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Detection and molecular characterization of Giardia and Cryptosporidium in Canadian dairy cattle

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Detection and molecular characterization of *Giardia* and *Cryptosporidium* in Canadian dairy cattle

Tatjana Coklin

A thesis Submitted to the School of Graduate Studies
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In Partial Fulfillment of the Requirement for the Degree of Master of Science

Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine

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TO

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ABSTRACT

Giardia and *Cryptosporidium* are intestinal protozoan parasites that infect a wide range of host species, including humans. DNA sequencing of *Giardia* and *Cryptosporidium* isolates from human and animal sources has identified numerous species and genotypes, and has demonstrated that a number of *Giardia* and *Cryptosporidium* genotypes are shared between animals and humans. Therefore, livestock may act as a source of contamination of the food and water supply. The goal of this study was to optimize methods for the detection and molecular characterization of *Giardia* and *Cryptosporidium*. Achieving this objective involved the incorporation of immunomagnetic separation, as well as the evaluation of different methods, including microscopy, flow cytometry and polymerase chain reaction. A number of faecal samples from adult cattle and calves were collected from farms in Ontario and Prince Edward Island (PEI). Following DNA extractions from stool samples, a nested-PCR was used for *Giardia* to amplify a fragment of the 18S rRNA gene generating a 292-bp product. Nested-PCR protocol was also used for *Cryptosporidium* to amplify fragments of the heat-shock protein 70 (HSP-70) gene (ca. 325 bp). For *Giardia*, of 143 cattle samples analyzed by PCR, 32 (22.4 %) were positive. When IMS was incorporated into the methodology, 64 out of 143 (44.8 %) samples analyzed were positive for *Giardia*. For *Cryptosporidum*, out of 143 cattle faecal samples analyzed by the PCR method using the HSP-70 gene, 58 were positive (40.6 %), while using IMS, plus PCR, 60 samples were positive (42 %). Results from this study indicated that incorporation of IMS significantly improve the sensitivity of PCR for the detection of both *Giardia* ($p<0.01$) and *Cryptosporidium* ($p=0.02$). Among the other genes that were targeted, including the *β-giardin* gene and glutamate dehydrogenase (GDH) for *Giardia*, and the *Cryptosporidium*

oocyst wall protein (COWP) and 18S rRNA for *Cryptosporidium*, the18S rRNA for *Giardia*, and HSP-70 for *Cryptosporidium* were found to be the "best genes". When different methods, including microscopy, flow cytometry and PCR-IMS were compared, the PCR method showed the highest sensitivity in detecting both parasites. Genotyping done by DNA sequencing showed that there was a high prevalence of zoonotic genotypes (Assemblage A for *Giardia*, and *C. parvum* bovine genotype for *Cryptosporidium*) among the samples from both PEI and Ontario. In addition, a temporal study was done on calf samples from Ontario and showed that over time there was a decrease in *Cryptosporidium* infections, concomitant with an increase in *Giardia* infections.

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LIST OF ABBREVIATIONS

AIDS:	Acquired immunodeficiency syndrome
AVC:	Atlantic Veterinary College
CDC:	Centers for Disease Control and Prevention
CDF:	Cumulative probability function
COWP:	*Cyptosporidium* oocyst wall protein
DAPI:	4', 6-diamidino-2-phenylindole
DHFR-TS:	Dihydrofolate reductase thymidylate synthase
FITC:	Fluorescein isothiocyanate
gdh:	Glutamate dehydrogenase
GP:	Glycoprotein
HCl:	Hydrochoric acid
HIV:	Human immunodeficiency virus
HSP-70:	Heat shock protein 70
ICR:	Institute for Cancer Research
IMS:	Immunomagnetic separation
ITS:	Intergenic transcribed sequence
mAB:	Monoclonal antibody
NaOH:	Sodium hydroxide
ON:	Ontario
PBS:	Phosphate-buffered saline
PCR:	Polymerase chain reaction
PEI:	Prince Edward Island

PFGE:	Pulsed-field gel electrophoresis
qPCR:	Real-time PCR
RFLP:	Restriction fragment length polymorphism
RNR:	Ribonucleotide reductase
R-PE:	R-phycoerythrin-labelled
SCID:	Severe combined immune deficiency
SSCP:	Single-strand conformation polymorphism
tim:	Triose phosphate isomerase
TRAP:	Thrombospondin-related adhesive protein
TYI-S-33:	Trypticase, yeast extract, iron-serum
UK:	United Kingdom
US:	United States
VSP:	Variant-specific proteins

