.3.6.1.1. Cattle

A number of prevalence studies on *Giardia* in cattle have been reported, the majority of which were conducted in North America. The prevalence in pre-weaned dairy calves (n=407) examined from 14 farms in 7 states in the USA ranged from 9 to 93% with an average of 40%. The non-zoonotic Assemblage E accounted for 85% of positive samples while 15% were the zoonotic Assemblage A (Trout et al., 2004). In a recent study, the prevalence of *Giardia* in adult

dairy cattle was found to range from 3 to 64%, with an average prevalence of 27%. Genotyping revealed that 94% of the *Giardia* isolates were Assemblage E and 6% were assemblage A (Trout et al., 2007). Wade et al. (2000) found 8.9% of the 2943 fecal samples collected from 109 dairy herds in five counties of southwestern New York tested positive for *Giardia.* Cysts were most prevalent in calves younger than 6 months of age with 20% of the calves in this age group testing positive. The lower prevalence in this study as compared to the previous may be due to the difference in sample size of animals tested. *Giardia* species were detected at a prevalence of 6.5% from a random sample of adult beef cattle (Hoar et al., 2001).

In Canada, an overall prevalence of 29% from 104 beef cattle from six different locations was reported (Olson et al., 1997a). *Giardia* was also detected on 45.7% of dairy farms in Quebec (Ruest et al., 1998) and on all 20 dairy farms examined in the Fraser River Valley in British Columbia, with an overall prevalence of 73% for calves (Olson et al., 1997). In Southern Alberta, no adult cattle were infected but 22.7% of calves tested had cysts in their feces (Buret et al., 1990a). In another Alberta study, the prevalence and genotypes of *G. duodenalis* in beef calves from large cow-calf farms were determined. *Giardia* cysts were found on all farms and in 168 of the 495 fecal samples examined, with a prevalence ranging from 7 to 60% among farms. In the same study, 41 of the 42 samples genotyped belonged to Assemblage E and 1 belonged to Assemblage A (Appelbee et al., 2003).

In comparing the prevalence and genotypic characterization of *Giardia* in dairy calves from Western Australia (n=36) and Western Canada (n=28), 58% of Western Australian and 57% of Western Canadian calves were found to be positive for *Giardia.* Ten positive samples from Western Canada and 5 from Western Australia underwent genetic characterization. Eight of

the Western Canadian isolates were Assemblage E and 2 were Assemblage A while four of the Western Australian isolates were Assemblage E and 1 Assemblage A (O'Handley et al., 2000).

McAllister et al. (2005) reported an overall prevalence of 8.7% for *Giardia* in 669 beef cows from 39 farms located within 10 counties of Ontario. *Giardia* was detected on 64% of the farms. They also sampled 192 calves from 10 beef farms representing 4 watersheds, in British Columbia and found an overall prevalence for *Giardia* to be 36%.

Outside North America, 40.6% of fecal samples tested from calves up to 8 weeks old (n=715) in New Zealand, contained *G. intestinalis* cysts (Hunt et al., 2000), while in Spain an overall prevalence of 11.7% (65 of 554 bovine) from 16 of 30 (53.3%) farms surveyed was reported (Quilez et al., 1996a). *Giardia* cysts were detected in 26.6% of the 815 calves sampled randomly from various regions of Switzerland (Taminelli and Eckert 1989). In Italy, all isolates of *G. duodenalis* from dairy calves examined were found to correspond to the Assemblage E genotype (Berrilli et al., 2004).

Like *Cryptosporidium*, the majority of studies reporting on the presence of *Giardia* in ruminants have concentrated on calves that have traditionally been more susceptible to infections with microbial agents. However, it has been observed that infections with *Giardia* in ruminants, unlike *Cryptosporidium*, are most common toward the end of the neonatal period, and cysts can be excreted for at least 100 and 175 days for dairy and beef calves, respectively (O'Handley and Olson, 2006).

1.3.6.1.2. Pigs

Giardia infection has been documented in domestic pigs around the world. In a study by Olson et al. (1997a), an overall prevalence of 9% was reported in Canadian farms at different

geographical locations. This was followed by a large study in Alberta, involving 1602 pigs on 50 farms in which *Giardia* was found in 70% of the farms and 8.5% of fecal samples collected. The distribution of *Giardia* cysts were 3.8, 9.8, 10.8, 15.0, 5.7 and 4.1% for piglet, weaners, growers, finishers, boars and sows, respectively (Guselle and Olson, 1999).

In a Norwegian study, 10 of 684 (1.5%) litters of suckling piglets from 100 indoor swine herds tested positive for *Giardia* (Hamnes et al., 2007). In this study, 10 of the 100 (10%) herds had at least one positive litter. In Denmark, *Giardia* was found in 40% of weaner pigs (Maddox-Hyttel et al., 2006).

1.3.7. Distribution of *Giardia* species/genotypes in cattle and pigs and implications for zoonosis

Studies which have involved gene sequence analysis have all shown that the *G. duodenalis* isolated from cattle in all age groups mostly belongs to the non-zoonotic genotype Assemblage E (Trout et al., 2006) (Table 1.4). This has led to the general suggestion that *Giardia* carried by cattle may be of minimal zoonotic threat and therefore of low epidemiological significance. However, the zoonotic *G. duodenalis* assemblage A has been reported at within-herd prevalences of 2-59% (O'Handley RM et al., 2000; Appelbee et al., 2003; Trout et al., 2004; Trout et al., 2005; Trout et al., 2006; Geurden et al., 2008) and herd prevalences of 50-71% (Trout et al., 2004; Trout et al., 2005; Trout et al., 2006; Uehlinger et al., 2006). *G. duodenalis* assemblage B has also occasionally been detected in cattle (Coklin et al., 2007) (Table 1.4). Although the presence of *G. duodenalis* assemblages A and B in cattle suggests a possible risk, the threat of *Giardia* to environmental contamination from cattle leading to human infection, may not be as important as *Cryptosporidium*.

Previous reports have shown that the genotypes of *Giardia* in pigs are also predominantly the non-zoonotic assemblage E (Ey et al., 1997; Guselle and Olson, 1999). However recent studies have identified the zoonotic *G. duodenalis* assemblage A in pigs (Guselle and Olson 2005; Langkjaer et al., 2007) and therefore pigs can be a source of human infection with this parasite. Younger pigs are also more likely to be infected with assemblage A while assemblage E predominates in older animals (Zintl et al., 2007b; Armson et al., 2009). Like in cattle, the threat to environmental contamination with *Giardia* from pigs leading to human infection may not be as important as with *Cryptosporidium*.

Table 1.4. Selected studies of age-related prevalence of *Cryptosporidium* and *Giardia* in domestic livestock where isolates were genotyped

Species	Location (Study type)	Number of herds sampled (% Herd Prevalence)	Age group (#of animals)	*Crypto* Prevalence (%)	*Giardia* Prevalence (%)	# of samples analyzed by PCR (PCR method)	# Positive by PCR (%)	*Crypto* spp. Identified (% of PCR positive)	*Giardia* assemblage (% PCR positive)	Reference
Dairy Cattle	Canada; Australia (Cross-sectional)	1 (100) 2 (100)	2-10 weeks Canada - 28 Australia - 36	ND	57 58	10 6 (direct PCR of 16S)	10 (100) 5 (83)	ND	A (20); E (80) A (20); E (80)	(O'Handley et al., 2000)
	Western Australia (Longitudinal)	2 (100)	≤3 weeks (10) 4-7 weeks (28) ≥8 weeks (16)	20.7 3.4 0.0	17.8 52.3 29.0	NS (direct 16S PCR)/ (direct 18S PCR) *Giardia/ Crypto*)	31(NS) *Giardia* and 6 (NS) for *Crypto* from all ages	*C. parvum* (100)	E (100)	(Becher et al., 2004)
	USA (Cross-sectional)	14 (93)	3-11 months (464)	ND	31	456 (direct 16S PCR)	237 (52)	ND	A (13), E (87)	(Trout et al., 2005)
	USA (Cross-sectional)	14 (100)	12-24 months (571)	ND	36	571 (direct PCR of 16S)	204 (36)	ND	A (9), E (91)	(Trout et al., 2006)
	Zambia (Cross-sectional)	37 (75.7)	1-90 days (250)	42.8	ND	32. (nested PCR of both 18S and HSP70)	30 (93.7) for HSP70; 32 (100) for 18S	*C. parvum* (65.6); *C. bovis* (28.1) for Hsp70, *C. parvum* (68.8) and *C. bovis*	ND	(Geurden et al., 2006)

								(31.2) for 18S		
	Canada (Cross-sectional)	2 (100)	0.4-6 months (91) 1-2 years (6) >2 years (46)	39.6 50 0.0	45 100 28.3	91 6 46 (nested 16S PCR for *Giardia* & HSP70 for *Crypto*)	41 (45)/ 36 (39.6) 6 (100)/ 3 (50) 13 (28.3)/ 0 (0) *Giardia*/ *Crypto*	*C. parvum* (30.8); *C. bovis* (2.2) *C. parvum* (50) No *Crypto* detected	B (24), E (21) B (50), E (50) B (21.7), E (6.5)	(Coklin et al., 2007)
	Spain (Cross-sectional)	60 (48.3)/ (96.6) *Crypto/Giardia*	3-13 years (379)	8.4	26.6	32/101 *Crypto/Giardia* (Nested HSP70 & 18S PCR)/ (nested β-giardin & *gdh* PCR)	10 (31)/ 4 (4)	*C. parvum* for all 10 positive samples	*G. duodenalis* assemblage E for all 4 positive samples	(Castro-Hermida et al., 2007)
	Belgium (Cross-sectional)	100 (48)	≤10 weeks (499)	ND	22	49 for β-giardin; 28 for *tpi* genes (direct β-giardin & *tpi* PCR)	49 (100) for β-giardin; 28 (100) for *tpi*	ND	A (59) for β-giardin) & (39) for *tpi*; E (41) for β-giardin) & (61) for *tpi*	(Geurden et al., 2008)
Beef Cattle	Canada (Cross-sectional)	9 (100)	2-10 weeks (495)	ND	34.3	42 (nested 16S PCR)	42 (100)	ND	A (2), E (98)	(Guselle et al., 2003)
	Zambia (Cross-sectional)	25 (44.0)	24 days[1] (238)	8.0	ND	8 (nested 18S & HSP70 PCR)	8 (100) for Hsp70; 4 (80) for 18S	*C. parvum* 7 (87.5) & *C. suis* 1 (12.5) for HSP70, *C. parvum* 3 (37.5) & *C.*	ND	(Geurden et al., 2006)

								suis 1 (12.5) for 18S		
	Belgium (Cross-sectional)	50 (64)	≤10 weeks (333)	ND	45	44 for β-giardin; 24 for *tpi* genes (direct β-giardin & *tpi* PCR)	44 (100) for β-giardin; 24 (100) for *tpi*	ND	A (16) for β-giardin) & (9) for *tpi*; E (84) for β-giardin) & (91) for *tpi*	(Geurden et al., 2008)
Cattle*	Viet Nam (Cross-sectional)	NS	<3 months (31) 3-6 months (48) >6-12months (78) >12 months (109)	48.4 41.8; 6.3; 2.1 26.9; 2.6; 1.3 30.3; 9.2; 6.4	ND	NS (nested 18S PCR)	NS	*C. parvum* (48.4) *C.parvum* (41.8); *C. andersoni* (6.3); mixed *C. parvum* & *C. andersoni* (2.1) *C.parvum* (26.9); *C. andersoni* (2.6); mixed *C. parvum* & *C. andersoni* (1.3) *C.parvum* (30.3); *C. andersoni* (9.2); mixed *C. parvum* & *C. andersoni* (6.4)	ND	(Nguyen et al., 2007)
	Brazil (Cross-	NS	<30 days (8) 2 months (1)	100 100	ND	8 1	8 (100) 1 (100)	*C. parvum* 8 (100)	ND	(Thomaz et al., 2007)

	sectional)					(nested 18S PCR)		*C. bovis* 1 (100)		
TDNL	Zambia (Cross-sectional)	92 (15.2)	24 days (256)	6.3		5 (nested 18S & HSP70 PCR)	5 (100) for HSP70; 4 (80) for 18S	All *C. bovis*	ND	(Geurden et al., 2006)
Pig	Australia (Cross-sectional)	22 (NS)	<5 weeks (199) 5-8 weeks (447)	30.8 69.2	ND	7 21) (nested 18S PCR)	7 21	*C. suis* (71.4); PG II (28.6) *C. suis* 12 (57.1); PG II (71.4)	ND	(Ryan et al., 2003)
	Czech Republic (Cross-sectional)	8 (100)	≤5 weeks (3368) 6-13 weeks (835) <13 weeks (135)	5.7 24.1 0	ND	NS (direct 18S PCR)	NS	All *C. suis* All *C. suis* Not identified	ND	(Vitovec et al., 2006)
	Ireland (Cross-sectional)	5 (100)	Weaners (127) Finishers (121) Gilts (15) Sows (75) Boars (4)	15 9 6.7 13 0	ND	29 (direct 18S PCR)	18 (62) 5 (17.2) 1 (3.4) 5 (17.2) 0 (0)	*C. suis* (72.2); PGII (27.7) PGII (100) UN (100) *C. suis* (20); PGII (20); *C. parvum* (40) ; *C. muris* (20) Not identified	ND	(Zintl et al., 2007a)

	Denmark (Random selection of (oo)cysts containing samples from previous study)	NS	Sows (10)/(10) Weaners (356)/(190) Piglets (29)/(15) *Crypto/Giardia*	2 74 4		0/4 170/77 13/1 (nested 18S &/or HSP70 PCR)/ (nested 18S &/or *gdh* PCR)	NS	ND *C. suis* (24) ; PGII (76) *C. suis* (71) ; PG II (29)	E (100) A (12); D (1); NU (1) E (87) A (100)	(Langkjaer et al., 2007)
	Norway (Cross-sectional)	100 (31)/ (10) *Crypto/Giardia*	Suckling piglets, 4-33 days (684 litters). Pooled fecal samples	8.3 (IFA)	1.5	14/0 samples for (direct 18S PCR)	9 (64.3)	*C. suis* (66.6) ; PGII (33.3)	ND	(Hamnes et al., 2007)
	Spain (Cross-sectional)	24 (62.5)	Weaned (75) Fattening (42) Sows (25)	30.7 11.9 16	ND	23 5 4 (nested 18S PCR)	26 (81.2)	*C. suis* (35); PGII (50) PGII (11.5) *C. suis* (3.8)	ND	(Suarez-Luengas et al., 2007)
	Australia (Cross-sectional)	4 (100)	Pre-weaned (123); Post-weaned (156); Sows (10)	10.6 32.7 0	ND	123 156 10 (nested 18S PCR & RFLP)	13 (10.6) 51 (32.7) 0 (0)	*C. suis* (53.8) *C. suis* (11.7); PGII (62.7) 0	ND	(Johnson et al., 2008)
	Czech Republic (Cross-	1 (100)	Pre-weaned (119) Weaned	21.8 29.0	ND	26 38	18 (69.2) 32 (84.2)	*C. suis* (100) *C. suis* (81.3); PGII (15.6) ;	ND	(Kvac et al., 2009a)

	sectional)		(131) Fattening (123) Sows (40)	17.1 2.5		21 1 (nested 18S PCR & RFLP)	19 (90.5) 0 (0)	*C. muris* (3.1) *C suis* (5.2) ; PGII (89.5) ; *C. muris* (5.3) ND		
	Australia (Cross-sectional)	4 (100)	Pre-weaned (123) Post-weaned (156) Sows (10)	ND	18.7 41 30	23 64 3 (direct 16S PCR)	17 (74) 39 (61) 1 (33.3)	ND	A (47); E (53) A (28); E (69.2); F(2.6) E (100)	(Armson et al., 2009)

PGII - *Cryptosporidium* pig genotype II
ND - Not determined
NS - Not stated
TDNL - Traditional husbandry farms where calves remain with the cow on pasture
16S - 16S rRNA
18S - 18S rRNA
tpi - Triose phosphate isomerase
gdh - Glutamate dehydrogenase
HSP70 – Heat shock protein 70
UN - Unidentified

1.4. Risk factors

Preventing *Cryptosporidium* and *Giardia* infections in domestic livestock would be advantageous because of their potential as zoonotic agents. To achieve this, it is essential that pertinent risk factors are defined. Of particular importance is that currently there is no effective therapy for cryptosporidiosis, so identifying risk factors for *Cryptosporidium* infection plays an essential role in effectively reducing exposure to infectious oocysts. Previous studies have investigated the factors associated with *Cryptosporidium* (Garber et al., 1994; Maddox-Hyttel et al., 2006; Starkey et al., 2006; Trotz-Williams et al., 2007) and *Giardia* (Xiao et al., 1993; Ruest et al., 1998; Wade et al., 2000). In these studies, a number of demographic and farm management factors that predispose livestock to infection with *Cryptosporidium* and/or *Giardia* species are taken into account. Demographic factors may include: location, size of the farm, herd size, age distribution of animals sampled, and other species of animals present on the farm. Management factors relate to general management, maternity, pre-weaning or post-weaning pen management, type of housing, bedding material, general sanitation, provision of colostrum to calves, etc. (Wade et al., 2000). While some studies rely on a questionnaire, others collect the data at the time of sampling (Wade et al., 2000). Also, results vary between studies, which are likely attributed to different approaches in data collection, study population, and design or geographical variation. For example, Wade et al. (2000) found that animals were at a higher risk of infection with *Giardia* species in the winter, while Xiao et al. (1993) found that the risk was rather higher in the summer. The study by Xiao et al. (1993) was an outbreak investigation on two farms in Ohio, while the study by Wade et al. (2000) was a cross-sectional study on a larger number of farms over a longer period of time. Also in the US, calves were more likely to be infected with *Cryptosporidium* on farms with larger herds (more than 100 cows) (Garber et al., 1994), while in

Spain, the risk of infection with *Cryptosporidium* in cattle, was lower on farms with a larger number of cattle (Castro-Hermida et al., 2002). While it is likely that larger herds produce higher numbers of calves, which become infected with *C. parvum*, increasing environmental contamination. The authors in the latter study attributed the lower risk of *Cryptosporidium* infection on those farms with a larger number of cattle to the particular circumstances found in rural Galacia, Spain, where housing and hygiene conditions on large farms tend to be better

In the present study, information was obtained during sampling of cattle fecal specimens to determine the risk of infection with *Cryptosporidium* and *Giardia* with respect to age of the animal, location of the farm, and housing type for dairy herds, and age of the animal and location of the farm for beef herds. The effect of age and season on *Cryptosporidium* shedding in humans was also investigated.

1.5 *Cryptosporidium* and *Giardia* on Prince Edward Island

On Prince Edward Island (PEI), only a handful of studies have been conducted on *Cryptosporidium* and *Giardia* in domestic livestock, particularly cattle. All the studies either involved teaching herds in the Atlantic Veterinary College of the University of Prince Edward Island or a small number of convenience samples obtained from herds within the vicinity of the University (Uehlinger et al., 2011, in press). These studies have not only reported high prevalences of *Cryptosporidium* and *Giardia*, but have also found zoonotic species and genotypes in the cattle herds examined. PEI is largely a rural region with a significant agrarian economy in an area with abundant rainfall and groundwater. The Province has the highest cattle-to-human ratio in Canada. Cattle husbandry and manure management practices have an impact on the concentration of (oo)cysts in surface water (Keeley and Faulkner, 2008), manifested

through direct fecal input, runoff from agricultural lands, and waste from abattoirs (Bodley-Tickell et al., 2000). Animal agricultural activities might therefore serve as important sources for contaminating the watersheds with zoonotic species and genotypes of *Cryptosporidium* and *Giardia.* This could have public health consequences for the human population on PEI. To date, however, there are no studies that have extensively determined the epidemiology of *Cryptosporidium* and *Giardia* in domestic livestock and the transmission dynamics for the infection of these parasites to humans in this Province.

1.6 Aim of the Study

1. Determine the prevalence of *Giardia* and *Cryptosporidium* in livestock in PEI
2. Determine whether PEI cattle and pigs are infected with zoonotic *Giardia* and *Cryptosporidium.*
3. Determine if the presence of cattle and pigs increases the concentation of *Giardia* and *Cryptosporidium* in water sources in the vicinity of these farms.
4. Determine if humans on PEI are infected with the same *Giardia* and *Cryptosporidium* species and genotypes as cattle and pigs.

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2.0. *GIARDIA* AND *CRYPTOSPORIDIUM* ON DAIRY FARMS AND THE ROLE THESE FARMS MAY PLAY IN CONTAMINATING SOURCE WATER IN PRINCE EDWARD ISLAND, CANADA

Abstract

Cattle represent a reservoir for *Giardia* and *Cryptosporidium,* and may contaminate source water through run-off from cattle operations, manure storage or field application of manure. The objective of this study was to determine the prevalence and genotypes of *Giardia* and *Cryptosporidium* on dairy farms and the water sources associated with the the farms on Prince Edward Island (PEI), Canada. Fecal samples were obtained from 752 animals randomly selected from 20 dairy farms. Ground and surface water samples in and around the farms were also collected. Prevalence was determined by direct immunofluorescence assay, while genotyping /species determination was by PCR and DNA sequencing. Thirty-two percent and 14% of fecal samples, and 100 and 55% of herds, were positive for *Giardia* and *Cryptosporidium*, respectively. Of 73 samples genotyped for *Giardia*, *Giardia duodenalis* assemblage E was predominant among all cattle age groups, while only 6 and 4% of isolates belonged to assemblages A and B, respectively. Assemblage B was only detected in three of 31 samples genotyped from pre-weaned calves (<2 mo of age). Of all the 41 samples genotyped *Cryptosporidium bovis* predominated (51%) while *C. andersoni* was detected mostly in samples genotyped from adult cattle (53% of 17 animals). *C. ryanae* was found in all age groups, while only calves <2 mo of age were positive for *C. parvum* (2 of 12 preweaned calf samples genotyped). *Giardia* cysts and *Cryptosporidium* oocysts were detected in 46 and 85% of surface water samples respectively, while only one groundwater sample was positive for

Cryptosporidium. All four genotyped *Cryptosporidium* isolates from water were *C. parvum*. These study findings suggest that, dairy cattle on PEI are predominantly infected with non-zoonotic species/genotypes of *Cryptosporidium* and *Giardia*; however, some surface water sources were contaminated with the zoonotic *C. parvum* and may represent a threat to the watershed and subsequent disease transmission to humans.