1 GENERAL INTRODUCTION

1.1 *Giardia* Introduction

Despite its early discovery, *Giardia* only recently became universally studied. It infects a wide range of hosts, including humans, throughout the world (Dixon, 2003). *Giardia* is a protozoan parasite which belongs to the phylum Protozoa, Subphylum Sarcomastigophora, Superclass Mastigophora, Class Zoomastigophora, Order Diplomonadida, Family Hexamitidae (Health Canada, 2004). *Giardia duodenalis* is a causative agent of giardiasis, a gastrointestinal disease mostly recognized among travelers (particularly in the developing world), children in day-care centres and homosexual males. It is believed that *Giardia* was initially discovered and described by van Leeuwenhoek in 1681. In 1882, Kunstler gave the generic name *Giardia* to a flagellate from the intestine of tadpoles of amphibians, but controversy about the names and numbers of *Giardia* species lasted for years. Standard classification models for the organism had assumed that different hosts had their own *Giardia* species. This resulted in the description of over 40 different species, and that was later considered an overestimation. In 1952, Filice proposed three species names to be used, based on morphology of the median body (Adam, 2001): *G. duodenalis* (wide range of domestic and wild mammals, including humans), *G. agilis* (amphibians) and *G. muris* (rodents). During the 1970s, the species name *G. lamblia* was used, while in the 1980s it was *G. duodenalis*, followed in the 1990s by *G. intestinalis*.

With the use of electron microscopy, additional species were described, i.e., *G. psittaci* from parakeets, and *G. ardeae* from herons. The description of a sixth species, *G. microti* (voles and muskrats), was possible through molecular characterization.

1.2 Biology of *Giardia*

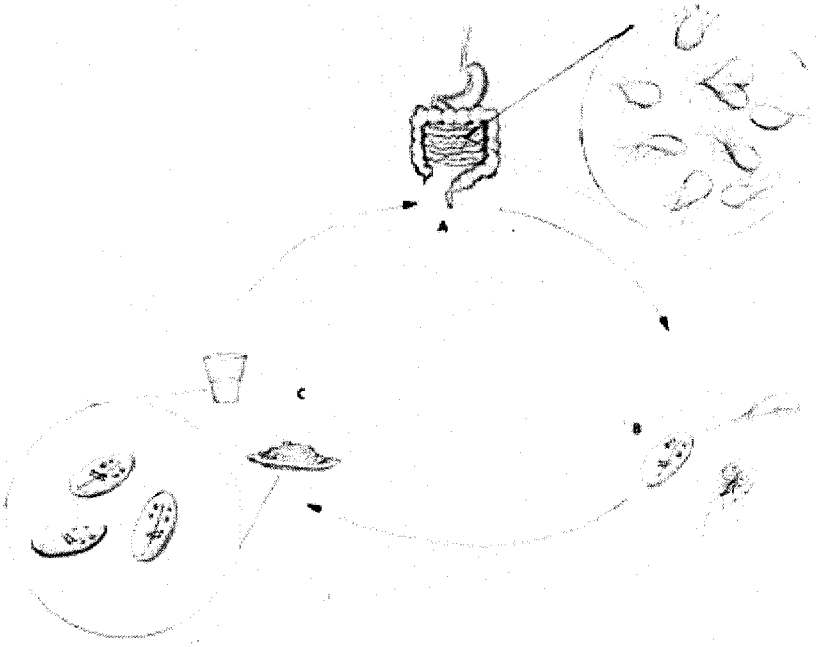
The small, flagellated protozoan parasite *Giardia* lives in the small intestines of animals and humans. It has two developmental stages, the trophozoite and the cyst (Figure 1). Mostly inert and very resistant to environmental factors, the cyst is the infectious form of *Giardia*. The cysts can survive for several months if humidity and temperature (such as water at 4-10° C) are appropriate (Wolfe, 1992).

Transmission usually occurs through cyst-contaminated food and water or via the direct faecal-oral route. Humans are likely the most important reservoir for *Giardia* (Wolfe, 1992), but a number of mammals (domestic animals and pets), have a significant role in zoonotic transmissions. In addition, wild animals, such as beavers and muskrats, represent an important source of cysts in raw surface waters (Dixon et al., 1997). The fact that some *Giardia* isolates are not host specific (infect more than one animal species), while others are host adapted (infect only one animal species), indicates that some *Giardia* strains have a higher potential for human infection than other strains.

Ingestion of as few as 10 cysts can lead to an infection (Dixon, 2003). After ingestion, due to the effects of acidic gastric pH, as well as chymotrypsin and trypsin (the pancreatic enzymes), the cyst undergoes a process of excystation in the duodenum. As a result, two vegetative form trophozoites, are produced from each cyst. They inhabit the duodenum and upper jejunum where they replicate and reproduce asexually by binary fission. Consequently, due to cholesterol starvation or exposure to bile salts in the ileum, the process of encystation of some of the trophozoites occurs (Gillin et al., 1988; Lujan et al., 1996). The cysts are ovoid in shape, ranging from 8-14 μm in length and 7-10 μm in width. They have two or four nuclei, axonemes and median bodies, as well as the remnants of

Figure 1. Life cycle of *Giardia duodenalis*

A- Cysts are excysted in the duodenum, and trophozoites released. B- Cysts and trophozoites are excreted with faeces to the environment. C- Water and food are contaminated with infectious cysts. (The figure is taken from Ortega and Adam, 1997, Clin. Infect. Dis., with kind permission from The University of Chicago Press, Appendix I).



organelles visible (Gillin et al., 1996). This is why *Giardia* is classified as a primitive eukaryote, as no mitochondria, peroxisomes, endoplasmic reticulum or nucleoli have been identified (Adam, 1991). The trophozoites or feeding stage, are pear-shaped bodies with two nuclei, two slender median rods, eight flagella in four pairs, a pair of darkly staining median bodies, and a large ventral sucking disc which allows mechanical attachment to the intestinal mucosa (Health Canada, 2004).

1.3 Literature review on taxonomy of *Giardia*

Multilocus enzyme electrophoresis (typing of organisms based on the migration of a set of enzymes on a starch gel in the presence of an electric field) was the first study of the molecular differences of *G. duodenalis*, followed by RFLP analysis. Andrews et al., (1989) used the technique of allozyme electrophoresis on 29 Australasian stocks and 48 clones of *G. duodenalis* from humans. They examined 50 different enzymes, and 26 loci were found to be suitable for use as genetic markers, with data showing the presence of four genetic groups within *G. duodenalis*. Their findings suggested that *G. duodenalis* is a species complex. De Jonckheere et al. (1990) used *Giardia* isolates from primates and rodents to grow the parasites axenically and compare them by different electrophoretic techniques. Using Southern blots hybridized with an rDNA probe, they found two profiles of RFLP in *Giardia* to be similar to previously found strains isolated from humans. A few other studies have used RFLP analysis for molecular classification of *Giardia* (Ey et al., 1993; Ey et al., 1996).

For studying *Giardia* chromosomal patterns, pulsed-field gel electrophoresis (PFGE) was also used (Adam et al., 1988), but with less success due to common chromosome rearrangements. Nash et al. (1988) used surface antigens in an attempt to classify *Giardia*.

They used clones of the WB isolate of *G. duodenalis* in combination with the cytotoxic monoclonal antibodies (mAb) 6E7, which reacts with surface antigens. Most of the cloned *Giardia* were killed, but a few survived and demonstrated the presence of a new set of surface antigens. In addition, when they exposed *Giardia* to another cytotoxic mAb, a third set of antigens appeared, demonstrating that a single trophozoite can generate organisms with varying surface antigens. Classification by surface antigens was also limited because of the antigenic variation of the variant-specific proteins (VSPs).

More quantitative comparison of *Giardia* isolates was followed in subsequent studies by sequence comparisons of the different genes. Characterization of this genetic diversity suggested that *G. duodenalis* consisted of at least two genetically distinct groups, and as many as four. These groups were assigned different pseudonyms by different researchers (Table 1).

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

Current Assemblages	Nash (Nash et al., 1985)	Mayrhofer, Andrews (Andrews et al., 1989; Mayrhofer et al., 1995)	Homan (Homan et al., 1992)	Host range
A- I	Group 1	Assemblage A (Group 1)	Polish	Variety of mammals[a]
A- II	Group 2	Assemblage A (Group 2)	Polish	Humans
B- III	Group 3	Assemblage B (Group 3)	Belgium	Humans, beavers, dogs
B- IV	Group 3	Assemblage B (Group 4)	Belgium	Humans, beavers, dogs
C				Dogs
D				Dogs
E				Livestock
F				Cats
G				Rats

[a] Humans, livestock, dog, cats, beavers.