2.1. Introduction

Cryptosporidium and *Giardia* have been recognized as common intestinal parasites responsible for causing gastrointestinal disorders in both humans and animals including domestic livestock (Fayer, 1997). Transmission of these parasites occurs through the fecal-oral route or through the consumption of contaminated water or food. The (oo)cysts are small, immediately infective, environmentally persistent, and chemically resistant which allows them to easily breach conventional filtration and chemical methods used in drinking water treatment (Simmons et al., 2001). Ingestion of as few as ten *Cryptosporidium* oocysts or *Giardia* cysts can elicit infection in humans (Olson et al., 1999).

Many animal species, including cattle, are known to be human sources of *Cryptosporidium* and *Giardia* infection.(Fayer, 2004; Thompson, 2004) Cattle, particularly calves, have come under considerable attention given the reported high prevalence and the intensity of shedding of *Cryptosporidium* oocysts or *Giardia* cysts (up to 2.6 x 10^7/g) in their feces (Bradford and Schijven, 2002). Demographic and on-farm management factors, such as the farm geographic location, age of animals, and type of housing, can have effects on the level and intensity of infection with *Cryptosporidium* and *Giardia* in livestock (Wade et al., 2000a; Trotz-Williams et al., 2007). Human *Cryptosporidium* and *Giardia* infections are predominantly waterborne and the parasites have been detected in both raw and drinking water (Wallis et al.,

1996). While wildlife are thought to contribute to the contamination of pristine waters (Hansen and Ongerth, 1991), much of the potable water contamination is suspected to be from animal agriculture, through direct fecal input, runoff from agricultural lands, and waste from abattoirs (Bodley-Tickell et al., 2000). Cattle husbandry and manure management practices, therefore, have an impact on the concentration of (oo)cysts in surface water (Keeley and Faulkner, 2008).

Four species of *Cryptosporidium* are routinely detected in cattle: the zoonotic species, *C. parvum* and the non-zoonotic species *C. andersoni*, *C. bovis* and *C. ryanae*, following a complex age-related distribution. *C. parvum* primarily infects pre-weaned calves (Santin et al., 2004), *C. andersoni* predominantly infects juvenile and mature cattle (Anderson, 1998; Lindsay et al., 2000), *C. bovis* is mainly found in 2-11-mo old calves (Santin et al., 2004), and *C. ryanae* predominates in both pre-weaned and 2 to 11-mo old animals (Santin et al., 2004).

The *Giardia* genotype most commonly found in cattle is the host-adapted *G. duodenalis* assemblage E, while the zoonotic assemblages A and B have occasionally been detected (O'Handley RM et al., 2000; Uehlinger et al., 2006; Coklin et al., 2007).

Since zoonotic *Cryptosporidium* and *Giardia* occur in cattle, an understanding of the epidemiology and transmission routes by which (oo)cysts may gain access to water sources is fundamental to mitigate the risks of environmental contamination with these parasites from livestock farms. The objectives of this study were as follows: (1) to determine the prevalence and genotypes of *Cryptosporidium* and *Giardia* isolates from cattle on dairy farms on PEI, Canada, (2) to investigate the potential for contamination of water sources with zoonotic species/genotypes associated with these farms, and (3) to determine risk factors for animal infection with these parasites.

2.2. Materials and Methods

2.2.1. Fecal sample collection and processing

Twenty dairy farms were selected from around PEI based on the presence of surface water within 500m of the barn, accessibility of animals for sampling, and the owner's willingness to participate in the study. Between January and August 2006, fecal samples were randomly taken (ear tags numbers drawn from a container) once from 20 cows (>6 mo of age) and 20 calves (≤6 mo of age) on each farm. Calves were further grouped into those <2 mo of age and those 2 to 6 mo of age. If farms had less than 20 cows or 20 calves in each age group, samples were collected from all animals available.

Fecal samples were collected rectally, or freshly voided, using sterile disposable latex glove and placed in clean plastic containers. Fecal samples were transported to the laboratory in a cooler and stored for short periods (maximum one week) at 4°C until processing, or at -20°C if processing was delayed more than one week.

For each farm, data were collected on animal and management factors (Table 2.1) that were hypothesized to be associated with the risk of *Cryptosporidium* and/or *Giardia* infection.

Fecal analysis was performed according to a previously described procedure (O'Handley et al., 1999) with slight modifications. Twenty gram fecal samples from adults and 5g samples from calves were added to 35 and 10ml, respectively, of phosphate-buffered saline (pH 7.4) (PBS), and thoroughly mixed with an applicator. The suspension was then passed through four layers of surgical gauze (Nu-gauze, 4 ply, Johnson & Johnson, Montreal, QC). The strained fecal sample homogenate was gently layered over either 15ml (adults) or 5ml (calves) of 1M sucrose (Sigma-Aldrich Canada Ltd., Oakville, ON) solution in a clean polypropylene conical tube for adult and young animals, respectively

Table 2.1. Variables examined for the association between farm and management factors and the risk of infection with *Giardia* species or *Cryptosporidium* species

Variable	Detail
County	
	Kings
	Queens
	Prince
Age	
	Cattle (>6 months)
	Calves (≤6 months)
Housing	
	Calves from tie-stall farms
	Calves from free-stall farms
	Cows tie-stall
	Cows free-stall

Samples were centrifuged at 800 x g for 5 min in a fixed-angle rotor centrifuge. With a disposable pipette, the upper filtrate layer and sucrose surface were transferred into a clean 50ml centrifuge tube for the adult samples and 15ml tubes for the calf samples, and again centrifuged at 800 x g for 5 min. The resulting supernatant was discarded and the pellet re-suspended in PBS to approximately 1.5ml.

2.2.2. Oocyst and cyst examination from fecal samples

The concentrate was applied in 20µl volumes to two wells on a fluorescence microscopy slide (Waterborne, New Orleans, LA) and dried for 10 min at 37°C on a slide warmer. A *Cryptosporidium* or *Giardia*-specific fluorescein isothiocyanate (FITC)-labeled monoclonal antibody solution (0.04ml) (Crypt-a-glo™ or Giardi-a-glo™, Waterborne Inc, New Orleans, LA) was added to each of the two wells, respectively. The slide was placed in a humid chamber at room temperature for 40 min. Once removed from the humid chamber, the slide was briefly

rinsed with PBS, air dried at room temperature, and mounted with a fluorescent antibody mounting fluid (AquaPolymount, Polysciences, Warrington, PA). Each well was then covered with a 22 mm^2 coverslip (VWR Scientific Inc., Media, PA) and kept in the dark. *Cryptosporidium* oocysts and *Giardia* cysts were examined and enumerated at 200 x and 100 x magnifications, respectively, under an epifluorescence microscope (Leica DM 2500, Leica Microsystems, Wetzlar, Germany). The number of oocysts and cysts per gram of feces was determined as previously described (O'Handley et al., 1999). The sensitivity of this method was determined previously to be 66 oocysts or cysts per gram of feces (O'Handley et al., 1999).

2.2.3. Water sample collection and processing

Water samples were collected from ground (well) and surface water (rivers, streams, creeks and brooks) from locations within the vicinity of the farms (within 500m from the animal housing facility) between September 2006 and August 2007. Water samples from ponds on the farms were originally included as surface water samples but were excluded in the analysis since the easy access for cows and their frequent presence in the ponds could bias the results of the study. Surface water, was obtained from 13 farms, and collected both upstream and downstream from the perceived edges of the pasture land, while a sample of ground water was obtained from each of the 20 farms. Groundwater samples positive for *Cryptosporidium* and/or *Giardia* were re-sampled within about two months of obtaining the initial results and testing repeated for the presence of the parasites as well as background indicator bacteria such as coliform/*E. coli* counts.

Water samples were passed through a Filta-Max® (IDEXX, Westbrook, ME) foam filter system at a flow rate of 3.3 l min^{-1} according to the manufacturers' recommendation and described in the US-EPA method1623(USEPA, 2003) manual. One hundred litres of water was

filtered from a ground source. A 20l volume of surface water was similarly collected where there was no difficulty in the filtration process. However, if a 20l volume of water had not been filtered in 30 min due to high turbidity (turbidity was not measured but crudely implied by the difficulty or slowness of the filtration process) of the sample, a 10l grab water sample was obtained and filtered in the laboratory for analysis. This normally required two or more Filta-Max filters, as well as a multiple of membrane filters for the sample concentration step. The Filta-Max filters were transferred to clean plastic bags, sealed and transported in a cooler to the laboratory for further processing.

At the laboratory, samples were processed for parasite isolation and enumeration according to US-EPA method 1623 (USEPA, 2003). Briefly, oocysts or cysts were eluted using the Filta-Max® wash station procedure and separated from debris by immunomagnetic separation (IMS) with a Dynabeads-GC Combo kit (Dynal Biotech, Brown Deer, WI, USA). The sample was mounted onto slides (Superstick slides, Waterborne Inc, New Orleans, LA, USA), fixed with methanol and stained with fluorescent labeled (FITC) monoclonal antibodies (Waterborne Inc, New Orleans, LA) and a nucleic acid stain [DAPI, 2-(2-amidinophenyl)-6-indolecarbamidine dihydrochloride]. Slides were then cover slipped and stored in the dark to dry. Upon drying, the edges of the cover slip were sealed with clear nail polish and stored in the dark until viewed. Parasites were enumerated by epifluorescence microscopy (Leica Axioscope fluorescence microscope equipped with a blue filter block - excitation wavelength, 490 nm; emission wavelength, 510 nm). Samples in which oocysts or cysts fulfilled defined morphological criteria according to the US-EPA 1623 protocol were considered positive. The number of oocysts or cysts detected per litre was calculated according to a previously described method (Farias et al., 2002) as follows:

Number of oocysts or cysts in pellet = Number of oocysts or cysts in an analyzed drop x total mL of the pellet/volume of analyzed drop

Number of oocysts or cysts /l = Number of oocysts or cysts in pellet/number of litres filtered or flocculated

Concentrations of oocysts or cysts from upstream locations of surface water were subtracted from those of corresponding downstream sites to achieve a net concentration of oocysts or cysts. A positive result was interpreted as the positive impact of the specific farm on the contamination of the water source with *Cryptosporidium* oocysts or *Giardia* cysts.

2.2.4. Oocyst or cyst recovery from microscope slides

Oocysts or cysts were extracted from samples on microscope slides using a modification of a procedure previously described (Nichols et al., 2006). Briefly, slides were placed on absorbent tissue, and a cotton swab moistened with nail polish remover was applied to soften the varnish on the perimeter of the coverslip and the opposite end of the swab was used to scrape the softened varnish from the coverslip-slide interface onto the absorbent tissue. A clean scalpel blade was used to lever a corner of the coverslip from the slide surface, and then the coverslip was gently lifted off the slide. The coverslip was inverted and placed onto the absorbent tissue. The Teflon-coated area of the slide surrounding the well was dried with a small piece of folded absorbent tissue, and then 25 µl of lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.5% sodium dodecyl sulfate) was pipetted onto the well of the slide. The entire surface of the

well was scraped with a sterile 1-µl bacteriological inoculation loop (Nunc, United Kingdom). Residual lysis buffer was aspirated by tilting the slide to an angle of about 45° from the horizontal towards the operator and aspirating the fluid that collected at the bottom of the well by placing the tip of a P20 Gilson pipette fitted with a filter-tipped pipette tip close to the fluid. The scraped sample in lysis buffer was pipetted into an appropriately labeled 0.5-ml screw-cap microcentrifuge tube. A further 25µl volume of fresh lysis buffer was deposited onto the sample well using a clean pipette tip, and the sample was scraped using the same inoculation loop. All liquid was removed from the well as described above, and then the slide was rotated through 180° and the slide-scraping steps were repeated two more times. The final volume of the sample amounted to ~100µl. The tube was capped and the suspension centrifuged at 4,000 x g for 60 sec. Using a pipette, 50µl of the supernatant was removed and discarded. The rest of the sample was re-suspended by gentle vortexing. Once scraped, the slides and coverslips were retained and re-examined by epifluorescence microscopy to determine the efficiency of the removal procedure.

2.2.5. DNA extraction

DNA extraction was performed on at least five randomly selected direct immunofluorescence antibody assay (DFA) positive processed fecal samples from both cows and calves from each farm, and on all DFA positive water samples. DNA was extracted using the Qiagen DNeasy Tissue Kit (Qiagen Inc., Mississauga, ON) according to the manufacturer's instructions. Briefly, a total of 50µl of processed feces or water sample was suspended in 180µl of ATL buffer, and thoroughly mixed by vortexing. Proteinase K (920mg/ml) was added to the suspension, thoroughly mixed, and 200µl of AL buffer was added after overnight incubation at 55°C. DNA was eluted in 100µl of AE buffer.

2.2.6. Polymerase chain reaction and DNA sequence analysis

Modifications of previously described nested PCR protocols were used for the amplification of the β-giardin (Caccio et al., 2002) and 16S ribosomal DNA (rDNA) (Appelbee et al., 2003) from *Giardia* and the heat shock protein 70 (HSP70) (Guselle et al., 2003) and 18S rDNA (Fayer et al., 2006) from *Cryptosporidium*.

For the β-giardin gene, the primary reaction, a 753 bp amplicon was amplified using the forward primer G7 (5' – AAGCCCGACGACCTCACCCGCAGTGC-3' and the reverse primer G759 (5' GAGGCCGCCCTGGATCTTCGAGACGAC-3'). In the sequential nested PCR reaction, a 384 bp amplicon was amplified using forward primer G376 (5' CATAACGACGCCATCGCGGCTCTCAGGAA-3') and the reverse primer G759. The PCR mix consisted of 1x buffer containing 1.5 nM $MgCl_2$, 200 μM of each dNTP, 10 pmol of each primer, 5 units of Hot Start Taq DNA polymerase (Qiagen Inc., Mississauga, ON)), 2μl of 1% BSA (an addition to the original protocol) and 5 μl of purified DNA in a final volume of 50μl. PCR for both cycles were performed as follows: after an initial denaturation step of 5 min at 95°C, a set of 35-40 cycles was run, each consisting of 30 s at 94°C, 30 s at 65°C and 30 s at 72°C, followed by a final extension of 10 min at 72°C.

The 16S rDNA PCR protocol utilized the initial primers Gia2029 (5'–AAGTGTGGTGCAGACGGACTC-3') and Gia2150c (5'– CTGCTGCCGTCCTTGGATGT – 3') which amplified a 497 bp amplicon, and secondary primers RH11 (5'–CATCCGGTGGATCCTGCC-3') and RH4 (5'–AGTCGAACCCTGATTCTCCGCCAGG-3') generating a 292 bp amplicon. The PCR mix consisted of 1x buffer containing 2.0 mM $MgCl_2$, 200 μM of each dNTP, 5% DMSO, 12.5 pmol of each primer, 5 units of Hot Start Taq DNA

polymerase, and 2-5 µl of purified DNA in a final volume of 50µl. PCR was performed as follows: after an initial denaturation step of 2 min at 96°C, a set of 35-40 cycles was run, each consisting of 45 s at 96°C, 30 s at 58°C and 45 s at 72°C, followed by a final extension of 4 min at 72°C. The sequential nested-PCR cycle was the same except the annealing temperature was reduced to 55°C.

For the HSP70 gene, a 448 bp amplicon was amplified in the primary reaction using the forward primer; (5'–GGTGGTGGTACTTTTGATGTATC-3') and the reverse primer (5'–GCCTGAACCTTTGGAATACG-3'). The secondary reaction primers were forward primer (5'–GCTCATGATACTCACTTGGGTGG-3') and the reverse primer (5'–CTCTTGTCCATACCAGCATCC-3'), generating a 325 bp amplicon. The PCR mix consisted of 1x buffer containing 1.5 mM $MgCl_2$, 120-160 µ of each dNTP, 2.5ul BSA, 12.5 pmol of each primer, 1-2 units of Hot Start Taq DNA polymerase, and 5 µl of purified DNA in a final volume of 50 µl. PCR was performed as follows: after an initial denaturation step of 5 min at 95°C, a set of 35 cycles was run, each consisting of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C, followed by a final extension of 4 min at 72°C. The sequential nested-PCR cycle was the same except the annealing temperature was increased to 58°C for 30 s, and 1 µl of DNA template was used from the first reaction.

For the 18S rDNA amplification, an 830 bp PCR product was amplified first by primary PCR with the forward primer (5' –TTCAGAGCTAATACATGCG-3') and the reverse primer (5' –CCCATTTCCTTCGAAACAGGA-3'). In the secondary PCR step, a PCR product of 819-825 bp long (depending on the species) was amplified by using the forward primer (5'–GGAAGGGTTGTATTTATTAGATAAAG-3') and the reverse primer

(5'–AAGGAGTAAGGAACAACCTCCA-3'). The PCR mix consisted of 1x buffer containing 3 mM $MgCl_2$, 200 µM of each dNTP, 2.5µl BSA, 12.5 pmol of each primer, 2.5 units of Hot Start Taq DNA polymerase, and 1-5 µl of purified DNA in a final volume of 50 µl. PCR was performed as follows: after an initial denaturation step of 3 min at 94°C, a set of 40 cycles was run, each consisting of 30 s at 94°C, 90 s at 58°C and 2 min at 72°C, followed by a final extension of 7 min at 72°C. The sequential nested-PCR was the same except that in the PCR mix the $MgCl_2$ concentration was 1.5 mM.

All amplification products were visualized in 1.5% agarose gels with ethidium bromide. Positive PCR amplicons were sequenced in both directions, using the same PCR primers used to produce the amplicons, at the Genome Quebec Innovation Centre at McGill University, Montreal, QC. To determine the genotypes of *Giardia* and species of *Cryptosporidium*, sequences were compared with similar published sequences by BLAST – analysis (Altschul et al., 1990)

Each *Giardia* sequence was compared to the GenBank sequences of *Giardia* assemblages A (accession number AF113902), B (accession number DQ7891121) and E (accession number AY655701). Similarly, each *Cryptosporidium* sequence was compared to the GenBank sequences of *Cryptosporidium* as follows: *C parvum* (accession number: AF093490), *C. bovis* (accession number: AY741305) *C. ryanae* (accession number: AY587166) and *C. andersoni* (accession number: AB089285). Sequences were aligned using the software BioEdit (Hall, 1999).

2.2.7. Data collection and statistical analysis

The herd prevalence and within-herd prevalence were obtained from the DFA results. The within-herd prevalence was calculated as the number of positive animals over the total number of animals tested for the farm of interest. The herd prevalence was calculated as the number of farms with at least one positive animal over the total number of farms.

A generalized linear mixed model (random effects logistic regression) was used to test for significance of region (county on PEI) (Fig. 2.1), housing system for both older animals (> 6 mo of age) and young animals (≤ 6 mo of age) (Table 2.1), and age of animals as fixed systematic environmental effects for *Cryptosporidium* and/or *Giardia* infection, accounting for herd random effects (Dohoo et al., 2009).

A negative binomial regression model was used to determine if there was any significant difference in the number of *Cryptosporidium* oocysts or *Giardia* cysts/ml between the upstream and downstream samples for surface water.

In all instances, a *P*-value < 0.05 was considered statistically significant. All analyses were performed using the statistical software package STATA 11.0 (Stata Corporation, College Station, TX).

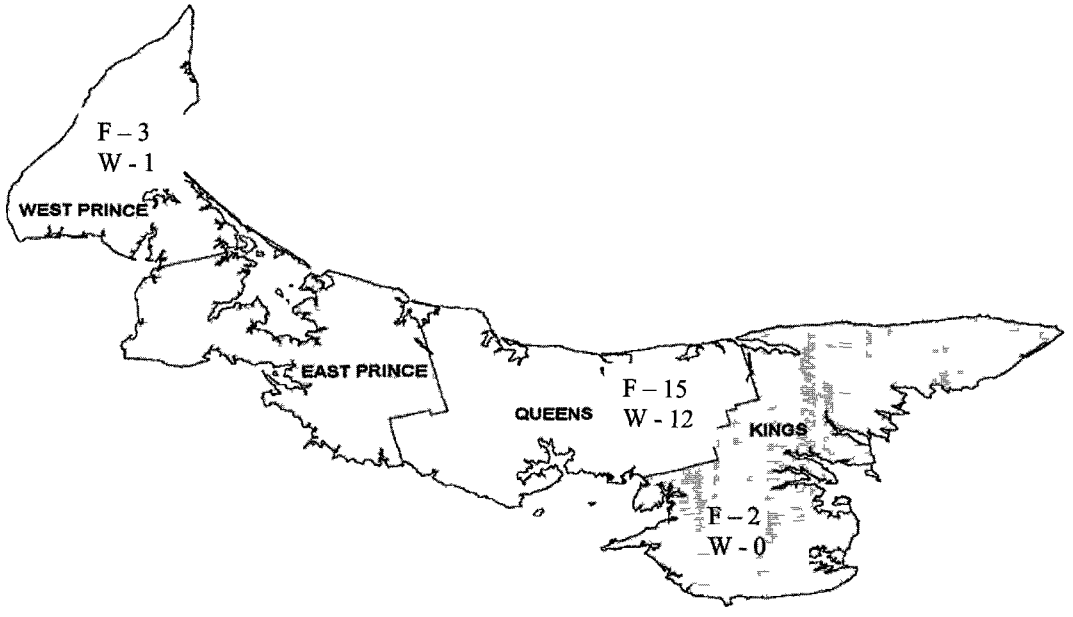

Figure 2.1. Map of Prince Edward Island (PEI), Canada, showing the three counties: Prince (East and West), Queens, and Kings from where animal fecal and water samples were obtained. F - number of dairy herds sampled; W - number of surface water obtained.
Source: PEI Statistics Bureau. Department of Finance and Municipal Affairs.