Assemblage A and B are most commonly used to describe the two genetically distinct populations of *G. duodenalis* that infect humans. Furthermore, analysis of Assemblage A and B isolates revealed the genetic distance between the two, suggesting that *G. duodenalis* is a species complex. As axenic cultures had limitations, PCR targeting conserved regions of ribosomal and housekeeping genes were done using DNA from the cysts of infected hosts.

Lu et al. (1998) compared the triose phosphate isomerase (*tim*) sequences of the three genotypes (Groups 1, 2, and 3) described by Nash (1992) and showed that Groups 1 and 2 are similar while Group 3 is different, indicating that Group 1/2 and Group 3 correspond to the two major genotypes found by other researchers. They also used three isolates from different parts of China and showed that partial *tim* sequences fit within the genotypes from other parts of the world. Two isolates fit into Group 3, while the third was a mixture of isolates from Groups 1 and 3.

Monis et al. (1999) did a comparison study of *G. duodenalis* of all known genetic groups with other *Giardia* species, namely, *G. ardeae*, *G. muris*, and *G. microti*. They used the segments from four housekeeping genes, i.e., glutamate dehydrogenase (*gdh*), *tim*, elongation factor 1α, and 18S ribosomal RNA, that were examined by analysis of nucletoide sequences determined from DNA amplified by PCR. Isolates were also compared by allozymic analysis of electrophoretic data for 21 enzymes representing 23 gene loci. The results supported the monophyly of *G. duodenalis* and also showed that this species includes genotypes that represent at least seven deeply rooted lineages, designated as Assemblages A to G. *G. ardeae* and/or *G. muris* were used as outgroups (outgroup is a closely related taxon or group of taxa which, from prior biological knowledge, can be presumed to form a sister group or to be ancestral to the ingroup of interest). *G. microti* was included in the analysis of

18S rRNA and demonstrated monophyly of *Giardia* but not of *G. duodenalis*, placing *G. microti* within *G. duodenalis*.

All these studies confirmed the division of *G. duodenalis* human isolates into two major genotypes. The *tim* nucleotide sequences of group 1 and 2 were different by 1% in the protein coding region and 2% in the flanking regions. Divergence between groups 1 and 3 was 19%, while the flanking regions were so dissimilar that no alignment was possible (Adam, 2001). The 18S rRNA sequences only showed a 1% divergence between Groups 1 and 3, demonstrating its highly conserved nature. Groups 1 and 3 showed other biological differences, with group 3 being more pathogenic for humans than group 1 (Adam, 2001). The other difference between the two groups (1 and 3) was in their culturing abilities. A measurement of *in vitro* growth in TYI-S-33 medium for 12 of the 15 isolates of *G. duodenalis* from human and animal stool samples, revealed three phenotypes: 'rapid', 'medium-rate' and 'slow' growers (Karanis and Ey, 1998). These phenotypes were characterized by generation times of 9-11 h (5 isolates), 12-15 h (5 isolates) and 18-20 h (2 isolates), respectively. Group 1 or group 2 (Assemblage A) genotypes correlated with the *in vitro* growth rates, of the 'rapid' and 'medium-rate' cultures, while both Assemblage B isolates were 'slow growers'. PCR was used to amplify segments of VSP or the enzyme *gdh*, followed by RFLP, and this identified genotypes belonging to three genetic groups. Group 1- a zoonotic genotype from Assemblage A, involving humans and animals was found in seven isolates from humans, calf and a chinchilla. Six isolates, all from humans, belonged to group II, Assemblage A characteristic for humans only. Only two isolates, from human and a monkey, were classified as Assemblage B genotypes. Based on their findings, Karanis and Ey (1998) concluded that genetically based metabolic differences may determine how rapidly *G. duodenalis* isolates can grow in axenic culture.

A number of additional Assemblages (genotypes) have been suggested for *Giardia* isolates from mammals other than humans. These additional genotypes are morphologically the same as human *G. duodenalis*, but their sequences of protein-coding regions differ. Molecular characterization allowed identification of a dog isolate which is genetically different from human *G. duodenalis*. *G. duodenalis* isolates from dogs were difficult to grow axenically as compared to human isolates, suggesting that this would be a different Assemblage. In order to further address the zoonotic potential of dog *Giardia*, Monis et al. (1998) established a suckling-mouse model using isolates obtained from 11 infected dogs and, based on sequence analysis, found that the dog isolates belonged to Assemblages C and D. Hopkins et al. (1997) did a genetic characterization of *Giardia* isolates from dogs and humans living in the same locality. They compared 18S rRNA sequences from 13 human and 9 dog isolates, and found four different genetic groups. Groups 1 and 2 contained human isolates, and groups 3 and 4 consisted only of *Giardia* isolates from dogs. One dog sample contained sequences from both groups 2 and 3. These findings suggested that the zoonotic transmission of *Giardia* infections between humans and dogs does not occur frequently. This was the first report of dog-associated 18S rRNA sequences, suggesting a new *G. duodenalis* subgroup.

Livestock Assemblage E, cat Assemblage F and rodent Assemblage G are also recognised, largely as a result of molecular characterization experiments. Studies of *Giardia* from cattle demonstrated that some of the isolates belong to the livestock Assemblage E, and some to Assemblage A (subgenotype 1, infecting various mammals including humans). One of the studies addressed the prevalence of *G. duodenalis* infections in Western Canadian and Western Australian dairy calves (O'Handley et al., 2000). A PCR-based method amplifying a fragment of 18S rRNA gene was used, followed by sequence comparison from *Giardia*

isolates, both to each other and to previously sequenced isolates. Of 10 calves from Western Canada, 8 aligned with the proposed 'Hoofed livestock' (Assemblage E) genotype, whereas for the five isolates from Western Australian calves, four sequences were identical to the 'Hoofed livestock' genotype. Two isolates from the Western Canadian calves and one isolate from the Western Australian calves had the identical genetic sequence to the genotype (Assemblage) A sequence, a common human genotype. This suggested that *Giardia* infections in calves may pose a risk to public health, regardless of geographical location.

Currently, Assemblages C through G have not yet been isolated from humans, suggesting that some genotypes of *G. duodenalis* have a broad range of host specificity, while others appear to be more host specific.

1.4 Symptoms, diagnosis and treatment of giardiasis

The incubation period of giardiasis is 1-2 weeks (Ortega and Adam, 1997), and individuals are often asymptomatic. Acute infections result in diarrhea, weight loss, abdominal discomfort and nausea. The illness is usually self-limiting, lasting for 2-4 weeks, but if untreated symptoms can last for weeks or months. *Giardia* can especially affect growth and development in young children, as it produces persistent diarrhea and intestinal malabsorption (Miotti et al., 1986; Ortega and Adam, 1997).

The diagnosis of giardiasis is based on clinical history and/or history of travel, and laboratory diagnoses involve stool examinations for the presence of cysts. As cysts and trophozoites are present in stool samples but not constantly excreted, at least three examinations are often needed. Stool examinations are done either on fresh samples or on formalin or polyvinyl alcohol preserved samples (Vesy and Peterson, 1999). Cysts are

concentrated by centrifugation, or flotation, and then visualized microscopically using a wet-mount, with or without Lugol's iodine. Currently, many clinical laboratories are using fluorochrome-conjugated monoclonal antibodies specific for the cyst wall (Dixon, 2003).

Several prescription drugs are available for treating *Giardia* infections. The most commonly used are metronidazole and tinidazole, which are nitroimidazole derivatives. They represent the first line of medications in treating giardiasis, as the treatment periods are short and compliance good (Dixon, 2003).

1.5 Introduction to *Cryptosporidium*

Along with several other protozoan genera, *Cryptosporidium* belongs to the Phylum Apicomplexa, Class Coccidea, Order Eucoccidiorida, Family Cryptosporidiidae (Fayer, 1997). In developing countries, the disease occurs most often in young children, while in industrialized countries, cryptosporidiosis occurs in adults by means of foodborne or waterborne transmission. Other modes of transmission include the faecal-oral route, person-to-person (usually due to poor hygiene in hospitals, daycares, etc.), sexual transmission, and nosocomial infection. Zoonotic spread from animals to humans is an important means of transmission, as calves, rodents, puppies, kittens and many other animals can serve as a reservoir of *Cryptosporidium* (Dixon, 2003). Ingestion of as few as 10 oocysts can cause infection (Okhuysen et al., 1999). With the emergence of acquired immune deficiency syndrome (AIDS), cryptosporidiosis has attracted more attention as an opportunistic infection in human immunodeficiency virus (HIV) infected patients.