2.3. Results

2.3.1. Fecal samples

Giardia cysts were detected on all farms (100%; 95% CI: 100-100%). The within-herd prevalence of *Giardia*-positive cattle ranged from 2.5 to 52.5% (median 32%). Of a total of 752 animals tested, 240 (32%; 95% CI: 29-35%) were infected with *Giardia.* Calves, <2 mo of age, had a higher (P<0.01) percentage of *Giardia*-positive (58%; 95% CI: 48-67%) samples than calves between 2 and 6 mo of age (40%; 95% CI: 32-48%) or animals older than 6 mo (25%; 95% CI: 20-29%) (Table 2.2). Genotyping data was available for 33 isolates with the β-giardin gene only and 64 with the 16S rDNA gene only. The distribution of *Giardia* genotypes in dairy cattle based on the combined sequencing data from the β-giardin and 16S rDNA gene loci are presented in Table 2.3. Overall, for samples successfully genotyped (n=73), 6, 4 and 90% of *G. duodenalis* isolates belonged to assemblage A, B and E, respectively (Table 2.3). Assemblage E was commonly identified in all the age groups, occurring in 84, 95 and 95% of samples genotyped in ages < 2 mo, 2 to 6 mo and >6 mo, respectively. The zoonotic assemblage A was identified in two calves <2 mo, while one each was found in calves 2 to 6 mo, and animals >6 mo of age. All isolates (3) of the zoonotic assemblage B were detected in calves <2 mo of age.

PCR positive specimens sequenced in the present study shared 100% sequence similarity with the GenBank sequences of *Giardia* assemblages A (accession number AF113902), B (accession number DQ7891121) and E (accession number AY655701).

Cattle >6 mo of age were less likely to be infected with *Giardia* species than animals ≤6 mo of age (P= 0.001). There was no significant association between either the location of the farm (P= 0.10), or the type of housing (P=0.37), and infection with *G. duodenalis.*

Cryptosporidium oocysts were detected on 11 (55%; 95% CI: 31-79% of the 20 farms. The within-herd prevalence of *Cryptosporidium*-positive cattle ranged from 2.5% to 88.9% (median 3.8%). Of 752 animals tested, 108 (14%; 95% CI: 12 - 17%) were *Cryptosporidium*-positive. Prevalence of *Cryptosporidium* was 17% (95% CI: 11-25%), 14% (95% CI: 9-21%) and 15% (95% CI: 12-19%) in cattle < 2, 2 to 6, and > 6 mo, respectively (P=0.20) (Table 2.2). Genotyping data were available for 24 isolates with the HSP70, and 21 isolates with the 18S rDNA genes. The distribution of *Cryptosporidium* species based on the combined sequencing data from the HSP70 and 18S rDNA genes (n=41) is presented in Table 2.3. Overall, for isolates successfully genotyped, *C. bovis* had the highest prevalence (51%), followed by *C. andersoni* (27%), *C. ryanae* (17%) and *C. parvum* (5%) (Table 2.3). *C. bovis* was in 58% of samples genotyped in pre-weaned calves (< 2 mo), 81% in post-weaned (2 to 6 mo) calves, and 29% in older animals (> 6 mo of age). *C. andersoni* was the predominant species found in genotyped samples in adult cattle (> 6 mo) (53%), while only one isolate was found in pre-weaned calves. Two *C. ryanae* isolates were found in genotyped samples in each of animals aged < 2 mo and 2 to 6 mo, while 3 isolates were detected in animals > 6 mo of age. The zoonotic *C. parvum* was only found in pre-weaned calves (< 2 mo).

PCR positive specimens sequenced in the present study shared 100% sequence similarity with genotypes in GenBank as follows: *C parvum* (GenBank accession number: AF093490), *C. bovis* (GenBank accession number: AY741305) *C. ryanae* (GenBank accession number: AY587166) and *C. andersoni* (GenBank accession number: AB089285)

Cattle >6 mo of age in free-stall housing were more likely (P=0.002) to be infected with *Cryptosporidium* than other types of housing. There was no significant association between age of the animal and infection with *Cryptosporidium* species (P= 0.48). The prevalence of

Cryptosporidium spp. infection tended to be higher in farms located in Queens County (P= 0.08) than in Kings and Prince Counties (Fig 2.1).

Table 2.2. Age-related prevalence of *Cryptosporidium* spp. and *Giardia* spp. based on protozoal specific direct immunofluorescence antibody (DFA) assay

Age of Animal	Number of Animals	# Positive	Overall prevalence (95% CI)	Age-related Prevalence (95% CI)
Cryptosporidium	752	108	14 (12, 17%)	
<2 mo	119	20		17 (11, 25%)
2 - 6 mo	145	21		14 (9, 21%)
>6 mo	411	62		15 (12, 19%)
Unknown	77	5		6 (0.9, 12%)
Giardia	752	240	32 (29, 35%)	
<2 mo	119	69		58 (48, 67%)
2 - 6 mo	145	58		40 (32, 48%)
>6 mo	411	101		25 (20, 29%)
Unknown	77	12		16 (7, 24%)

Table 2.3. Distribution of *Cryptosporidium* and *Giardia* species/genotypes in dairy cattle based on the overall sequencing data from the HSP70 and 18S rDNA genes for *Cryptosporidium* and the β-giardin and 16S rDNA genes for *Giardia*.

Category	*Cryptosporidium* spp. positive					*G. duodenalis* assemblages positive			
	n	*C. parvum*	*C. bovis*	*C. ryanae*	*C. andersoni*	n	A	B	E
Combined	41	2 (5)	21 (51)	7 (17)	11 (27)	73	4 (6)	3 (4))	66 (90)
<2 mo	12	2 (17)	7 (58)	2 (17)	1 (8.3)	31	2 (7)	3 (10)	26 (84)
2 - 6 mo	11	-	9 (81)	2 (18)	-	22	1 (5)	-	21 (95)
>6 mo	17	-	5 (29)	3 (18)	9 (53)	20	1 (5)	-	19 (95)
Unknown	1	-	-	-	1 (100)	-	-		-

n – Total number of samples genotyped; numbers in parentheses are (%)

2.3.2. Water Samples

Surface water was obtained from 13 farms, and 20 (one from each farm) domestic (well) water samples were obtained from the homes on the farms. For surface water, eight had samples taken from both the upstream and downstream locations while five samples were taken from only the downstream locations. Seven of the samples taken from both the upstream and downstream locations, and four samples from the downstream locations only, were positive for *Cryptosporidium* oocysts, for a farm prevalence of 85% (11 of 13). The concentration of oocysts in the positive surface water samples ranged from 0.05 to 10.5 oocysts/l. At 6 of the 7 farms (86%) with both downstream and upstream samples, a positive net change in *Cryptosporidium* oocyst concentration was found, and at one farm (farm 10) no net change was found (the upstream and downstream samples had the same concentration of oocysts), while at only one farm a negative net change was found (Fig. 2.2). On average, a positive net change of 3.02 oocysts/l (95% CI: 1.60-5.70) was found (P=0.001).

Genotyping was only successful for *Cryptosporidium* isolates from 4 surface water samples from 4 different farms; all 4 were identified as *C. parvum*. Only one of the farms with a *C. parvum*-positive water sample was also positive for *C. parvum* in cattle. *Cryptosporidium* oocysts were detected in one groundwater sample with an oocyst concentration of 8.83/l, but this isolate was not amenable to genotyping. The surface water associated with this farm was also positive for *Cryptosporidium* but not amenable to genotyping

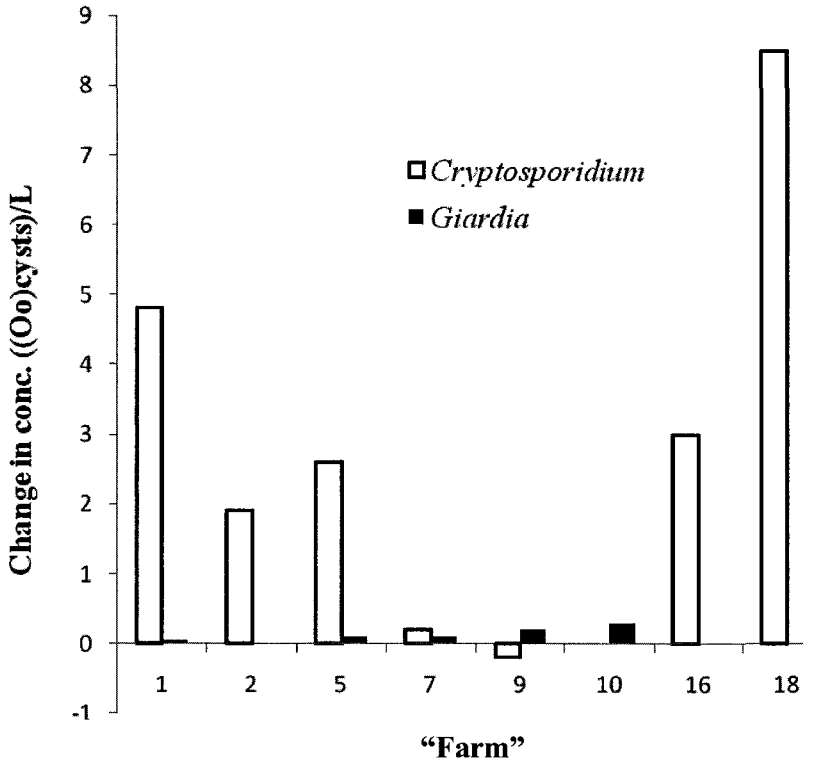

Figure 2.2. Net change in concentration from upstream to downstream sampling of *Cryptosporidium* oocysts and *Giardia* cysts in surface water (with both upstream and downstream values) within ~500 meters of dairy farms sampled on Prince Edward Island, Canada

Giardia cysts were detected in 6 (46%) of 13 surface water samples. Five of the six samples were obtained from both the upstream and downstream locations, with positive net changes in the concentration of cysts from upstream-to-downstream sampling (Fig. 2.2). The concentrations of *Giardia* cysts in the surface water were much lower than *Cryptosporidium* oocysts, ranging from 0.05 to 0.6 cysts/l. *Giardia* cysts were not found in any ground water sample. Genotyping was not successful for any of the *Giardia*-positive surface water isolates.

2.4. Discussion

Giardia and *Cryptosporidium* are highly prevalent in dairy herds on PEI. For *Cryptosporidium*, the relatively high herd-level prevalence of 55% identified in a one-time sample in the present study is in keeping with a number of studies which have reported up to 100% prevalence (Olson et al., 1997b; Starkey et al., 2006). The overall animal prevalence of *Cryptosporidium* in dairy cattle has previously been reported to range from 2-59% from other locations within North America (Garber et al., 1994; Olson et al., 1997a; Sischo et al., 2000; Wade et al., 2000b; Starkey et al., 2006; Coklin et al., 2007; Trotz-Williams et al., 2007; Coklin et al., 2009). The animal prevalence found in this study (14%) falls within the lower end of this range. In previous studies which have reported a higher overall animal prevalence, more than 60% of the animals sampled were ≤6 mo of age, and therefore within the age group known to demonstrate the highest prevalence for *Cryptosporidium* infection (Garber et al., 1994; Olson et al., 1997a; Sischo et al., 2000; Coklin et al., 2007; Trotz-Williams et al., 2007). In our study and those that have reported lower values, including Starkey et al (Starkey et al., 2006) and Wade et al (Wade et al., 2000a) with a prevalence of 3.2 and 2% respectively, more than 50% of the animals involved were above 6 mo of age.

In the current study, *C. parvum* was only detected in pre-weaned calves. *C. bovis* was the predominant species, occurring mostly in calves 2-6 mo of age. Similar results have been reported in other studies (Starkey et al., 2006; Feng et al., 2007). A small percentage of isolates was also identified as *C. ryanae* in each age group, similar to other studies (Santin et al., 2004; Fayer et al., 2006; Feng et al., 2007). To our knowledge, this represents the first report of *C. ryanae* in dairy cattle on PEI, Canada. *C. andersoni* was predominantly found in adult cattle. The

Cryptosporidium species/genotype distribution among the different age groups is consistent with previous reports (Fayer et al., 2007).

Giardia was detected on all 20 farms (100% farm prevalence). This is consistent with a similar but smaller study involving 11 dairy farms conducted on PEI (Uehlinger et al ., 2011, in press) and previous point prevalence studies in North America (Olson et al., 1997a; Trout et al., 2006; Coklin et al., 2007). The overall prevalence of *Giardia* (32%) found in this study is also similar to that observed by Olson et al (Olson et al., 1997b) (31%). The highest prevalence of *Giardia* infections was observed in pre-weaned calves < 2 mo old (58 %) with the intensity of infection declining through post-weaned calves 2-6 mo of age (40%) and animals older than 6 mo (25 %). This trend is in accordance with the findings of Trout et al (Trout et al., 2004; Trout et al., 2005; Trout et al., 2006). Other studies (O'Handley et al., 1999; Huetink et al., 2001; Becher et al., 2004) indicated that the prevalence of *Giardia* reached peak levels from about 4 weeks to 4-5 mo of age.

Overall, DNA sequence analysis revealed 90% of the *Giardia* isolates successfully genotyped belonged to assemblage E, while only 6% and 4% belonged to the zoonotic genotypes assemblages A and B, respectively. Generally, assemblage E and assemblage A were distributed among all age groups. Assemblage B, that has been identified in both ruminants and humans, was only detected in calves < 2 mo of age. In a similar study, a high prevalence of assemblage E in dairy cattle was also reported on PEI (Uehlinger et al., 2011, in press). In the same study, assemblage A was only detected concomitantly with assemblage E at 12% in younger animals (≤6mo of age), while in our study, assemblage A was equally detected at 5.7 and 5.0% for samples genotyped in animals ≤6mo and >6mo of age, respectively. Previous studies have also reported the relatively constant occurrence of assemblage A at 6 and 7% for pre-weaned and

post-weaned animals, respectively, for dairy cattle (Trout et al., 2004; Trout et al., 2005). Differences in the distribution of assemblage A in dairy cattle in the two studies on PEI might be due to the difference in sampling. While farms from which fecal specimens were obtained from calves in the first study were convenience samples of herds located close to the Atlantic Veterinary College on PEI, samples from our study were obtained from herds located across the Province. In other regions of Canada, assemblage E is also the predominant genotype found in cattle, but assemblage A is occasionally found (O'Handley et al., 2000; Appelbee et al., 2003). Few studies have also reported the presence of assemblage B in dairy cattle (Coklin et al., 2007). Calves infected with *Giardia* and *Cryptosporidium* commonly shed up to 2.6×10^7 (oo)cysts/g of feces (Bradford and Schijven, 2002). Hence, even a small population of calves infected with the zoonotic genotypes of these parasites could have a significant impact on public health.

For *Giardia*, our findings are consistent with studies (Quilez et al., 1996; Wade et al., 2000a) in which the risk of infection decreases with the age of the animal. There was no significant association between infection with *Giardia* and either the regional location of the farm or the type of housing. No regional differences were associated with *Giardia* infection in dairy cattle in other studies (Ruest et al., 1998). The lack of any association between geographic location and the infection with *Giardia* could be attributed to the small size of PEI and the proximity of the different farms. In another study conducted recently on PEI (Uehlinger et al., 2011, in press), housing was also not associated with *Giardia* infection in dairy cattle, which may indicate a regional difference or difference in management of dairy cattle on PEI.

Several studies have reported the correlation of increased concentrations of *Cryptosporidium* oocysts and/or *Giardia* cysts in surface water associated with animal agricultural activities (Rose et al., 1989; Hansen and Ongerth, 1991; Ong et al., 1996). In the

current study, *Cryptosporidium* oocyst concentration increased from the upstream to the downstream samples. The absence of *C. parvum* in the feces of animals on most of the farms with *C. parvum*-positive surface water samples has also been reported in other studies (Ong et al., 1996). The different sampling times for the fecal samples and water samples in our study might account for the discrepancy in the results from the farms and the surface water associated with them. Animal fecal specimens were obtained between January and August 2006, while water sampling began in September of the same year. Most of the farms were visited in the winter months when the surface water sources would have been frozen. Other reasons include the fact that some of the locations of the streams/rivers from which surface water samples were collected had slow moving or stagnant water and *C. parvum* from pre-weaned calves might have been transported earlier to the water through runoff events and deposited in the water basin. As this was a point prevalence study, the incidence of *Cryptosporidium* could have been impacted by intermittent shedding in the animals or sampling when infection in the animals was not high. Only two out of 20 farms were sampled in May which had the highest prevalence of *Cryptosporidium* infections in animals. More than 50% of fecal specimens in this study were obtained from animals >6 mo of age, reflecting a population characterized by lower prevalence of *Cryptosporidium* spp. *C. parvum* is not specific to calves but generally considered a parasite of domestic and wild ruminants and humans (Xiao et al., 2004), meaning that the occurrence of the species in the watersheds in this study could be a result of agriculture as well as wildlife, or human sewage. However, PEI lacks wild ruminants (deer and moose), leaving agriculture or human sewage as the only possible known explanations.

Cryptosporidium oocysts were detected in one out of 20 groundwater (well) samples in our study. Groundwater was once thought to be protected from protozoal contamination given

the ability of soil to filter microorganisms. However, in a US study, between 9.5 to 22% of groundwater samples were positive for *Cryptosporidium* (Hancock et al., 1998). In follow-up re-sampling of the positive ground water in our study, *Cryptosporidium* was again present concurrently with low levels of coliform counts. Unfortunately, we were unable to genotype the isolates to determine whether they were zoonotic. Regular consumers of contaminated groundwater, which usually contains low levels of *Cryptosporidium* oocysts or *Giardia* cysts (Hancock et al., 1997), may have developed immunity that does not extend to visitors (Morris and Tyson, 2003). Age deterioration, poor well-head hygiene, or defects in design may provide localized pollution pathways from run-off, nearby septic tanks or unlined livestock slurry pits (Morris and Tyson, 2003).

All of the five *Giardia*-positive surface water samples obtained from the upstream and downstream locations had positive net changes in the concentration of cysts detected. No water sample from domestic sources was positive for *Giardia* cysts. Since none of the *Giardia* positive samples was successfully genotyped, it is not possible to comment on their zoonotic potential. Although there is a positive trend for water contamination with *Giardia*, the fact that most genotypes in the cattle were non-zoonotic suggests that this is unlikely to be a major public health concern. However, as we were unable to obtain genotype information on the *Giardia* isolates from the water samples, it is difficult to confirm this. On the other hand, *Cryptosporidium* contamination of surface water was increased with farm exposure, and based on genotyping data from these water samples, *C. parvum* was common, which suggests that a zoonotic potential exists.

Cattle >6 mo of age in free-stall housing were more likely to be infected with

Cryptosporidium spp. than those in other types of housing. In confined housing, cows that are free to roam in a free-stall barn are more likely to contaminate the environment and potentially the feed and water with *Cryptosporidium* oocysts compared to cattle mostly confined to their stall in a tie stall barn. Cows have been shown to be an important source of infection with *Cryptosporidium* for other animals, including their own offspring (Faubert and Litvinsky, 2000). The age of an animal was not associated with the risk of shedding *Cryptosporidium* oocysts, in contrast with other studies (Olson et al., 1997a; O'Handley et al., 1999; Wade et al., 2000b). While it seems counter-intuitive, this may be attributed to the effect of other risk factors that were not considered in this study. However, based on genotyping, there appears to be an age distribution to specific species of *Cryptosporidium*. Previous studies have observed regional differences in the prevalence of *Cryptosporidium* infection in dairy cattle (Duranti et al., 2009). In this study, the prevalence of *Cryptosporidium* infection in dairy cattle tended to be higher in Queens County (Fig. 1) than Kings and Prince Counties. The differing geography of these counties may influence various climatological factors such as temperature, humidity and precipitation, which in turn greatly influence the biologic environment (Mortimer and Cherry, 2004). The life cycles and reservoir mechanisms of many pathogens including parasites are dependent on appropriate temperature and humidity (Mortimer and Cherry, 2004). However, PEI is Canada's smallest province and as such, its geographical area (5,684 km^2) might be too small to have any significant impact on specific pathogen distribution.

In conclusion, this study found that while the role of dairy cattle in contaminating the watershed with zoonotic species of *Giardia* may be negligible, these animals are clearly a potential source of contamination of the watershed on PEI with *C. parvum.*

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3.0. *GIARDIA* AND *CRYPTOSPORIDIUM* ON BEEF FARMS AND THE ROLE THESE FARMS MAY PLAY IN CONTAMINATING SOURCE WATER IN PRINCE EDWARD ISLAND, CANADA

Abstract

The common occurrence of *Cryptosporidium* and *Giardia* in domestic cattle is a matter of concern for public health, given the potential to contaminate water through field application of manure and pasture run-off. The objectives of this study were to determine the prevalence and genotypes of *Giardia* and *Cryptosporidium* on beef farms in Prince Edward Island (PEI), Canada, including the water sources associated with the farms, and to determine risk factors for infection of cattle with these parasites. Twenty beef farms were selected based on the presence of surface water <500m from the barn. Prevalence was determined by direct immunofluorescence microscopy, while genotyping and species determination was by nested-PCR and DNA sequencing. *Giardia* and *Cryptosporidium* were detected in 42% and 17% of 739 fecal samples, and 100 and 80% of farms, respectively. *G. duodenalis* livestock genotype assemblage E predominated (89%). The zoonotic assemblages A and B were found in 4 and 7% of the fecal samples that were genotyped (n=100), respectively. The *Giardia* genotypes were evenly detected between the cows and calves examined. Of the genotyped samples (n=55) the most common *Cryptosporidium* species detected in this study was *C. andersoni* (49%), predominantly found in cattle >6mo of age, while most *C. bovis* and *C. parvum* isolates were detected in calves ≤6mo of age. All *C. ryanae* isolates (four) were found in calves. *Giardia* cysts and *Cryptosporidium* oocysts were detected in 14 and 93% of surface water samples of 14 farms, respectively.

Cryptosporidium oocysts were detected in three (15%) ground water samples of 20 farms. One *Cryptosporidium*-positive water sample, which was the only surface water sample amenable to genotyping, contained *C. parvum*. The farm-level risk factors investigated in this study, age of animals, and location of the farm were not associated with the risk of infection in cattle with either *Cryptosporidium* spp. or *G. duodenalis*.

We conclude that beef cattle may represent a potential threat to watersheds and subsequent disease transmission to humans on PEI from *Cryptosporidium* spp. and *G. duodenalis*.

3.1. Introduction

Cryptosporidium oocysts and *Giardia* cysts commonly occur in the aquatic environment and there have been a significant number of reports of waterborne outbreaks of cryptosporidiosis and giardiasis worldwide (LeChevallier et al., 1991; Mackenzie et al., 1994; Hoogenboezem et al., 2001). Surface waters are most susceptible to contamination with *Cryptosporidium* oocysts and *Giardia* cysts (Rose et al., 2002). Groundwater is usually free of these organisms, but occasional contamination has been reported at low concentrations (Hibler, 1988; Hancock et al., 1997). Several studies have demonstrated the presence of *Cryptosporidium* and *Giardia* in raw and treated water sources across Canada (Isaac-Renton et al., 1987; Wallis et al., 1995) and contamination with these parasites is one of the most serious and challenging threats to the safety of Canada's drinking water supplies.

Studies which have looked at the impact of land use activities in watersheds show that the occurrence of *Cryptosporidium* oocysts and *Giardia* cysts is high both in frequency and concentration in waters impacted by cattle ranch operations (Ong et al., 1996; Bagley et al.,

1998; Keeley and Faulkner, 2008). This, together with the wide distribution of cattle feeding operations in watersheds, gives rise to the common notion in the public health community that cattle play a significant role in contaminating source water with *Cryptosporidium* and *Giardia*. In order to determine the importance of cattle in the potential contamination of watersheds with public health consequences, knowledge of the prevalence and capacity of the animals to load the watershed with zoonotic species of these parasites is required. Such awareness will also be of strategic importance in watershed protection efforts. Dairy cattle have been the subject of most prevalence studies of *Cryptosporidium* and *Giardia* in cattle in Canada (O'Handley et al., 1999; Huetink et al., 2001). The reasons for the rather large volume of studies in dairy cattle might stem from the ease of sampling animals in the confined husbandry system of the dairy operation as opposed to the mostly pasture model for beef cattle. In the Atlantic region of Canada, there have only been a few studies done on the epidemiology of *Cryptosporidium* and *Giardia* in dairy cattle (Uehlinger et al., 2006; Coklin et al., 2009; Uehlinger et al., 2011, in press) and none in beef cattle.

We undertook this study to determine the prevalence of *Giardia* and *Cryptosporidium* in beef cattle, and the role that beef cattle operations might play in contaminating source water with zoonotic genotypes and species of these parasites.

3.2. Materials and methods

3.2.1. Fecal sample collection and processing

Fecal samples were collected between March and August, 2006 from 739 cattle between the ages of 1 month and 4 years old, selected from 20 beef farms on Prince Edward Island, Canada. On each farm, samples were randomly taken (ear tags numbers drawn from a container)

from 20 cows >6 mo of age, and 20 calves ≤6 mo of age. On farms that had less than 20 animals in each group, samples were collected from all animals available. Fecal samples were obtained rectally if possible, using a sterile disposable latex glove, or collected from the ground immediately after defecation. Samples were placed in clean plastic containers, transported to the laboratory on ice, and stored for short periods (maximum 1 week) at 4 °C until processing, or at -20 °C if processing was delayed more than one week. Data on the geographic location of each farm (i.e., Queens, Kings and Prince Counties, PEI) (Fig.3.1) was also collected.

Cryptosporidium oocysts and *Giardia* cysts were isolated from fecal samples according to a previously described procedure (O'Handley et al., 1999), with slight modifications. Briefly, fecal samples consisting of 20 g for adults and 5 g for calves were mixed with 35 and 10 ml of phosphate-buffered saline, pH 7.4 (PBS), respectively, passed through 4 layers of surgical gauze (Nu-gauze, 4 ply, Johnson & Johnson, Montreal, QC). The strained fecal sample homogenate was gently layered over 15 ml and 5 ml of 1M sucrose (Sigma-Aldrich Canada Ltd., Oakville, ON) solution (sp. gr. 1.13) in a clean polypropylene conical tube for adult and young animals, respectively. Following centrifugation at 800 x g for 5 min in a fixed-angle rotor centrifuge, the upper filtrate layer and sucrose surface were transferred into a clean 50 ml centrifuge tube for the adult samples and 15 ml tubes for the calf samples, and recentrifuged as above. The resulting supernatant was discarded and the pellet re-suspended in PBS to approximately 1.5 ml.

3.2.2. Direct immunofluorescence antibody (DFA) (oo)cyst examination of fecal samples

Twenty µl volumes of the fecal suspensions were air dried to fluorescence microscopy slides (Waterborne, New Orleans, LA), methanol fixed, and stained with *Cryptosporidium* or

Giardia-specific fluorescein isothiocyanate (FITC)-labeled monoclonal antibody solution (0.04mL) (Crypt-a-glo™ or Giardi-a-glo™, Waterborne Inc, New Orleans, LA). After incubation at room temperature for 40 min in a humid chamber, the slide was briefly rinsed with PBS, air dried at room temperature and mounted, using fluorescent antibody mounting fluid (AquaPolymount, Polysciences, Warrington, PA), with a 22 mm^2 coverslip (VWR Scientific Inc., Media, PA). *Cryptosporidium* oocysts and *Giardia* cysts were examined and enumerated at 200 x and 100 x magnifications, respectively, under an epifluorescence microscope (Leica DM 2500, Leica Microsystems, Wetzlar, Germany). The number of oocysts and cysts per gram of feces was determined as previously described (O'Handley et al., 1999). The sensitivity of this method was determined previously to be 66 oocysts or cysts per gram of feces (O'Handley et al., 1999).

3.2.3. Water sample collection and processing

Water samples were collected from ground water (well) and surface water (rivers, streams, creeks and brooks) from locations within the vicinity of the farms (within 500 m from the animal housing facility) between October 2006 and August 2007. Ponds, which were the only surface water sources on some farms, were excluded from the analysis since cows had easy access, and their frequent presence in the ponds could bias the results of the study. Surface water samples, obtained from 14 farms, were collected from both the upstream and downstream sites of the pasture land, while a sample of ground water was obtained from each well present on the 20 farms. Groundwater samples positive for *Cryptosporidium* and/or *Giardia* were re-sampled within about two months of obtaining the initial results and tesing repeated for the presence of the parasites as well as for background indicator bacteria such as coliform/*E. coli* counts.

Water samples were filtered through a Filta-Max® (IDEXX, Westbrook, ME) foam filter system at a flow rate of 3.3 l/min according to the manufacturers' instructions. One hundred litres of water was filtered from ground sources. Twenty litre volumes of surface water were similarly filtered where there was no difficulty in the filtration process. However, if a 20 l volume of water had not been filtered within 30 min due to high turbidity (turbidity was not measured but crudely implied by the speed of the filtration process) of the sample, a 10 l grab water sample was obtained and filtered in the laboratory for analysis. The Filta-Max® filters from samples filtered on-site were transferred to clean plastic bags, sealed, and transported in a cooler to the laboratory for further processing

At the laboratory, samples were processed for parasite isolation and enumeration according to US-EPA method 1623 (USEPA, 2003) , using the Filta-Max® wash station procedure for elution of (oo)cysts. Parasites were enumerated by epifluorescence microscopy (Leica Axioscope fluorescence microscope equipped with a blue filter block having an excitation wavelength of 490 nm and an emission wavelength of 510 nm). Samples in which (oo)cysts fulfilled defined morphological criteria according to the US-EPA 1623 protocol were considered positive. The number of (oo)cysts detected per litre was calculated according to a previously described method (Farias et al., 2002) as follows:

Number of (oo)cysts in pellet = Number of (oo)cysts in an analyzed drop x total mL of the pellet/volume of analyzed drop

Number of (oo)cysts/L = Number of (oo)cysts in pellet/number of litres filtered or flocculated

Concentrations of (oo)cysts from upstream locations of surface water were subtracted from those of the corresponding downstream sites to achieve a net concentration of (oo)cysts. A positive result was interpreted as the positive impact of the specific farm on the contamination of the water source with *Cryptosporidium* oocysts or *Giardia* cysts.

3.2.4. (Oo)cyst recovery from microscope slides

(Oo)cysts were extracted from samples on microscope slides using a modification of a procedure previously described (Nichols et al., 2006), involving for a series of 4 washes of the slide with 25 µl volumes of fresh lysis buffer. The final volume of the sample amounted to approximately 100 µl. The suspension was then centrifuged at 4,000 x g for 60 sec. Using a pipette, 50 µl of the supernatant was removed and discarded. The rest of the sample was re-suspended by gentle vortexing and further processed for DNA extraction.

3.2.5. DNA extraction

DNA extraction was performed on at least five randomly selected DFA positive fecal samples from both cows and calves from each farm, and on all eluted slides from DFA positive water samples. DNA was extracted using the Qiagen DNeasy Tissue Kit (Qiagen Inc., Mississauga, ON) according to the manufacturer's instructions. Briefly, a total of 50 µl of processed feces or water sample was suspended in 180 µl of ATL buffer and thoroughly mixed by vortexing. Proteinase K (20 mg/ml) was added to the suspension, thoroughly mixed, and 200 µl of AL buffer added after overnight incubation at 55 °C. DNA was eluted in 100 µl of AE buffer.

3.2.6. Polymerase chain reaction and DNA sequence analysis

For *Giardia*, nested-PCR was performed using modifications of protocols previously described for the amplification of the β-giardin (Caccio et al., 2002) and 16S ribosomal DNA (rDNA) (Appelbee et al., 2003) gene fragments. In this study, the nested cycle of the PCR using the β-giardin gene fragment was the same as the initial cycle except that the annealing temperature was increased to 66 °C from 58 °C for 30 s, and for the 16S rDNA the annealing temperature of the nested cycle was increased to 58 °C from 55 °C. Nested-PCR was also performed for *Cryptosporidium* using modifications of protocols previously described for the amplification of the heat shock protein 70 (HSP70) (Guselle et al., 2003) and 18S rDNA (Fayer et al., 2006) gene fragments. Similarly, the annealing temperature for the nested-PCR cycle using the HSP70 gene fragment was increased to 58 °C from 55 °C in this study. For the 18S rDNA nested-PCR, the $MgCl_2$ concentration for the PCR mixture was decreased to 1.5 mM from 3.0 mM.

All amplification products were visualized in 1.5% agarose gels with ethidium bromide. Positive PCR amplicons were sequenced in both directions, using the same PCR primers used to produce the amplicons, at the Genome Quebec Innovation Centre at McGill University, Montreal, QC. To determine the genotypes of *Giardia* and species of *Cryptosporidium*, sequences were compared with similar published sequences by BLAST – analysis (Altschul et al., 1990)

Each *Giardia* sequence was compared to the GenBank sequences of *Giardia* assemblages A (accession number AF113902), B (accession number DQ7891121) and E (accession number AY655701). Similarly, each *Cryptosporidium* sequence was compared to the GenBank

sequences of *Cryptosporidium* as follows: *C parvum* (accession number: AF093490), *C. bovis* (accession number: AY741305) *C. ryanae* (accession number: AY587166) and *C. andersoni* (accession number: AB089285). Sequences were aligned using the software BioEdit (Hall, 1999).

3.2.7. Data collection and statistical analysis

The herd prevalence and within-herd prevalence were obtained from the DFA results. The within-herd prevalence was calculated as the number of positive animals over the total number of animals tested for the farm of interest. The herd prevalence was calculated as the number of farms with at least one positive animal, over the total number of farms. The generalized linear mixed model (random effects logistic regression) was used to test for significance ($P<0.05$) of region (county on PEI) and age of animals as fixed systematic environmental effects for *Cryptosporidium* and/or *Giardia* infection, accounting for herd random effects (Dohoo et al., 2009). A negative binomial regression model was used to determine if there was any significant difference in the number of *Cryptosporidium* oocysts/l between the upstream and downstream samples for surface water, accounting for herd random effects. A boxplot distribution was created to demonstrate the effect of the within-herd prevalence on having positive or negative resultant *Cryptosporidium* oocysts in the water positive samples associated with the farms. A student *t-test* statistic was also used to determine if there was a significant association ($P<0.05$) between the within-herd prevalence and having positive or negative resultant *Cryptosporidium* oocysts in the water positive samples associated with the farm. There was not sufficient data to do similar computations for *Giardia*-positive water

samples. All analyses were performed using the statistical software package STATA 11.0 (Stata Corporation, College Station, TX).

3.3. Results

3.3.1. Fecal samples

Of the 739 fecal samples collected, 310 (42%; 95% CI: 38-46%) were confirmed as positive for *Giardia* by DFA (Table 3.1). At least one *Giardia*-positive animal was identified on all farms (100% farm prevalence), and the within-herd prevalence ranged from 7 to 83% (median 44%). *Giardia* infection was equally distributed between calves ≤ 6 months of age (42%; 95% CI: 37-47%) and cattle > 6 months of age (42%; 95% CI: 36-47%). Genotyping was possible for 25 isolates with the β-giardin gene and 84 isolates with the 16S rDNA gene. Overall, for samples successfully genotyped (n=100), 4, 7 and 89%, of *G. duodenalis* isolates recovered belonged to assemblage A, assemblage B and assemblage E, respectively (Table 3.2). Assemblage E was found in 90% (54 of 60 isolates) of the *G. duodenalis* isolates that were successfully genotyped in calves, and 88% (35 of 40 isolates) of cattle (Table 3.2). Two isolates of assemblage A were found in each age group; four assemblage B isolates were found in calves, and three in cattle (Table 3.2).

No significant association was found between infection with *Giardia* species and either the age of the animal (P= 0.66), or the county (Fig. 3.1) from which the farm was located (P= 0.26).

Table 3.1. Age-related prevalence of *Cryptosporidium* and *Giardia* based on immunofluorescence assay in 20 Prince Edward Island, Canada, beef herds

Age of animal	Number of Animals	# Positive	Overall prevalence (95% CI)	Age-related Prevalence (95% CI)
Cryptosporidium	739	123	17 (14-19%)	
≤6 mo	397	71		18 (14-22%)
>6 mo	342	52		15 (11-19%)
Giardia	739	310	42 (38-46%)	
≤6 mo	397	168		42 (37-47%)
>6 mo	342	142		42 (36-47%)

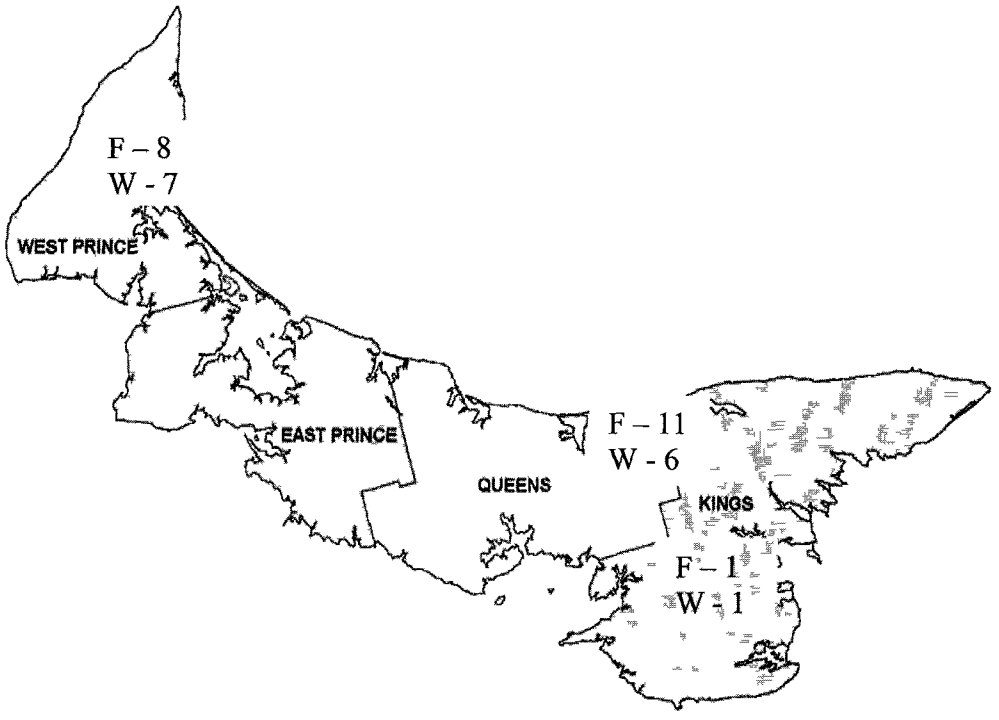

Figure 3.1. Map of Prince Edward Island (PEI), Canada, showing the three counties: Prince (East and West), Queens, and Kings from where animal fecal and water samples were obtained. F - number of beef herds sampled; W - number of surface water samples.
Source: PEI Statistics Bureau. Department of Finance and Municipal Affairs

Table 3.2. Distibution of *Cryptosporidium* and *Giardia* species/genotypes in beef cattle based on the combined sequencing data from the HSP70 and 18S rDNA genes for *Cryptosporidum* and the B-giardin and 16S rDNA genes for *Giardia*

Category	n	*Cryptosporidium*-positive				n	*Giardia*-positive		
		C. parvum	*C. bovis*	*C. ryanae*	*C. andersoni*		A	B	E
Combined*	55	13 (24)	11 (20)	4 (7)	27 (49)	100	4 (4)	7 (7)	89 (89)
≤6mo	46	12 (26)	10 (22)	4 (9)	20 (43)	60	2 (3)	4 (7)	54 (90)
□6mo	9	1 (11)	1 (11)	-	7 (78)	40	2 (5)	3 (8)	35 (88)

* positive by at least one gene locus

- = no isolates detected

n = total number of samples genotyped; numbers in parentheses are percentages

Of the 739 fecal specimens examined, 123 (17.0%; 95% CI: 14-19%) were confirmed as *Cryptosporidium*-positive by DFA (Table 3.1), and represented 16 of the 20 farms sampled (80% farm prevalence). The within-herd prevalence on *Cryptosporidium*-positive farms ranged from 2.5 to 82.4% (median 11%). *Cryptosporidium* was detected in 18% (95% CI: 14-22%) of calves and 15% (95% CI: 11-19%) of cattle (Table 3.1). Genotyping was determined for 24 isolates with the HSP70 gene and 39 isolates with the 18S rDNA gene. Overall (n=55) 24, 20, 7 and 49% of isolates genotyped belonged to *C. parvum, C. bovis*, *C. ryanae* and *C. andersoni*, respectively (Table 3.2). In the isolates successfully genotyped, *C. andersoni* was 78% of the genotyped isolates (7 of 9 isolates) of cattle, and 43% (20 of 46 isolates) of calves. The detection of *C. parvum*, *C. bovis* and *C. ryanae* was confined to calves, at the levels of 26, 22 and 9%, respectively (Table 3.2). No significant association was found between infection with *Cryptosporidium* species and either the age of the animal (P= 0.72) or the location of the farm (P= 0.64).

3.3.2. Water Samples

Surface water was obtained from 14 farms and 20 (one from each farm) domestic (well) water samples were obtained from the homes on the farms. For surface water, eleven farms had samples taken from both the upstream and downstream locations while three samples were taken from only the downstream location. Ten of the samples taken from both the upstream and downstream locations and all three samples from the downstream location only were positive for *Cryptosporidium* oocysts for a farm prevalence of 93% (13 of 14). The concentration of oocysts in the positive surface water samples ranged from 0.05 to 14.6 oocysts/L. At six of the 10 farms (60%) with *Cryptosporidium*-positive surface water sample(s) from both the upstream

and downstream locations, a positive net change in *Cryptosporidium* oocyst concentration was found, while at four farms a negative net change was found (Fig. 3.2). On average a positive net change of 1.14 oocysts/L (95% CI: 0.84-2.4) was found (P=0.19). A boxplot distribution of the within-herd prevalence and having a resultant positive or negative effect on the concentration of *Cryptosporidium* oocysts in the water-positive samples is presented in Fig 3.3. Despite the apparent differences between the distributions, prevalence in animals was not significantly associated with having a positive or negative change for *Cryptosporidium* oocysts (P = 0.56) in the surface water-positive samples.

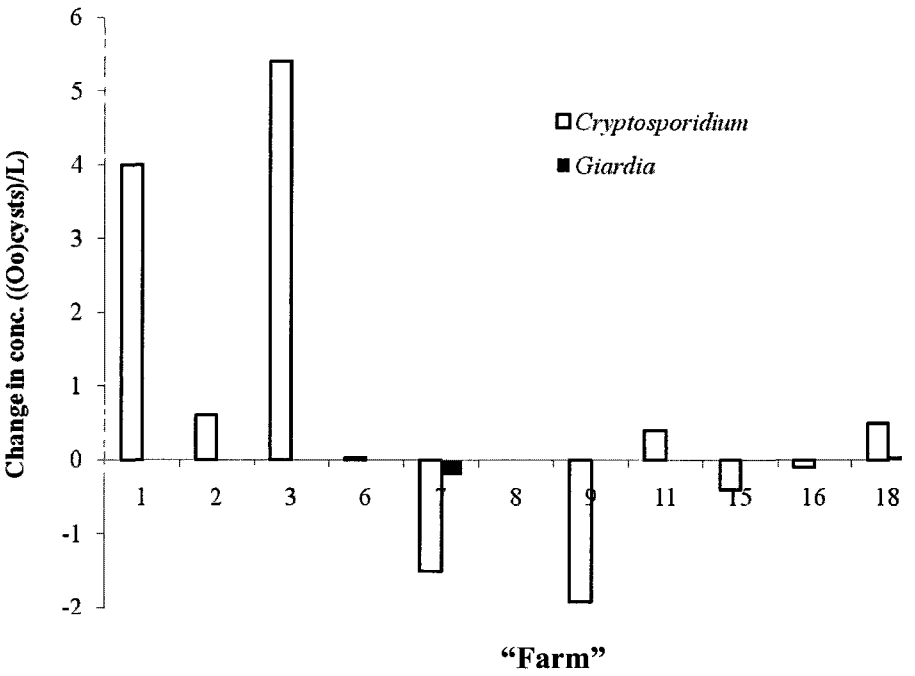

Figure 3.2. Prevalence and net concentration of *Cryptosporidium* oocysts and *Giardia* cysts in surface water (with both upstream and downstream values) within the vicinity (~500m) of beef farms on Prince Edward Island

Genotyping was only successful for one surface water sample, and this isolate was identified as *C. parvum*. *Cryptosporidium* oocysts were detected in three ground water samples, one each from three different farms, with oocysts concentrations ranging from 0.1 to 7.2/L. Two of the farms with *Cryptosporidium*-positive ground water were also positive for *Cryptosporidium* in livestock. No ground water samples were successfully genotyped.

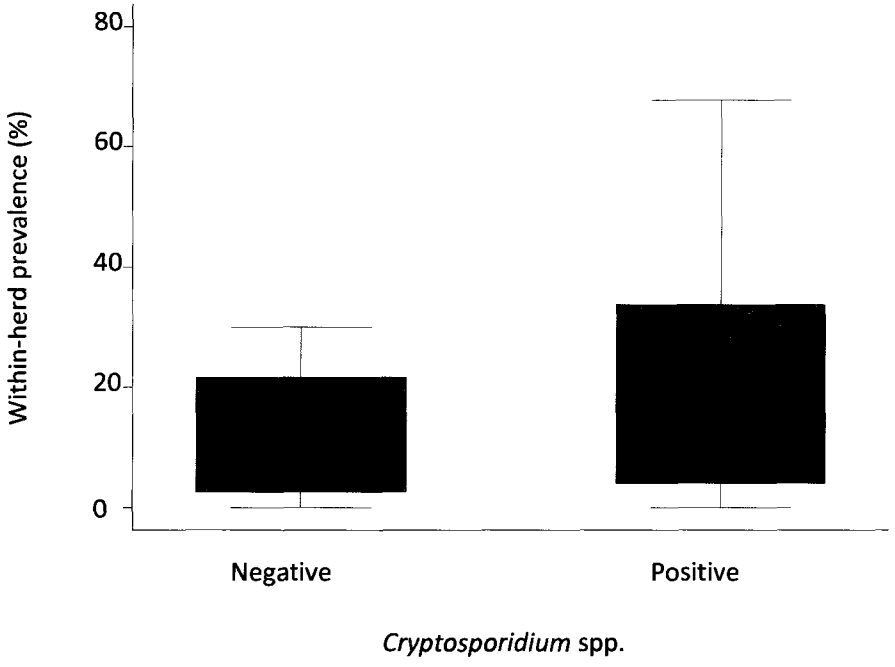

Figure 3.3. Boxplot of the within-herd livestock prevalences for farms with (n=6) or without (n=4) *Cryptosporidium* oocysts in water-positive samples. Upper and lower bounds of box denote the 75th and 25th percentiles. Upper and lower bounds of bars are the 90th and 10th percentiles. Line within the box is the median

Giardia cysts were detected in only 2 (14%) of the surface water samples, One sample had a positive net change and the other had a negative net change in the concentration of cysts

from upstream to downstream sampling (Fig. 3.2). The concentrations of *Giardia* cysts in the surface water were much lower than *Cryptosporidium* oocysts, ranging from 0.05 to 0.2 cysts/L. *Giardia* cysts were not found in any ground water samples. Genotyping was not successful for any of the *Giardia*-positive isolates from water samples.

3.4. Discussion

Reported *Cryptosporidium* prevalence rates in beef cattle in North America range from 7.1 to 43% (Atwill et al., 2003; McAllister et al., 2005; Gow and Waldner, 2006; Fayer et al., 2010), with 1.1 to 21% in post-weaned and adult cattle (Fayer et al., 2000; Hoar et al., 2001; Atwill et al., 2003; Feltus et al., 2008), and 3.1 to 38% in calves under 6 mo of age (Gow and Waldner, 2006; Feltus et al., 2008). The overall prevalence of *Cryptosporidium* in the beef cattle sampled on PEI in the present study (17%) was consistent with these studies, as was the prevalence reported in both calves (15%) and adult cattle (18%).

Among the isolates successfully genotyped in this study, *C. andersoni* was the predominant (49%) species of *Cryptosporidium*. Although the majority (78%) of successfully genotyped isolates from cattle >6 months of age were *C. andersoni*, it also accounted for 43% of the genotyped isolates from calves ≤6 mo of age. The present study demonstrates, therefore, that it is possible for *C. andersoni* to be highly prevalent in pre-weaned calves, and may indicate interaction between mature animals and calves resulting in the infection of susceptible calves at an earlier age than previously reported in both beef and dairy cattle (Fayer et al., 2006; Geurden et al., 2007; Feltus et al., 2008; Fayer et al., 2010). *C. parvum, C. bovis* and *C. ryanae* were only detected in calves ≤6 mo of age. This finding is in accordance with the complex age-related

distribution of *Cryptosporidium* species/genotypes found in beef cattle (Feltus et al., 2008), and in dairy cattle (Santin et al., 2004; Fayer et al., 2006; Fayer et al., 2007).

Studies on the prevalence of *Giardia* in beef cattle in North America have found overall point prevalences of between 6.5% and 34% (Hoar et al., 2001; Appelbee et al., 2003; McAllister et al., 2005), with prevalences ranging from 22 to 36% for calves under 6 mo of age (Olson et al., 1997; Hoar et al., 2001; Appelbee et al., 2003; McAllister et al., 2005; Gow and Waldner, 2006), and 6.5 to 17% for post-weaned and adult cows (Olson et al., 1997; Appelbee et al., 2003; McAllister et al., 2005; Gow and Waldner, 2006). In the present study, *Giardia* was detected in 42% of overall samples, including 42% in calves (<6 mo of age) and 42% in adult cattle (>6 mo of age). This is higher than previous reports and may indicate a regional difference in prevalence that reflects management of beef cow-calf operations on PEI, where animals tend to be more confined, compared to other regions of North America (PEI Department of Agriculture, Unpublished).

To date, only a handful of studies in North America have reported on the genotypes of *G. duodenalis* capable of infecting beef cattle. These studies have revealed the predominance of assemblage E in beef cattle, with point prevalences of up to 100% (Appelbee et al., 2003; Dixon et al., 2011; Uehlinger et al., 2011, in press). While assemblage A has also been detected in beef, albeit at low levels (0-2.4%) (Appelbee et al., 2003; Uehlinger et al., 2011, in press), to our knowledge, there have been no previous reports on the detection of assemblage B in beef cattle in North America. The low infection rate of zoonotic genotypes (assemblages A and B) of *Giardia* in beef cattle from previous studies leads to the suggestion that these animals may not be important in the epidemiology of human giardiasis (Caccio et al., 2005). In the present study, however, *G. duodenalis* assemblages A and B were found in 4 and 7% of PEI beef cattle,

respectively. These results demonstrate that the beef herds in this study may represent a reservoir for zoonotic *Giardia*, and a potential threat to the watersheds on PEI and to public health.

Cryptosporidium oocysts were highly prevalent (93%), while *Giardia* cysts were only occasionally (14%) present, in surface water associated with the farms in this study. A majority (60%) of *Cryptosporidium*-positive surface water samples obtained from both upstream and downstream locations had increases in oocyst concentrations from the upstream to the downstream sample. While genotyping was only possible for one of these samples, the identification of *C. parvum* in surface water was consistent with previous studies (Xiao et al., 2001). In this study, *Cryptosporidium* was not detected in any of the animal fecal samples from the farm with the *C. parvum*-positive surface water sample. Similar results were reported in other studies (Ong et al., 1996). The different sampling times for the fecal and water samples in our study might account for the discrepancy in the results from the farm animals and the surface water associated with them. Sampling of animals on the farms was completed in August 2006, while water sampling began in October of the same year. Other possible reasons include the intermittent shedding of oocysts by the animals, increasing the chances of missing possible infections in animals outside of the time of sampling in point prevalence studies. Also, manure management practices might be more important in terms of watershed contamination than infection rates determined in animals. In a comparative study of 11 dairy farms sampled monthly for 6 months in the northern US, the single most important factor in detecting *Cryptosporidium* in surface water was increased frequency of spreading manure on fields (Sischo et al., 2000). It is, therefore, possible that *Cryptosporidium* from earlier infections on the farms might account for the isolate detected in the water sample from farms where no oocysts were detected. *C. parvum* has a broad host range including domestic and wild ruminants and humans (Xiao et al.,

2004), meaning that the occurrence of the species in the watersheds in this study could also be a result of agriculture as well as wildlife, or human sewage. However, PEI lacks wild ruminants (deer and moose) leaving agriculture or human sewage the likley possible explanations.

It is less likely that groundwater will be contaminated, given the ability of soil to filter large concentrations of microorganisms. However, age deterioration, poor well-head hygiene, or defects in design may provide localized pollution pathways from run-off, nearby septic tank or unlined livestock slurry pits (Morris and Tyson, 2003). Three ground water samples in this study were positive for *Cryptosporidium* oocysts. In previous studies, between 9.5 to 22% of groundwater samples were positive for *Cryptosporidium* (Hancock et al., 1998). Although regular consumers of contaminated groundwater, which usually contains low levels of *Cryptosporidium* oocysts or *Giardia* cysts (Hancock et al., 1997) may have developed immunity, visitors (e.g. on-farm holiday accommodation and campsite situations) may be vulnerable (Morris and Tyson, 2003).

In follow-up analyses of the positive samples, they were again not only positive for *Cryptosporidium*, but had elevated levels of coliform/*Escherichia coli* counts, indicating fecal contamination of the wells which may have resulted from any of the sources indicated earlier. The presence of *Cryptosporidium* oocysts in ground water raises public health concerns, as potable water from this source will not normally undergo any form of treatment before consumption. Unfortunately, we were unable to genotype our *Cryptosporidium* isolates from ground water sources to determine whether they were zoonotic. We were also not able to demonstrate a significant association between prevalence of *Cryptosporidium* spp. in the cattle and a resultant positive or negative concentration for *Cryptosporidium* oocysts in the *Cryptosporidium*-positive water samples as there were too few samples to provide sufficient

power for the analysis. From the two *Giardia*-positive surface water samples, one had a positive change and the other a negative change in cyst concentration. None of the *Giardia* isolates were amenable to PCR. Previous studies have also reported on the inability to obtain PCR or sequencing results from *Cryptosporidium* oocysts and *Giardia* cysts obtained from surface water samples (Castro-Hermida et al., 2009). This has been attributed to the possible presence of inhibitors in the surface water, and many studies have demonstrated the reduction or suppression of PCR amplification of DNA extracted from *Cryptosporidium* oocysts and *Giardia* cysts from water sources by inhibitors (Sluter et al., 1997; Guy et al., 2003). The application of immunomagnetic separation (IMS) for the isolation of (oo)cysts prior to the extraction of DNA as performed in this study, is currently the standard procedure for removing PCR inhibitors in the PCR detection of *Cryptosporidium* and *Giardia* from water. Although IMS may improve DNA amplification, residual PCR inhibitors can still be present in DNA from (oo)cysts purified by this method (Jian et al., 2005). This is one of the most serious challenges to the PCR detection of *Cryptosporidium* and *Giardia* from water sources, and requires urgent resolution.

The results from this study suggest that beef cattle may represent a potential risk for contamination of the watershed in Prince Edward Island, Canada, with zoonotic *Cryptosporidium* and *Giardia*, and subsequent transmission to humans.

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4.0. PREVALENCE AND MOLECULAR CHARACTERIZATION OF *GIARDIA* AND *CRYPTOSPORIDIUM* IN PIGS ON PRINCE EDWARD ISLAND, CANADA

Abstract

In a cross-sectional study of 633 pigs from 21 herds on Prince Edward Island, Canada, the prevalence of infection with *Cryptosporidium* and *Giardia,* and the genotypes and species of isolates, were determined in order to establish the zoonotic potential of pigs in this region. Eighteen herds (86%) and 163 animals (26%) tested positive for *Cryptosporidium*, while just 3 herds (14%) and 6 animals (1%) tested positive for *Giardia*. *Cryptosporidium* spp. isolates were detected in 39% of weanlings (1-3 mo of age) and 9% of sows (> 8 m of age). Molecular characterization using the 18S rDNA and HSP70 gene fragments revealed the presence of *Cryptosporidium* sp. pig genotype II, *C. suis*, *C. parvum*, and *Cryptosporidium* sp. mouse genotype. Among the isolates of *Cryptosporidium* spp. successfully genotyped, pig genotype II (61%) predominated, with *C. suis* (36%) being the next most prominant isolate. *C. parvum* (2%; two isolates) and *Cryptosporidium* sp. mouse genotype (0.9%; one isolate) were only occasionally isolated. The only two *Cryptosporidium*-positive genotyped isolates from sows included one each of *C. suis* and *Cryptosporidium* sp. pig genotype II. All but one of the six *Giardia* positive isolates were detected in weanling pigs. None of the *Giardia*-positive isolates was amenable to PCR. This study demonstrates that *Cryptosporidium* spp. and *Giardia duodenalis* in pigs on PEI, Canada, are found mostly in weanlings (1-3 mo of age). Furthermore, the pigs are primarily infected by the host-specific genotypes and species, *Cryptosporidium* sp.

pig genotype II and *C. suis*, whereas the zoonotic *C. parvum* is rare. These findings suggest that domestic pigs on PEI, Canada, likely do not pose a significant health risk to humans from these parasites.

4.1. Introduction

There has recently been considerable interest in the role that livestock may play in the foodborne and waterborne transmission of *Cryptosporidium* and *Giardia* to humans. This interest is fueled by studies which have shown that these parasites are highly prevalent in livestock. Although cattle have been the subject of most of these studies (Olson et al., 1997; Wade et al., 2000; Becher et al., 2004; Uehlinger et al., 2006), *Cryptosporidium* and *Giardia* in pigs have been reported worldwide, and in all age groups (Guselle and Olson, 1999; Vitovec et al., 2006; Langkjaer et al., 2007; Zintl et al., 2007; Armson et al., 2009) .

Pigs are most frequently infected with host-specific species and genotypes of *Cryptosporidium*, namely *C. suis* and *Cryptosporidium* sp. pig genotype II. However, they can also be naturally infected with *C. muris*, *Cryptosporidium* mouse genotype 1, *Cryptosporidium* sp. Eire w65.5, and the zoonotic *C. parvum* (Morgan et al., 1999; Xiao et al., 2006; Chen and Huang, 2007; Zintl et al., 2007). Like cattle and other ruminants, *Giardia* infections in pigs are predominantly *G. duodenalis* assemblage E, but infections with the zoonotic genotype assemblage A have also been reported (Langkjaer et al., 2007; Armson et al., 2009) .

The application of swine manure in the cultivation of food and forage crops, as well as the spreading of swine slurry onto pasture and tillage land for the sole purpose of disposal, are common practices (Hutchison et al., 2004). Swine manure can be highly prevalent for *Cryptosporidium* spp. For example, in a study on livestock farms in Ontario, Canada, 26% of all

swine liquid manure samples tested positive for *Cryptosporidium*, compared with 8% for dairy solid manure and 7.3% for dairy liquid manure (Fleming, 1999). Proper composting of manure at ≥55° C for 3-15 days can yield safe fertilizer by destroying a number of different pathogens (Guan and Holley, 2003), however, and while *Giardia* cysts are degraded in swine manure holding tanks (Guselle and Olson, 1999), *Cryptosporidium* was detected in 25 of 56 treated swine slurry samples in another study (Xiao et al., 2006). The presence of *Cryptosporidium* oocysts or *Giardia* cysts in swine manure can manifest as direct contamination of produce and indirect contamination of water supplies through agricultural run-off.

The infection of pigs with zoonotic species and genotypes, including *C. parvum* and *G. duodenalis* assemblage A, indicates that they may play a potential role as sources of infection for humans. Studies on *Cryptosporidium* and *Giardia* infections in pigs have been conducted in other regions of Canada, however no such studies have been reported in the Atlantic region. The aim of this study, therefore, was to determine the prevalence and genotypes of *Cryptosporidium* and *Giardia* in domestic pigs on Prince Edward Island, Canada, and to determine if these animals represent a source for the potential zoonotic transmission of these parasites.

4.2. Materials and Methods

4.2.1. Selection of farms

Twenty-one swine farms were selected from all three counties: Queens, Kings and Prince on Prince Edward Island (PEI). A list of swine farmers was obtained from the Farm Services Section of the Department of Health Management, Atlantic Veterinary College, University of Prince Edward Island in Charlottetown, Canada. Farms were selected based on accessibility of animals for sampling and the owner's willingness to participate in the study.

4.2.2. Sample collection

At each of the 21 farms, fecal samples were collected between February 2007 and November 2007 from 20 adult animals (sows >8mo of age) and 20 weanlings (1-3mo of age). If farms had less than 20 animals in each age group, samples were collected from all animals available. Fecal samples were collected once from freshly voided feces using a sterile disposable latex glove and placed in clean plastic containers. A total of 633 fecal samples were collected, 277 from sows and 356 from weanlings. Samples were transported on ice to the laboratory and stored for short periods (maximum one week) at 4°C until processing, or at -20°C if processing was delayed more than one week.

4.2.3. Sample analysis

Feces were screened for *Cryptosporidium* oocysts and *Giardia* cysts using a quantitative sucrose gradient immunofluorescent antibody assay previously described (O'Handley et al., 1999). The assay was modified slightly only for adult animal samples in this study. Briefly, for adults, 20 g fecal samples were added to 35ml of phosphate-buffered saline pH 7.4 (PBS) and thoroughly mixed. The suspension was then strained through four layers of surgical gauze (Nu-gauze, four ply, Johnson & Johnson, Montreal, QC). The fecal sample homogenate was gently layered over 15 ml of 1M sucrose (Sigma-Aldrich Canada Ltd., Oakville, ON) solution (sp. gr. 1.13) in a clean polypropylene conical tube. Upon centrifugation at 800 x g for 5 min, the interface and the upper layer of liquid were transferred by pipette to a clean 50 ml centrifuge

tube and re-centrifuged. The resulting supernatant was discarded and the pellet re-suspended in PBS to approximately 1.5 ml.

4.2.4. (Oo)cyst examination

The pellet re-suspension was applied in 20 µl volumes to two wells on a fluorescence microscopy slide (Waterborne Inc., New Orleans, LA), dried for 10 min at 37°C on a slide warmer, methanol fixed, and stained with a *Cryptosporidium* or *Giardia*-specific fluorescein isothiocyanate (FITC)-labeled monoclonal antibody solution (0.04 mL) (Crypt-a-glo™ or Giardi-a-glo™, Waterborne Inc, New Orleans, LA). After incubation, the slide was briefly rinsed with PBS, air-dried, and mounted with glycerol (AquaPolymount, Polysciences, Warrington, PA) and a coverslip. *Cryptosporidium* oocysts and *Giardia* cysts were examined and enumerated at 200 x and 100 x magnifications, respectively, using an epifluorescence microscope (Leica DM 2500, Leica Microsystems, Wetzlar, Germany). The number of oocysts and cysts per gram of feces was determined as previously described (O'Handley et al., 1999). The sensitivity of this method was determined previously to be 66 oocysts or cysts per gram of feces(O'Handley et al., 1999).

4.2.5. DNA extraction

DNA extraction was performed on at least five randomly selected IFA-positive fecal samples from both sows and post-weaned piglets from each farm. DNA was extracted using the Qiagen DNeasy Tissue Kit (Qiagen Inc., Mississauga, ON) according to the manufacturer's instructions. Briefly, a total of 50 µl of processed feces was suspended in 180 µl of ATL buffer and thoroughly mixed by vortexing. Twenty microlitres of Proteinase K (20mg/ml) was added to

the suspension, thoroughly mixed, and 200µl of AL buffer added after overnight incubation at 55°C. DNA was eluted in 100 µl of AE buffer.

4.2.6. Polymerase chain reaction and DNA sequence analysis

Modifications of previously described nested-PCR protocols were used for the amplification of the β-giardin (Caccio et al., 2002) and 16S ribosomal DNA (rDNA) (Appelbee et al., 2003) from *Giardia*, and the heat shock protein 70 (HSP70) (Guselle et al., 2003) and 18S rDNA (Fayer et al., 2006) from *Cryptosporidium*.

For *Giardia*, the nested amplification by PCR using the β-giardin gene fragment, was the same as the initial amplification except that the annealing temperature was increased to 66°C from 58°C for 30 s, and for the 16S rDNA, the annealing temperature of the nested amplification was increased to 58°C from 55°C. Similarly, the annealing temperature for the nested-PCR amplification for *Cryptosporidium* using the HSP70 gene fragment was increased to 58°C from 55°C. For the 18S rDNA gene fragment, the nested-PCR protocol was the same as the initial amplification except that the $MgCl_2$ concentration for the PCR mixture was decreased to 1.5 mM from 3.0 mM.

All amplification products were visualized on 1.5% agarose gels stained with ethidium bromide. Positive PCR amplicons were sequenced in both directions with the same PCR primers used to produce the amplicons at the Genome Quebec Innovation Centre at McGill University, Montreal, QC. Sequences were compared with similar published sequences by BLAST – analysis (Altschul et al., 1990), and aligned using BioEdit (Hall, 1999).

4.2.7. Data collection and statistical analysis

The herd prevalence and within-herd prevalence were obtained from the IFA results. The within-herd prevalence was calculated as the number of positive animals over the total number of animals tested for the farm of interest. The herd prevalence was calculated as the number of farms with at least one positive animal, over the total number of farms visited. All analyses were performed using the statistical software package STATA 11.0 (Stata Corporation, College Station, TX).

4.3. Results

Cryptosporidium oocysts were microscopically detected in the feces of 163 pigs (26%; 95% CI: 22-29%) from 18 (86%; 95% CI: 69-102%) farms (Table 4.1). Infected animals included 138 weanlings (39%; 95% CI: 34-44%) and 25 sows (9.0%; 95% CI: 6-13%).

Table 4.1. Age-related prevalence of *Cryptosporidium* spp. and *Giardia* spp. on 21 Prince Edward Island, Canada, swine herds based on specific direct fluorescent antibody (DFA) assays.

Age	Number of Animals	*Cryptosporidium*		*Giardia*	
		# Positive	Prevalence (%)	# Positive	Prevalence (%)
1 - 3 months	356	138	39	5	1
> 8 months	277	25	9	1	0.4
Total	633	163	26	6	1

Genotyping data were available for 83 isolates with the HSP70 gene, and 60 with the 18S rDNA gene (Table 4.2), and the following species and genotypes were identified: *C. suis*, pig genotype II, *C. parvum*, and the mouse genotype. The distribution of *Cryptosporidium* species and

genotypes based on the combined sequencing data from the HSP70 and 18S rDNA genes is presented in Table 4.3. Overall, pig genotype II had the highest prevalence (61%), followed by *C. suis* (36%), *C. parvum* (2%), and the mouse genotype (0.9%). The number of isolates successfully genotyped from older animals (sows) was low (one isolate each of *C. suis* and pig genotype II). The zoonotic species, *C. parvum*, was only detected in weanlings (two isolates) (Table 4.3)

Table 4.2. Distribution of *Cryptosporidium* species in different age groups on 21 Prince Edward Island, Canada, swine herds based on individual sequencing data from HSP70 and 18S rDNA genes.

Age group	HSP70 only*					18S rDNA only*				
	n	*C. parvum*	*C. suis*	PG II	MG	n	*C. parvum*	*C. suis*	PG II	MG
1 - 3 mo	81	2 (3)	36 (44)	43 (53)	-	59	-	16 (27)	42 (71)	1 (2)
> 8 mo	2	-	1 (50)	1 (50)	-	1	-	-	1(100)	-
Total	83	2 (2)	37 (45)	44 (53)	-	60	-	16 (27)	43 (72)	1 (2)

n = number of samples genotyped, number in parentheses are (%)

- no isolates detected

PG = Pig genotype II

MG = Mouse genotype

*the number of *Cryptosporidium* species simultaneously detected with both the HSP70 and 18S rDNA genes: *C. suis* = 12 (≤ 3 mo of age), Pig genotype II = 18 (≤ 3 mo of age = 17, > 3 mo of age = 1)

Giardia cysts were detected on 3 farms (14%). Of 633 animals tested, *Giardia* cysts were detected in only six (1%) (Table 3.1), including five weanling piglets and one older animal. None of the *Giardia*-positive samples were amenable to PCR.

Table 4.3. Distribution of *Cryptosporidium* species and genotypes in different age groups of pigs based on the combined sequencing data from the HSP70 and 18S rDNA genes.

Age	n	*Cryptosporidium* species/genotype (%)			
		C. parvum	*C. suis*	PGII	MG
Overall*	113	2 (2)	42 (36)	69 (61)	1 (0.9)
1 - 3 mo	111	2 (2)	40 (36)	68(61)	1 (0.9)
> 8 mo	2	-	1 (50)	1 (50)	-

*Positive by at least one gene locus
n - Total number of samples genotyped by at least one gene locus
- No isolates detected
PGII - Pig genotype II
MG - Mouse genotype

4.4. Discussion

Cryptosporidium and *Giardia* infections are a common occurrence in pigs, having been found in all ages groups worldwide (Zintl et al., 2007; Armson et al., 2009; Kvac et al., 2009). *Cryptosporidium* has been reported in several studies, with prevalences ranging between 1.4 to 89% for individual pigs (Quilez et al., 1996; Wieler et al., 2001; Maddox-Hyttel et al., 2006; Vitovec et al., 2006; Johnson et al., 2008), and between 31 and 100% for swine herds (Maddox-Hyttel et al., 2006; Hamnes et al., 2007; Suarez-Luengas et al., 2007) . The overall prevalence of 26% found in our study is considerably higher than other studies reported in North America, where prevalence of *Cryptosporidium* in pigs ranged from 2.8to 11% (Sanford, 1987; Xiao et al., 1994; Guselle and Olson, 1999). The herd-level prevalence of *Cryptosporidium* (86%) is also relatively higher than the 34 to75% range previously reported in other North American studies

(Guselle and Olson, 1999). These differences may be due primarily to differences in farm management systems. This was exemplified by a Ohio, USA, study where *Cryptosporidium* infection only occurred in weanling piglets on the farm with good hygiene management, while on the more poorly managed farm, *Cryptosporidium* infection was found in both nursing piglets and weanlings (Xiao et al., 1994).

In pigs, *Cryptosporidium* is more prevalent in weanlings than in other age groups (Maddox-Hyttel et al., 2006). The high prevalence in weanlings is attributed to a reduction in immunity as they lose the maternally conferred immunity while their own immunity still needs to develop (Maddox-Hyttel et al., 2006). Some studies suggest an age-specific distribution of *Cryptosporidium* species and genotypes in pigs, where pig genotype II is more prevalent in weanlings, while *C. suis* is predominantly detected in nursery pigs (Langkjaer et al., 2007). In a recent study, newly designed porcine *Cryptosporidium* species/genotypes-specific primers were used to obviate the problem of the limitation of current protocols in detecting mixed *Cryptosporidium* infections in pigs (Martina et al., 2011). Mixed infections result in the predominance of one *Cryptosporidium* spp. over another, which may result in the lack of detection or the inaccurate detection of age-specific distribution of *Cryptosporidium* species/genotypes. In the study by Martina et al. (2011) *C,suis* was found in all tested categories of pigs (1-12 week of age and sows) while pig genotype II was detected only in animals older than 6 weeks of age (Martina et al., 2011). We were unable to observe any age dependence in our samples as all but a few isolates were detected in animals 1-3 months of age. *C. parvum* was only detected in two weanling animals in this study. Similar results in other studies have led to the conclusion that *C. parvum* is not widespread in pigs (Langkjaer et al., 2007). *Cryptosporidium* mouse genotype was detected in a single weanling piglet in our study. The

detection of *Cryptosporidium* mouse genotype in domestic pigs was reported in China (Chen and Huang, 2007). However, we are not aware of any similar finding in North America. This indicates that cryptosporidiosis might be transmitted between rodents and pigs (Chen and Huang, 2007). Although sporadic cases of *C. suis* in humans have been reported (Xiao et al., 2002; Cama et al., 2003), its contribution to the epidemiology of cryptosporidiosis in humans, is limited. The predominance of *C. suis* and pig genotype II suggests that isolates of *Cryptosporidium* from domestic pigs have little to no bearing on human infections on PEI.

Although less studied than *Cryptosporidium*, *Giardia* infections in pigs also appear to be widespread, ranging in overall prevalence from 0.1 to 31% (Hamnes et al., 2007; Armson et al., 2009), and 0 to 100% at the herd level (Hsu et al., 2007; Armson et al., 2009). Like *Cryptosporidium*, *Giardia* is more prevalent in weanlings than in other age groups (Xiao et al., 1994; Maddox-Hyttel et al., 2006; Armson et al., 2009). In the present study, the overall prevalence of *Giardia* species (1%) is lower than the 8.5 to 14.5% reported in other North-American studies (Xiao et al., 1994; Olson et al., 1997; Guselle and Olson, 1999, , 2001). However, a very similar prevalence of 0.8% was reported in pigs in Western Australia (Ryan et al., 2003). The majority of the *Giardia* isolates in this study, 5 out of 6, were obtained from weanling pigs and may be due to a reduction in immunity in weanlings, as suggested for *Cryptosporidium*. The positive isolates were obtained from three farms (14%). Comparatively, much higher herd level prevalences ranging between 67% to 80% have been reported in studies in other locations within North America (Olson et al., 1997; Guselle and Olson, 1999, , 2001). The differences in *Giardia* prevalences among different studies may also be attributed primarily to differences in farm management systems as reported for *Cryptosporidium* infection in pigs in the study by Xiao et al. (1994). Unfortunately, we cannot comment on the genotypes, as none of

the *Giardia* isolates could be amplified by PCR therefore no products were available for sequencing. Similar difficulties in genotyping *Giardia* isolates from swine were reported in a study where *Giardia* isolates were stained with 4'6-diamidino-2-phenylindole (DAPI) to confirm the presence of nuclei (DNA), but the results showed insufficient nucleated *Giardia* cysts for PCR (Hamnes et al., 2007) .

Our findings suggest that *Giardia* is less prevalent in pigs on PEI than elsewhere, and, while pigs on PEI are frequently infected with *Cryptosporidium*, the species are predominantly host-specific, and most infections are found in younger animals. Pigs on PEI have, therefore, a negligible role in the epidemiology of cryptosporidiosis and giardiasis in humans.

4.5. References

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5.0. MOLECULAR EPIDEMIOLOGY OF *CRYPTOSPORIDIUM* AND *GIARDIA* IN HUMANS ON PRINCE EDWARD ISLAND, CANADA: EVIDENCE OF ZOONOTIC TRANSMISSION FROM CATTLE

Abstract

Cryptosporidium and *Giardia* are the most common protozoal parasites in humans responsible for significant morbity and mortality worldwide. In Prince Edward Island (PEI), Canada, 658 human fecal specimens that had been submitted to the Queen Elizabeth Hospital diagnostic laboratory were obtained and screened for *Cryptosporidium* and *Giardia*. Overall, 143 (22%) samples were *Cryptosporidium*-positive, while three (0.5%) were positive for *Giardia*. Successful genotyping of 25 *Cryptosporidium* isolates by sequence analysis of the HSP70 gene, revealed that 28 and 72% were *C. hominis* and *C. parvum,* respectively. *Cryptosporidium* isolates from humans and previously genotyped *C. parvum* from beef cattle were sub-genotyped by sequence analysis of the GP60 gene. Sub-genotyping identified three subtypes belonging to the family IIa. All three subtypes IIaA16G2RI (55%), IIaA16G3RI (22%) and IIaA15G2RI (22%) were found in the animal isolates, while two of the subtypes found in the animals, IIaA16G2RI (80%) and IIaA15G2RI (20%), were also identified in the human isolates. *Cryptosporidium* infection in humans peaked in April-June. Molecular epidemiological analysis of the human data showed a *C. parvum* peak in the spring and a relatively smaller peak for *C. hominis* in July-September. The majority (57%) of human *Cryptosporidium* isolates were found in children between 5 and 10 years. All three *Giardia* isolates were identified as *G. duodenalis* assemblage A. Results of this study suggest that *Cryptosporidium* is prevalent in humans on PEI

who had their feces submitted for diagnostic evaluation to the provincial diagnostic laboratory. Further, the presence of the same zoonotic *C. parvum* subtypes in cattle and human isolates implies that transmission is likely zoonotic and cattle may be a source of sporadic human infections on PEI. Although *Giardia* in humans on PEI might have a zoonotic route of transmission, the presence of *Giardia* in humans on PEI is uncommon.

5.1. Introduction

Cryptosporidium and *Giardia* are among the most prevalent enteric parasites of humans and farm animals around the world. Human infections with *Cryptosporidium* and *Giardia* may result in gastrointestinal problems including severe diarrhoea, with neonates and immunosuppressed individuals being particularly at risk (Eckmann, 2003; Wielinger et al., 2008). Both parasites have a broad host range and can be transmitted by the fecal-oral route, or the contamination of food or water supplies (Xiao et al., 2004; Smith et al., 2006). While cattle are major hosts for *C. parvum*, with frequent infections in pre-weaned calves (Xiao and Feng, 2008; Uehlinger et al.,2011, in press), this parasite has a relatively lower prevalence in other farm animals, and is found only occasionally in companion animals (Giangaspero et al., 2006). *C. parvum* and *C. hominis* are the major species reported in humans (Hunter and Thompson, 2005), although, occasionally other species, including *C. felis, C. meleagridis*, *C. canis*, *C. andersoni* and *C. suis*, have been implicated in human illness (Leoni et al., 2006). Twenty-one species of *Cryptosporidium* are currently recognized, but *C. hominis* and *C. parvum* appear to be most widely distributed (Nuchjangreed et al., 2008). While *C. hominis* appears largely responsible for human-to-human transmission, *C. parvum* is typically responsible for animal-to-human transmissions (Nuchjangreed et al., 2008). *C. parvum* is highly prevalent in cattle,

particularly calves, leading to suggestions that cattle could be important in the epidemiology of cryptosporidiosis in humans. In spite of this, little is known about the role of cattle in the zoonotic transmission of the parasite to humans.

In order to understand transmission dynamics, sub-genotyping within a species is essential. Sub-genotyping analysis is more informative than genotyping as it can elucidate genetic variation on a finer scale (Peng et al., 2001). In particular, the *C. parvum* 60 kDa glycoprotein (GP60) gene-based PCR DNA sequencing has revealed at least 10 subtype allele families, IIa-IIj, among *C. parvum* isolates from humans and animals (Sulaiman et al., 2001; Alves et al., 2003; Peng et al., 2003a; Peng et al., 2003b; Sulaiman et al., 2005; Abe et al., 2006; Akiyoshi and Tzipori, 2006; Alves et al., 2006; Trotz-Williams et al., 2006). Among these subtype families, only IIa and IId have been detected in cattle (Alves et al., 2003; Peng et al., 2003b; Abe et al., 2006; Trotz-Williams et al., 2006). This suggests that not all *C. parvum* isolates are equally infectious to humans (Mallon et al., 2003; Alves et al., 2006; Hunter et al., 2007). In addition, the exclusive anthroponotic transmission of *C. parvum* subtypes has been demonstrated and seems to be responsible for most human *C. parvum* infections in the developing world (Xiao and Feng, 2008). Therefore, to determine the proportion of *C. parvum* infections in humans attributable to zoonotic transmission, sub-genotyping of this species is crucial, as the source of *C. parvum* in humans can be of bovine or of human origin (Xiao and Feng, 2008).

In the case of giardiasis, only *Giardia duodenalis* is recovered from humans and most other mammals (Adam, 2001). *G. duodenalis* is composed of at least 7 genetically distinct but morphologically similar assemblages (A to G). To date, only assemblages A and B have been convincingly identified in humans, while assemblages C to G appear to have host-specific

preferences (Yang et al., 2010). Although assemblage B has been reported to be an anthroponotic assemblage, it has also been reported in wild animals and companion animals (Thompson et al., 2000; Berrilli et al., 2004) but rarely in livestock (Bertrand and Schwartzbrod, 2007). However, results of some studies support the role of livestock as a potential reservoir of assemblage A cysts (Bertrand and Schwartzbrod, 2007). Two sub-genotypes have been identified both in assemblage A (A1 and A2) and in assemblage B (B3 and B4). Genotype A1 is generally found in animals, whereas genotype A2 is mainly found in humans. Assemblage A2 has, however, been found in animals (Yang et al., 2010). *Giardia* cysts have been found in run-off from agricultural areas (Slifko et al., 2000; Fayer et al., 2004). A previous study in Canada showed significant associations of giardiasis rates with manure application on agricultural land, and livestock density, suggesting that giardiasis may be a zoonosis (Odoi et al., 2003).

Prince Edward Island (PEI) has the highest cattle-to-human ratio in Canada (Statistics Canada, 2001, 2009, 2010). However, there is no detailed molecular epidemiological information on the zoonotic potential of *Cryptosporidium* and *Giardia* from cattle on PEI. In the current study, *Cryptosporidium* species and subtypes isolated from human fecal samples submitted to the clinical diagnostic laboratory of the Queen Elizabeth Hospital, PEI, were characterized and compared with isolates from bovine fecal samples from an earlier study to evaluate the potential for zoonotic transmission. The study also aimed at determining the genotypes of *G. duodenalis* in humans in the region, to elucidate the epidemiology of this parasite.

5.2. Materials and Methods

5.2.1. Fecal sample collection

Human fecal samples, in 50 ml screw-capped plastic containers, more than half of which contained preservatives submitted, for diagnostic testing, to the Queen Elizabeth Hospital microbiology laboratory in Charlottetown, PEI, were examined in this study. A total of 658 samples were collected between January and December, 2008. The samples were submitted to the diagnostic laboratory of the hospital for various clinical reasons and were not specifically submitted for cases of diarrhea or for the diagnosis of *Cryptosporidium* or *Giardia*. Samples were stored at 4°C and processed within one week of sampling. The samples were anonymized, with the only information provided by the laboratory being the age of the patient (mean 59.3 years; median 63 years; range 1-90 years).

Thirteen *Cryptosporidium*-positive isolates from beef cattle fecal samples, genotyped as *C. parvum* at both the HSP70 and 18S rRNA gene loci from a previous study, were used in this study. Fecal samples were obtained from beef cattle on PEI between March and August, 2006. Homogenates of isolates were stored at -20°C.

5.2.2. Purification and microscopic examination

Cryptosporidium oocysts and *Giardia* cysts were isolated from the fecal samples according to a previously described procedure (O'Handley et al., 1999), with slight modifications. Briefly, 5g of feces was added to 10ml of phosphate-buffered saline, pH 7.4 (PBS), and passed through four layers of surgical gauze (Nu-gauze, 4 ply, Johnson & Johnson, Montreal, QC). The strained fecal sample homogenate was gently layered over 5ml of 1M sucrose (Sigma-Aldrich Canada Ltd., Oakville, ON) solution (sp. gr. 1.13) in a clean

polypropylene conical tube. Following centrifugation at 800 x g for 5 min, the upper filtrate layer and sucrose surface were transferred into a clean 15ml tube and re-centrifuged. The resulting supernatant was discarded and the pellet re-suspended in PBS to approximately 1.5ml. Specimens were determined to be positive or negative for *Cryptosporidium* and *Giardia* by microscopy, using fluorescein isothiocyanate (FITC)-labeled monoclonal antibody staining (Crypt-a-glo and Giardi-a-glo, Waterborne Inc, New Orleans, LA).

5.2.3. Genotyping

DNA extraction was performed on fecal samples from all animals (n=13), and on direct fluorescent antibody (DFA) microscopy-positive human fecal samples (n=143), using the Qiagen DNeasy Tissue Kit (Qiagen Inc., Mississauga, ON) according to the manufacturer's instructions. For *Cryptosporidium*, genotyping of human samples was carried out by amplification of the HSP70 gene fragment using a modified nested-PCR as previously described (Guselle et al., 2003). Briefly, a 448 bp amplicon was amplified using the forward primer 5'–GGTGGTGGTACTTTTGATGTATC-3' and the reverse primer 5'–GCCTGAACCTTTGGAATACG-3' in the primary reaction. The secondary reaction used the forward primer 5'–GCTCATGATACTCACTTGGGTGG-3' and the reverse primer 5'–CTCTTGTCCATACCAGCATCC-3', generating a 325 bp amplicon. Initial amplification was performed in 50 µl volumes with 5 µl of template DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 120-160 µM of each dNTP, 200 nM of each primer, 1-2 U of Hot Start Taq polymerase (Qiagen Inc., Mississauga, ON) and 2.5 µl of non-acylated bovine serum albumin (BSA). Cycling conditions for the PCR consisted of an initial denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 4

min. The sequential nested-PCR cycle was the same except that the annealing temperature was increased to 58°C for 30 s, and 1 µl of DNA template was used from the first reaction.

Genotyping of *Giardia* was accomplished by the amplification of the β-giardin gene using a modified nested-PCR as previously described (Caccio et al., 2002). In the primary reaction, a 753 bp amplicon was amplified using the forward primer

G7 (5'–AAGCCCGACGACCTCACCCGCAGTGC-3') and the reverse primer

G759 (5'–GAGGCCGCCTGGATCTTCGAGACGAC-3'). In the sequential nested-PCR reaction, a 384 bp amplicon was amplified using forward primer

G376 (5'– CATAACGACGCCATCGCGGCTCTCAGGAA-3') and the reverse primer G759. The PCR mix consisted of 1× buffer containing 1.5 nM $MgCl_2$, 200 µM of each dNTP, 10 pmol of each primer, 5 U of Hot Start Taq DNA polymerase, and 5 µl of purified DNA in a final volume of 50µl. PCR was performed as follows: after an initial denaturation step of 5 min at 95°C, a set of 35-40 cycles was run, each consisting of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C, followed by a final extension of 10 min at 72°C. The sequential nested-PCR was the same except that the annealing temperature was increased to 66°C for 30 s. Secondary products of the expected sizes were sequenced in both directions with the forward and reverse primers used in the secondary PCR. Sequencing was performed at the Genome Quebec Innovation Centre at McGill University, Montreal, QC, Canada. Sequences were compared with similar published sequences by BLAST – analysis (Altschul et al., 1990), and aligned using BioEdit (Hall, 1999).

5.2.4. Sub-genotyping of *C. parvum*

Sub-genotyping was performed for all 13 cattle and 25 human isolates genotyped as *C. parvum*. Sub-genotyping was performed by sequence analysis of the GP60 gene according to a

previously described protocol (Alves et al., 2003). Briefly, in a nested-PCR, a 900 bp amplicon was amplified using the forward primer AL 3531 (5'-ATATGTCTCCGCTGTATTC-3') and reverse primer AL 3535 (5'-GGAAGGAAGGATGTATCT-3') in the primary reaction. In the sequential reaction an 800-850 bp amplicon was amplified using forward primer AL 3532 (5'-TCCGCTGTATTCTCAGCC-3') and the reverse primer AL3534 (5'-GCAGAGGAACCAGCATC-3'). Amplifications were performed in 50 µl volumes with 1 µl of template DNA, 1× PCR buffer, 3 mM $MgCl_2$, 200 µM of each dNTP, 200 nM of each primer, 2.5 U of Hot Start Taq polymerase. Cycling conditions for each PCR consisted of an initial denaturation at 95°C for 5 min followed by 35 cycles at 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. Secondary products of the expected size were sequenced in both directions with the forward and reverse primers used in the secondary PCR. DNA sequencing, and genotyping, were performed as previously described.

5.2.5. Statistical analyses

The prevalence of *Cryptosporidium* and *Giardia* in humans was defined as the number of samples positive for oocysts or cysts over the total number of samples examined. The effects of season and age group on shedding of *Cryptosporidium* spp. was analyzed using ordinary logistic regression. Logistic regression was also used to determine if there was a significant difference between the two age groups (5-10 and 45-50 years) with the highest peaks for *Cryptosporidium* oocysts shedding. Probability values of $P < 0.05$ were considered significant. There was not sufficient data to compute the association of similar risk factors for the shedding of *G. duodenalis*. All analyses were performed using the statistical software package STATA 11.0 (Stata Corporation, College Station, TX).

5.3. Results

5.3.1. *Cryptosporidium* and *Giardia* in humans

Of the 658 human fecal samples examined, 143 (22%; 95% CI: 19 - 25%) were positive for *Cryptosporidium* spp., and three (0.5%; 95% CI: .0.09 - 1.3%) for *G. duodenalis* by DFA microscopy. The prevalence of shedding *Cryptosporidium* oocysts decreased with increasing age (P = 0.02) (Fig. 5.1).

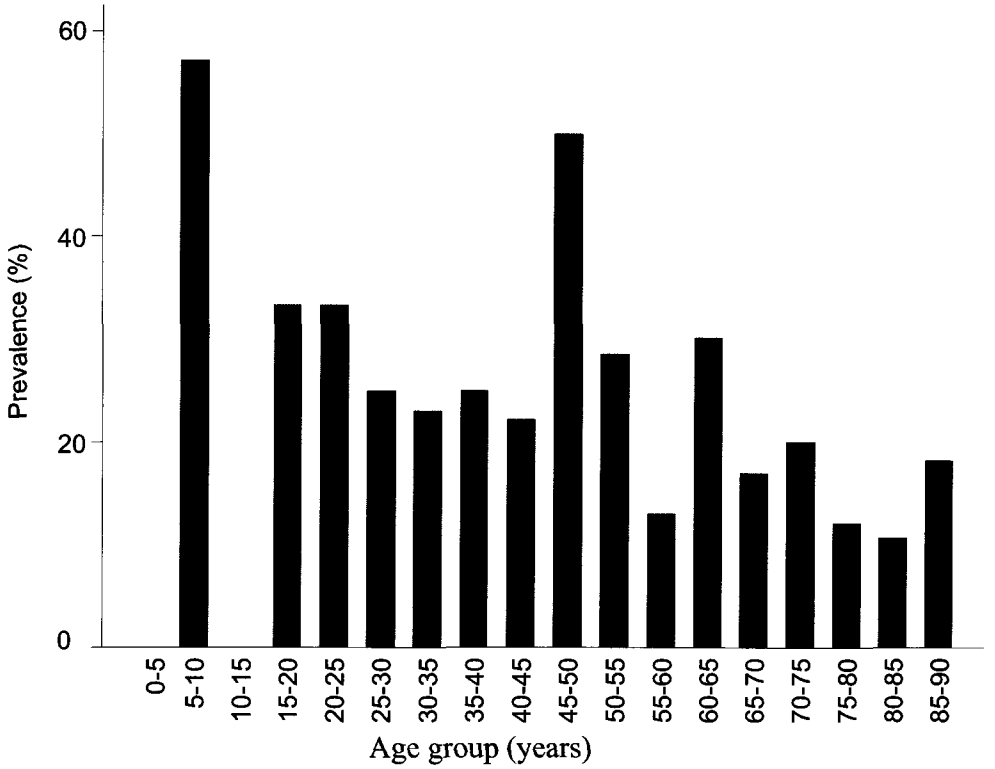

Figure 5.1. Age-dependent distribution of *Cryptosporidium* spp. infections in humans on Prince Edward Island, Canada, as determined by oocyst detection in fecal samples using direct fluorescence antibody staining.

The age distribution of infection showed higher peaks for two age groups, one composed of children between 5 and 10 years old and a second group of adults between 45 and 50 years old

(Fig. 5.1). The difference between these two peaks was not significant (P = 0.74). The majority (57%) of *Cryptosporidium* positives were observed in fecal samples from children. *Cryptosporidium* shedding peaked in the period April-June (P = 0.001) (Fig. 5.2). Otherwise, *Cryptosporidium* shedding was evenly spread over the year

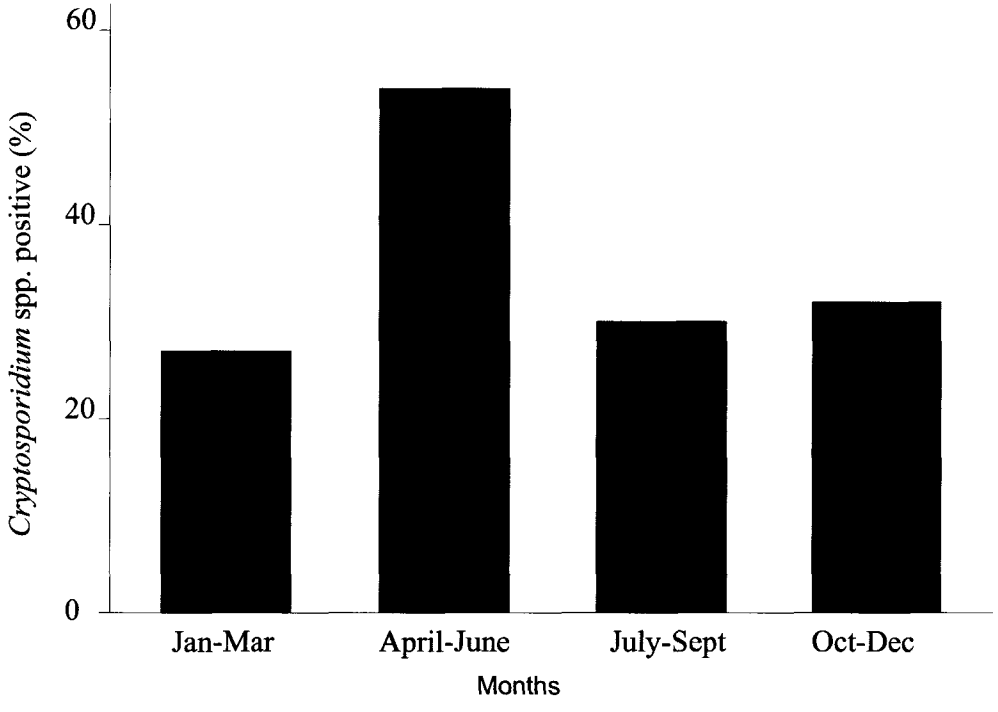

Figure 5.2. Seasonality of *Cryptosporidium* infections in humans on Prince Edward Island, Canada.

5.3.2. *Cryptosporidium* and *Giardia* species/genotypes in humans

Degradation of *Cryptosporidium* DNA from preserved human fecal samples led to genotyping of only a fraction of the samples (25 out of 143). Among the 25 isolates from which genotyping data was available, the majority (64%) were identified as *C. parvum*, followed by *C. hominis* (36%) (Table 5.1). The seasonal distribution of the total numbers of samples positive for *C. hominis* and *C. parvum* is presented in Table 5.1.

Table 5.1. Seasonal distribution of *C. hominis* and *C. parvum* in humans on Prince Edward Island, Canada.

Cryptosporidium species*	n	Season			
		Jan - March	April - June	July - Sept	Oct - Dec
C. parvum	16	2 (13)	11 (69)	3 (19)	0 (0)
C. hominis	9	1 (11)	1 (11)	5 (55)	2 (33)
Total	25	3 (12)	12 (48)	8 (32)	2 (8)

**Cryptosporidium* isolates which could be genotyped by sequence analysis of the HSP70 gene. Numbers in brackets represent percentages per species of *Cryptosporidium* isolated in the season

A distinct spring peak of *C. parvum* shedding coincided with the peak in the total annual incidence of *Cryptosporidium* sheddings (Fig. 5.2). *C. hominis*, on the other hand, was most frequently detected between the months of July to September (Table 5.1). We were unable to determine the association between age and occurrence of either *Cryptosporidium* species given the limited number of samples. All three *Giardia* isolates from humans belonged to *G. duodenalis* assemblage A. Two of the isolates were detected between the months of January to March, while the other was detected between the months of October to December. No further analyses were done on the *Giardia* isolates.

5.3.3 Sub-genotyping of *C. parvum*

Sub-genotyping was only performed for isolates genotyped as C. *parvum*. Sub-genotyping by sequence analysis of the GP60 gene was possible for nine of 13 *C. parvum* isolates from beef cattle, and five of 18 human isolates.

Table 5.2. GP60 sub-genotypes identified within *Cryptosporidium parvum* from beef cattle and humans on Prince Edward Island, Canada.

Isolate identifier	GP60 sub-genotypes
Cattle samples	
CB1	IIaA16G2RI
CB3	IIaA16G2RI
CB4	IIaA16G2RI
CB5	IIaA16G2RI
CB8	IIaA16G2RI
CB2	IIaA16G3RI
CB6	IIaA16G3RI
CB7	IIaA15G2RI
CB9	IIaA15G2RI
Human Samples	
H101	IIaA16G2RI
H103	IIaA16G2RI
H104	IIaA16G2RI
H105	IIaA16G2RI
H102	IIaA15G2RI

Sub-genotyping identified three different subtypes belonging to the subtype family IIa (Table 5.1). All three sub-genotypes IIaA16G2RI (55%), IIaA16G3RI (22%) and IIaA15G2RI

(22%) were present in the cattle isolates. Two of the sub-genotypes found in the cattle, IIaA16G2RI (4 of 5) and IIaA15G2RI (1 of 5), were also found in humans (Table 5.2).

5.4. Discussion

Studies on *Cryptosporidium* infection in humans have focused mainly on determining the prevalence of *Cryptosporidium* species/genotypes in stool samples from patients with microscopically diagnosed cryptosporidiosis from diagnostic laboratories in hospitals (Feltus et al., 2006; Llorente et al., 2007), or from specific populations such as children (O'Brien et al., 2008) or HIV patients (Cama et al., 2003; Nuchjangreed et al., 2008). From the handful of studies where samples have been obtained from individuals from the general population, the prevalence ranged from 1.5 to 10.8% (Chai et al., 1996; Park et al., 2006; Mirzaei, 2007; Cheun et al., 2010). In the present study, the overall *Cryptosporidium* prevalence in humans was at 22%. The Queen Elizabeth hospital on PEI, from where we obtained our human fecal specimens, is the central and largest public hospital in the province and is the primary diagnostic laboratory for the province. Although the samples were not specifically submitted for cases of diarrhea or for the diagnosis of *Cryptosporidium* or *Giardia*, it is likely that at least some of these samples were obtained from symptomatic individuals, and therefore, did not represent a cross section of the population. In this instance, the source of the sample introduces a bias which might have influenced our results. Other factors which may have contributed to the high prevalence in our study, compared to previous studies, could include differences in the methods of screening for *Cryptosporidium* oocysts and the geographic location of sampling. In previous reports that found a lower prevalence, testing for *Cryptosporidium* oocysts was done using acid-fast staining (Chai et al., 1996; Park et al., 2006; Mirzaei, 2007; Cheun et al., 2010), whereas, in our study, a

method was used that concentrated oocysts and identification was based on monoclonal antibody capture (direct fluorescence antibody staining). This method is considered more sensitive and specific, and it therefore reduces the likelihood of false-negative and false-positive results (Garcia et al., 1987). Given that many of the human fecal samples were preserved, *Cryptosporidium* DNA may have degraded and could not be amplified, resulting in the low number of isolates amenable to genotyping (Cheun et al., 2010). Other studies have also reported difficulties in determining *Cryptosporidium* species and genotypes, specifically attributed to difficulties encountered in obtaining human fecal samples free of preservatives (Trotz-Williams et al., 2006).

All human *Cryptosporidium* infections detected in this study were identified as either *C. hominis* or *C. parvum*, similar to the findings in other countries (Hunter et al., 2004; O'Brien et al., 2008). Although *C. hominis* is commonly detected in human outbreaks of cryptosporidiosis in North America (Feltus et al., 2006), *C. parvum* has been found to be the dominant species in some human cryptosporidiosis cases (Feltus et al., 2006). Some studies have found suggestive evidence that *C. parvum* and *C. hominis* cluster in rural and urban areas, respectively (McLauchlin et al., 2000; Feltus et al., 2006; Ng et al., 2008). In the study by Feltus et al. (2006) in Wisconsin, known for its significant dairy industry, the authors could not determine whether the population from which their samples were drawn was rural or urban. However, as none of the samples came from highly populated areas in the state, the samples likely have originated from rural, sparsely populated, areas with intensive dairy husbandry. The majority of *Cryptosporidium* isolates from humans in the present study were identified as *C. parvum*. Not only is PEI the

second most rural Canadian province (Robb and Holman, 2011), but it also has the highest cattle-to-humans ratio in Canada. The results of the present study reflect those which have been reported from other rural locations (Nuchjangreed et al., 2008; Zintl et al., 2009). The high prevalence of infection with *C. parvum* in rural areas is presumed to stem from intensive husbandry of ruminants, especially cattle (Xiao and Feng, 2008).

Overall, the proportion of *Cryptosporidium*-positive samples had a significant seasonal pattern, with a distinct peak in the spring. It should be noted, however, that as the results of this study reflect data obtained from a single year, it is not possible to know if this can be generalized to other years. Long-term studies are needed to draw definitive conclusions regarding seasonality. The distribution of genotypes also showed discernible seasonal differences. The proportion of *C. parvum* infections peaked from April to June, coinciding with the period during which the prevalence of *Cryptosporidium*-positive samples was highest, while *C. hominis*-positive samples peaked between July and September. Other studies have also reported a peak incidence of *Cryptosporidium* spp. in humans in the spring (Zintl et al., 2009), as well as a bimodal distribution with *C. parvum* in the spring and a second smaller peak for *C. hominis* in the late summer and fall (McLauchlin et al., 2000). The peak incidence in spring may indirectly reflect run-off from spring rains and farming events such as lambing and calving, and agricultural practices such as manure spreading, which tend to increase environmental contamination with *C. parvum* oocysts (McLauchlin et al., 2000; Lowery et al., 2001). In contrast, *C. hominis* infections are more common in patients with a history of foreign travel, or a result of increased recreational water activities during the late summer and fall (McLauchlin et al., 2000; Pedraza-Diaz et al., 2001; Hunter et al., 2004). *C. hominis* outbreaks have also been

linked to contact with another person with diarrhea, diapering of young children, and day-care nurseries (Chalmers et al., 2009).

Cryptosporidium has been found in persons from all age groups (Lowery et al., 2001). However, data suggest that children up to 15 years of age are more susceptible to infection (Mann et al., 1986; Lowery et al., 2001; Mirzaei, 2007; Wielinger et al., 2008; Cheun et al., 2010; Ng et al., 2010). In the present study, age was found to be significantly associated with the prevalence of *Cryptosporidium* shedding, with the highest prevalence being among children five to 10 years of age (57%). However, there was also a peak in the 45 to 50 year age group (50%). The latter peak may be due to *C. hominis* acquired through overseas travel, which is more common among people of this age group, and the time of year when such travel occurs. It could, however, also be a result of other risk factors for *C. hominis* mentioned previously.

Among the 10 GP60 alleles (subtype families) identified among *C. parvum* from humans and cattle (IIa to IIj), only IIa and IId appear to be zoonotic (Quilez et al., 2008). Worldwide, the most common *C. parvum* alleles identified in humans are IIa and IIc (Zintl et al., 2009). Allele IIc has only been detected in humans (Alves et al., 2003; Xiao et al., 2003; Xiao and Ryan, 2004). All beef cattle isolates in this study belonged to the allele IIa, specifically IIaA16G2R1, IIaA15G2R1 and IIaA16G3R1. As expected, the anthroponotic *C. parvum* allele IIc was not found in any of the bovine isolates, nor was it found in any of the human isolates in the present study. Although we were able to characterize only a few isolates from humans, due to the difficulty in amplifying DNA, two of the *C. parvum* sub-genotypes found in the bovine isolates, IIaA16G2R1 and IIaA15G2R1, were also found in the human isolates. The *C. parvum* subtype IIaA15G2R1 is the most commonly identified zoonotic infection in calves in the US and Canada and in humans from around the world, including the US, UK, Portugal, Slovenia, Australia,

Japan, and Kuwait (Xiao et al., 2007). In the present study, one human isolate belonged to sub-genotype IIaA15G2R1, and appears to be the first report of this *C. parvum* subtype in humans in Canada. The age of the individual, from which this sub-genotype was found (62 years old) belongs to the demographic group which makes up the majority of farm operators on PEI (Statistics Canada, 2009). The occupation of this person was not available to the investigators. Geographic segregation of *C. parvum* subtypes exists, and specific subtypes have been shown to be endemic to different localities (Xiao and Feng, 2008). For example, a majority of the IIa subtypes found in calves and humans in Michigan and in neighboring Ontario had 16 copies of the TCA repeat sequences in the GP60 gene, representing IIaA16G2R1 and IIaA16G3R1 (Xiao et al., 2007). This is similar to the present study where 55 and 22% of the *C. parvum* isolates sub-genotyped for cattle belonged to IIaA16G2R1 and IIaA16G3R1, respectively, while subtype IIaA16G2R1 also accounted for 80% of isolates in humans. The zoonotic *C. parvum* belonging to the subtype family IId has been found only in a few specimens from humans, cattle, sheep and water samples in Kuwait, Portugal, Spain, Serbia and Hungary (Quilez et al., 2008). The detection of this allele has not been reported in animals or humans in North America, suggesting that this group is not a major zoonotic pathogen in North America (Xiao et al., 2007). Our data suggest that the peak in human shedding is caused by zoonotic, and not anthroponotic, transmission of *Cryptosporidium.* Human-to-human transmission of the zoonotic allele is known to occur, however the finding that the same *C. parvum* strain found in humans was also found in cattle suggests that cattle may be a source for sporadic *Cryptosporidium* infections in humans on PEI, Canada.

Like *Cryptosporidium,* studies on the prevalence of *Giardia* in humans have focused on the determination of the proportions of assemblages A and B in *Giardia*-positive clinical samples

(Yang et al., 2010), or samples obtained from specific population groups at risk, such as children (Siwila et al., 2011) or people with HIV (Ana et al., 2009). In the handful of studies where samples have come from the general population, *Giardia* prevalence ranged from 2 to 24% (Capelli et al., 2003; Anthony et al., 2007; Mahdy et al., 2009). The prevalence of 0.05% in our study is comparatively lower and suggests that *Giardia* shedding on PEI is uncommon. In Canada, several human *G. duodenalis* outbreaks have been reported in the last two decades (Wallis et al., 2001) with the most predominant genotype being assemblage A (Caccio and Ryan, 2008). The exclusive detection of assemblage A in this study is consistent with the predominant occurrence of this assemblage in Canada. The results of this study suggest that there is potential for zoonotic transmission of *Giardia* to occur, but it is uncommon.

The results described here suggest that *C. parvum* is the most common species of *Cryptosporidium* in humans on PEI. Further the occurrence of the same subgenotypes of *C.parvum* in bovine as in humans, coupled with the spring peak of *C. parvum* which coincides with run-off from spring rains, farm events such as calving, and agricultural activies such as the spreading of manure on fields: activities likely to increase the environmental contamination of *C. parvum*, gives credence to livesock as the major source of human infections. The results of this study provide useful information needed to increase the understanding of the transmission dynamics accounting for human exposure and infections with zoonotic *C. parvum* on PEI. This information will be of strategic importance in pollution intervention strategies from farm animal sources, which should include reducing the burden of *Cryptosporidium* on-farm, as well as implementing measures aimed at decreasing the contamination of watersheds with oocysts.

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6.0. GENERAL DISCUSSION

The main aim of this thesis was to determine if domestic livestock are a source of *Cryptosporidium* and *Giardia* infectious to humans and if livestock are capable of contaminating ground and surface water with these parasites. More specifically, the molecular epidemiology of *Cryptosporidium* and *Giardia* was studied in dairy cattle (Chapter 2), beef cattle (Chapter 3), pig herds (Chapter 4), in the human population (Chapter 5) and in surface and ground water associated with the livestock operations on Prince Edward Island. In light of the fact that a zoonotic species found in humans may not necessarily originate from an animal source, molecular tools capable of discerning the genotype of an organism on a finer scale were used to explore the transmission dynamics of zoonotic *Cryptosporidium* genotypes (sub-genotypes) found in humans on PEI (Chapter 5). The results of these studies are discussed in relation to the literature. Lastly, suggestions for further research are presented.

6.1. Prevalence and zoonotic potential of *Cryptosporidium* and *Giardia* in dairy cattle on Prince Edward Island

The results of this thesis and other research demonstrate that *Cryptosporidium* and *Giardia* are a common occurrence in domestic livestock and these parasites are highly prevalent in the sampled dairy herds on PEI (Chapters 2) which was a convenience sample. The high herd-level prevalence of 55% identified on PEI is in keeping with a number of studies which have reported up to 100% prevalence (Olson et al., 1997b; Starkey et al., 2006). The overall animal prevalence of *Cryptosporidium* in dairy cattle has previously been reported to range from 2-59% from other locations within North America (Garber et al., 1994; Olson et al., 1997a; Sischo et al.,

2000; Wade et al., 2000b; Starkey et al., 2006; Coklin et al., 2007; Trotz-Williams et al., 2007; Coklin et al., 2009). The animal prevalence in sample cattle found in our research was 14%, with prevalence of *Cryptosporidium* generally higher in younger animals although, adult cattle were also found to have been infected with *Cryptosporidium.*

Since we decided that only one positive animal in a herd needed to be positive to call the herd positive, this could explain the high herd prevalence relative to the lower animal prevalences in our research. Coupled with this is the fact that we sampled equal numbers of young cattle (<6 mo of age) and adults while most of the previous studies focused their sampling on the calves. It is also possible that herds participating in this study had different management practices compared with herds in previous studies. Recent studies have identified a complex age-related distribution of *Cryptosporidium* species and genotypes in dairy cattle (Santin et al., 2004; Fayer et al., 2006; Fayer et al., 2007). Moreover, there seems to be geographical differences in the age-related prevalence of *Cryptosporidium* species and genotypes. For example, the majority of *Cryptosporidium* infections in pre-weaned calves and only a small percentage of infections in post weaned calves and heifers in the US, Belgium Ireland, Germany and the UK relate to *C. parvum* (Santin et al., 2004; Thompson et al., 2007; Xiao et al., 2007; Broglia et al., 2008; Brook et al., 2008; Geurden et al., 2008; Santin et al., 2008; Brook et al., 2009). Post-weaned calves were mostly infected with *C. bovis, C. andersoni*, and *C. ryanae* (Santin et al., 2004; Fayer et al., 2008; Santin et al., 2008). On the other hand, in China, India, Georgia and western North Dakota, *C. bovis* was the predominant *Cryptosporidium* species found in pre-weaned calves (Feng et al., 2007; Feltus et al., 2008). In other studies no significant correlation between age and the species/genotype of *Cryptosporidium* were found (Geurden et al., 2006; Langkjaer et al., 2007; Winkworth et al., 2008). In our research, the most prevalent species of *Cryptosporidium* in

dairy cattle was *C. bovis*, occurring mostly in samples genotyped in post-weaned calves. *C. ryanae* was detected in a small percentage of animals in all age groups and represents the first report of this *Cryptosporidium* species in cattle on PEI. *C. parvum* was only detected in pre-weaned calves. These findings are similar to those reported in the US (Geurden et al., 2006; Langkjaer et al., 2007; Winkworth et al., 2008) and clearly demonstrates, that on PEI, pre-weaned dairy cattle were the more important source of zoonotic *Cryptosporidium* versus mature dairy cattle.

Giardia was detected on all the sampled dairy farms. This is in agreement with previous studies which reported a herd prevalence of between 53 and 100% (Xiao et al., 1993; Xiao and Herd, 1994; Olson et al., 1997a; Wade et al., 2000; Hamnes et al., 2006; Maddox-Hyttel et al., 2006). The within-herd prevalence of *Giardia* was higher (32%) than that of *Cryptosporidium* and similar to the 14 to 100% reported in other studies (Olson et al., 1997b). Like *Cryptosporidium*, *Giardia* infections in animals are more predominant in younger dairy animals. The majority of cattle sampled in this thesis were infected with the non-zoonotic *G. duodenalis* assemblage E. This is conistent with the results of other studies from around the world which have reported the predominance of assemblage E in cattle (O'Handley et al., 2000; Appelbee et al., 2003; Becher et al., 2004; Berrilli et al., 2004; Trout et al., 2004; Trout et al., 2005; Trout et al., 2006; Langkjaer et al., 2007). While assemblage A was found in post-weaned and adult cattle, assemblage B was only detected in pre-weaned calves in the dairy study. Assemblage B was thought to be an anthroponotic species but it has been detected in other animals. Assemblage B in the pre-weaned calves may possibly be of human origin through handling of these young animals by farm workers or potentially contaminated water souces. Although prevalences of the zoonotic assemblages were low in the dairy cattle on PEI, their presence in calves is still a matter

of public health concern as calves tend to excrete more cysts per gram of feces than older cows (Huetink et al., 2001; Ralston et al., 2003; Maddox-Hyttel et al., 2006).

6.2. Prevalence and zoonotic potential of *Cryptosporidium* and *Giardia* in beef cattle on Prince Edward Island

The overall prevalence of *Cryptosporidium* in beef cattle (Chapter 3) is consistent with those reported in other studies (Atwill et al., 2003; McAllister et al., 2005; Gow and Waldner, 2006; Fayer et al., 2010). Confined practices in dairy cattle operations is the likely cause of higher *Giardia* and *Cryptosporidium* infection rates in dairy herds, as compared with the production of beef on open ranges (McAllister et al., 2005). However, on PEI, beef cattle are more intensively managed compared to the management system in Western Canada. *C. andersoni* was the predominant species found in beef cattle on PEI. The age-related distribution of *Cryptosporidium* species/genotypes has also been reported in beef cattle (Feltus et al., 2008) with *C. parvum* mostly confined to younger animals and *C. andersoni* predominantly detected in adult cattle. *C. andersoni* was also found in about 40% of young animals in beef cattle on PEI. This demonstrates that it is possible for pre-weaned calves on PEI to be infected with species typically associated with older cattle and may indicate unique interaction between mature animals and calves resulting in the infection of susceptible calves at an earlier age than previously reported in both beef and dairy cattle (Fayer et al., 2006; Geurden et al., 2007; Feltus et al., 2008; Fayer et al., 2010). Unlike in dairy cattle operations where calves are usually separated from their dams in the initial hours of birth, calves in beef herds on PEI stay with their dams. Also, beef cows on PEI are more confined than in beef cattle operations in other parts of North America, especially during the calving period (PEI Department of Agriculture.,

unpublished). *C. parvum* was detected in 26% of genotyped samples in calves while only one isolate from cows was positive for *C. parvum*. Calves in these beef herds are a potential reservoir for human infection with the zoonotic species of *Cryptosporidium*.

The prevalence of *Giardia* in beef cattle on PEI was found to be higher than those reported in other studies and may be a result of regional differences in management of beef cow-calf operations on PEI. A majority (89%) of the *Giardia* isolates in beef herds on PEI belonged to the non-zoonotic assemblage E. Other point prevalence studies have reported similar results (Appelbee et al., 2003; Uehlinger et al.,2011, in press). The zoonotic assemblages A and B were detected in 4 and 7% of isolates genotyped, respectively. In previous studies (Appelbee et al., 2003; Uehlinger et al.,2011, in press), assemblage A has only been detected at levels between 0-2.4% while there is no previous report of the presence of assemblage B in beef cattle in North America. This has prompted the conclusion that cattle may not be important in the epidemiology of giardiasis (Caccio et al., 2005). Like in dairy cattle, the presence of assemblage B in the beef cattle on PEI may originate from human sources. Beef cattle on PEI seem to be a reservoir of zoonotic *Giardia* for humans.

6.3. Prevalence and zoonotic potential of *Cryptosporidium* and *Giardia* in pigs on Prince Edward Island

Pig herds were found to have a high prevalence of *Cryptosporidium* (26%) while *Giardia* was rare (1%) in pig herds on PEI. Pigs were mostly infected with host specific species and genotypes of *Cryptosporidium*: *C. suis* and *Cryptosporidium* pig genotype II. The presence of *Cryptosporidium* mouse genotype in one pig isolate is an indication that there could be transfer

of *Cryptosporidium* from rodents to pigs. These findings suggest that domestic pigs likely do not pose a significant health risk to humans on PEI from these parasites.

6.4. The role of livestock in contaminating the watershed on PEI with zoonotic species/genotypes of *Cryptosporidium* and *Giardia*

Most surface water samples associated with cattle operations on PEI (85% dairy and 93% beef farms) were positive for *Cryptosporidium*. In the majority of *Cryptosporidium*-positive surface water, the concentrations of oocysts obtained in the downstream locations were higher than those from the upstream locations, indicating a positive effect from the farms. *Giardia* was only occasionally detected in surface water and was never found in ground water. Farm contamination of water sources with zoonotic *Gairdia* is unlikely. However, the only *Cryptosporidium* isolates obtained from surface water in the vicinity of cattle farms which were amenable to genotyping belonged to the zoonotic species *C. parvum*. The contamination of the watershed and therefore source water with zoonotic species of *Cryptosporidium* is a public health concern on PEI which requires further attention.

Intervention practices to prevent contamination of the watershed with *Cryptosporidium* and *Giardia* from livestock source, need to be implemented on the farm and within the watershed. More (oo)cysts present in animals, mean greater the risk that the manure from infected animals will contaminate the environment. Practical steps are required on the farm to reduce the carriage of (oo)cysts in livestock, which are discharged into the environment, and may eventually, contaminate water sources.

Watershed management strategies may include restricting livestock access to water by fencing stream banks, or providing bridges for water crossings or by providing alternative water

sources (drinking troughs) in order to prevent the direct deposition of feces into waterways (Sheffield et al., 1997; USEPA, 2001).

6.5. Molecular epidemiology of *Cryptosporidium* and *Giardia* in humans on Prince Edward Island, Canada: evidence of zoonotic transmission from cattle

In the human fecal samples examined, *Cryptosporidium* (22%) was more common than *Giardia* (0.5%). The source of the samples, the Queen Elizabeth Hospital (QEH) diagnostic laboratory on PEI, as well as the diagnostic method used (antibody capture staining), may have had an impact on the high prevalence. In other reported studies, the prevalences were lower, and the diagnostic methods used were mostly based on acid-fast staining (Chai et al., 1996; Park et al., 2006; Mirzaei, 2007; Cheun et al., 2010). The QEH is the largest hospital in the province where the majority of clinical cases and human fecal samples are received. The fecal samples were submitted to the diagnostic laboratory for various clinical reasons and not purposely for *Cryptosporidium* or *Giardia* testing. However, some of these samples were obtained from symptomatic individuals, and therefore, did not represent a cross section of the population, making this a biased sampling source. On PEI, children between the ages of 5-10 years were most likely (57%) to be infected with *Cryptosporidium*, while infections in people between the ages of 45-50 years were also high (50%). Worldwide, *Cryptosporidium* is mostly found in children up to 15 years of age (Mann et al., 1986; Lowery et al., 2001; Mirzaei, 2007; Wielinger et al., 2008; Cheun et al., 2010; Ng et al., 2010).

Most of the infections on PEI occurred in the spring months, similar to those reported in other studies (Zintl et al., 2009). In this thesis, all *Cryptosporidium* infections detected in humans were identified as either *C. hominis* or *C. parvum*, similar to the findings in other countries

(Hunter et al., 2004; O'Brien et al., 2008). While *C. parvum* is zoonotic and can be found in both animals and humans, *C. hominis* is exclusively anthroponotic. More *C. parvum* (64%) than *C. hominis* (36%) were found in the human specimens amenable to genotyping on PEI. The intent in this thesis was to determine the zoonotic potential of *Cryptosporidium* originating from domestic livestock sources. As a result, only *C. parvum* isolates from a representative sample of beef cattle and humans were subjected to sub-genotytpe analysis for comparison. GP60 analysis revealed three *C.parvum* sub-genotypes belonging to the zoonotic allele IIa, specifically IIaA16G2R1, IIaA15G2R1 and IIaA16G3R1 were identified in cattle. Two of these *C. parvum* sub-genotypes found in the cattle isolates: IIaA16G2R1 and IIaA15G2R1 were also found in the human isolates. The anthroponotic *C. parvum* sub-genotype allele IIc was not found in any of the human or cattle isolates.

In our research, the proportion of *C. parvum* infections peaked from April to June, coinciding with the period during which the prevalences of *Cryptosporidium*-positive samples were highest for both cattle and humans. On PEI, this peak incidence in spring indirectly reflects run-off from spring rains and snow melt, and farming events such as lambing, calving and agricultural practices such as manure spreading which tend to increase environmental contamination with *C. parvum* oocysts.

Given the peak occurrences of *Cryptosporidium* in both cattle in May and humans between April-June, coupled with the presence of the same zoonotic *C. parvum* sub-genotypes in cattle and human isolates, suggest that transmission could be largely zoonotic and cattle may be a source of sporadic human infections on PEI.

Giardia was only found in three human specimens, and all three isolates were determined to be assemblage A. No further analyses were done on the *Giardia* isolates, however, given the

fact that assemblage A is predominantly found in livestock these human isolates could have been transmitted from livestock, and therefore be zoonotic. The prevalence of *Giardia* assemblage A was low in cattle, suggesting their zoonotic impact is minimal.

Cryptosporidium parvum found in humans might have been acquired from cattle either by direct contact with infected animals or the consumption of contaminated food or water. Livestock can have a high prevalence of infection with *Cryptosporidium* and *Giardia*, while infected animals can shed a high number of oocysts and cysts (Fayer et al., 2000). During peak shedding, infected animals, particularly young animals, can excrete ~10^7 *Cryptosporidium* oocysts and *Giardia* cysts/g of feces for a number of days (Bradford and Schijven, 2002). Therefore, produce in the field may become contaminated when livestock defecate near crops, when crops are irrigated or washed with water accessed by livestock or contaminated through agricultural run-off, or when manure is intentionally applied to arable land (Schijven et al., 2004). In addition to produce, products of animal origin, such as milk and meats may also be subject to contamination with cysts and oocysts from livestock. The consumption of raw or undercooked seafood, such as bivalve molluscan shellfish, may also result in infection with *Cryptosporidium* or *Giardia.* Molluscan shellfish, tend to thrive in estuaries and marine coastal environments, where nutrient levels are high, and are likely contaminated by water bearing (oo)cysts from livestock effluent. Microorganisms occurring in these environments may be filtered by the gills during feeding, and become concentrated in the digestive tract/glands of the mollusk (Robertson, 2007). The ability of Eastern oysters to remove oocysts of *C. parvum* from contaminated seawater, retain them within hemocytes, on gills and within the body for at least one month after exposure, as well as retain infectivity in mice after residing in the oyster for one week, has been demonstrated (Fayer et al., 1997a). Numerous reports have documented

Cryptosporidium and *Giardia* (oo)cysts from mussels (Graczyk et al., 1999; Freir-Santos et al., 2000; Gomez-Bausta et al., 2000; Gomez-Couso et al., 2003a; Gomez-Couso et al., 2003b; Pritchard et al., 2007), oysters (Graczyk et al., 1998; Gomez-Couso et al., 2003a; Fayer et al., 1999), cockles (Graczyk et al., 1999) and clams (Graczyk et al., 1998a; Freir-Santos et al., 2001; Gomez-Couso et al., 2003b).

6.6. Risk factors for infection of cattle with *Cryptosporidium* and/or *Giardia* on PEI

On PEI, the trend for decreasing infection with *Giardia* with increasing age of dairy cattle is a phenomenon that has been reported in other studies. It stems from the fact that as animals age, their immune systems mature and are able to provide adequate defenses against microbial infections. Dairy cattle housed in free-stalls were most likely to be infected with *Cryptosporidium*, as in this province cattle have abundant contact with each other, which increases the chances of transmission from infected animals. The County location of a dairy farm did not influence the infection status of an animal with either *Cryptosporidium* or *Giardia.*

On PEI beef farms, the two risk factors investigated, age of the animal and location of the farm, were not associated with the risk of infection of animals with either *Cryptosporidium* or *Giardia* species. The lack of association of the age of the animal and the infection with either *Cryptosporidium* or *Giardia* is different from what has generally been seen in other studies. This might be due to other risk factors which were not considered in this thesis.

6.7. Conclusions

- Dairy cattle on PEI are predominantly infected with non-zoonotic species/genotypes of *Cryptosporidium* and *Giardia* (Chapter 2).

- Beef cattle on PEI are predominantly infected with non-zoonotic species/genotypes of *Cryptosporidium* and *Giardia* (Chapter 3).
- Some PEI surface water sources associated with farms are contaminated with zoonotic *C. parvum* and *Giardia duodenalis* and may represent a threat to the watershed and subsequent disease transmission to humans (Chapters 2 and 3).
- Pigs on PEI are primarily infected by the host-specific genotypes and species, *Cryptosporidium* sp. pig genotype II and *C. suis*, whereas the zoonotic *C. parvum* is rare. Pigs on PEI, likely do not pose a significant health risk to humans from these parasites (Chapter 4).
- *Cryptosporidium* was commonly found (22%) in human fecal samples submitted to the QEH hospital on PEI (Chapter 5).
- The primarily found *Cryptosporidium* species in humans was the zoonotic *C. parvum* and cattle may be a significant source of human infections on PEI (Chapter 5).
- Although *Giardia* in humans on PEI might have an animal source, the presence of *Giardia* in humans on PEI was uncommon (Chapter 5).

6.8. Future research

The results of this thesis have indicated the need to pursue research in other areas pertinent to the epidemiology of *Cryptosporidium* and *Giardia* on PEI. Most of the isolates in this thesis, from all sources sampled, were not amenable to PCR analysis. Of particular concern were those isolates from water and human sources. Although it was difficult to obtain PCR products from both *Cryptosporidium* and *Giardia* isolates, *Giardia* seemed to be more

problematic than *Cryptosporidium*. Determining the genotypes of *Cryptosporidium* and *Giardia* isolates from water and human samples remains one of the major stumbling blocks in surveillance and source tracking is necessary to obtain required information to implement cryptosporidiosis and giardiasis intervention strategies. This requires that studies be conducted to resolve the issue of PCR inhibitors and improved DNA extraction techniques which plague current methods.

The intent of this thesis was to determine if there were common species and genotypes of *Cryptosporidium* and *Giardia* in domestic livestock and humans, in order to elucidate the transmission dynamics of these parasites. As a result, particular attention was not given to the time of sampling for both livestock and water samples. Most livestock samples were collected in the winter months when the surface waters associated with the farms were frozen. The majority of the surface water which was collected mostly in the spring and summer months contained *Cryptosporidium* while *Giardia* was detected occasionally. Most of the animals on the farms with *Cryptosporidium*-positive and/or *Giardia*-positive surface water samples were not infected with these parasites. It is believed that this discrepancy was a result of the lack of concurrent fecal and water sampling on the farms. In order to better establish the connection between animal agricultural activities and the concentration of *Cryptosporidium* oocysts and *Giardia* cysts in PEI surface water, further studies are required that take into account the temporal effects on sampling.

The presence of *Cryptosporidium* and *Giardia* in water requires further investigation in a larger study spanning several years, to truly elucidate the contribution of contaminated water in potential cryptosporidiosis or giardiasis outbreaks on PEI. This study should include domestic

(well) water on livestock farms given the current finding of one well contaminated with *Cryptosporidium* and *E. coli.* concurrently.

A further study should address the prevalence and molecular characterization of *Cryptosporidium* and *Giardia* in fresh produce and molluscan shellfish, as these foodstuffs can potentially be exposed to contaminated irrigation water and livestock effluent on PEI.

Human fecal samples obtained from the QEH on PEI, for this thesis, indicated that *Cryptosporidium* is not uncommon and the majority of genotypes were zoonotic. The samples analyzed were submitted to the QEH diagnostic laboratory for various reasons and not all were specifically for the diagnosis of acute gastro-enteritis disease. Although the QEH is the biggest hospital serving the province, this is still a biased sample. Therefore, a sense of the true incidence or prevalence of sporadic cryptosporidiosis in humans on PEI and the confirmation of zoonotic transmission needs to be established through a larger, long-term study involving specimens from a random sampling of a non-biased population.

6.9. References

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