The lack of morphologic characteristics that clearly differentiate one *Cryptosporidium* sp. from another (Fall et al., 2003), the inability to culture the organisms in

large numbers, and confusion in the taxonomy of *Cryptosporidium* spp., have all been impediments to understanding the transmission of *Cryptosporidium*.

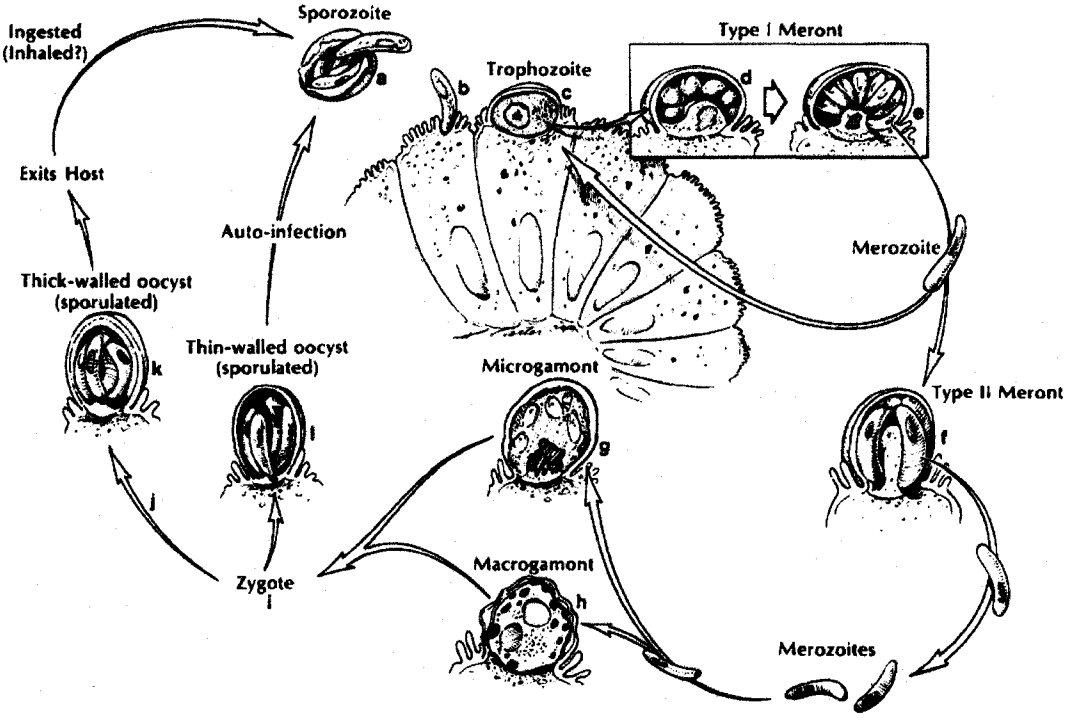
Cryptosporidium was first isolated and described by Tyzzer (Tyzzer, 1907, 1910) from the gastric glands of mice and named as *C. muris*. Later, a second species, *C. parvum,* was isolated from the small intestine of laboratory mice (Tyzzer, 1912), but *Cryptosporidium* was not recognized as an animal pathogen until 1955, during an outbreak in a turkey flock (Slavin, 1955). During this period of over 50 years, *Cryptosporidium* was commonly confused with other apicomplexan genera (subphylum Apicomplexa, are a large group of protozoa, characterized by the presence of an apical complex at some point in their life-cycle, exclusively parasitic, and completely lacking flagella or pseudopods except for certain gamete stages), namely members of the coccidian genus *Sarcocystis*. When the differences between *Cryptosporidium* and *Sarcocystis* were finally recognized, a number of new *Cryptosporidium* species were reported. Animal studies showed that *Cryptosporidium* isolates from different animals can be transmitted from one host species to another, which ended the practice of naming species based on host origin. Finally, in recent years, the use of molecular techniques is helping to clarify the confusion in *Cryptosporidium* taxonomy. *Cryptosporidium* species currently considered valid now include: *C. andersoni* (cattle), *C. baileyi* (chickens and some other birds), *C. felis* (cats), *C. galli* (birds), *C. hominis* (humans), *C. meleagridis* (birds and humans), *C. molnari* (fish), *C. muris* (rodents and some other mammals), *C. parvum* (ruminants and humans), *C. wrairi* (guinea pigs), *C. saurophilum* (major species in lizards and minor in snakes), and *C. serpentis* (major species in snakes and minor in lizards) (Xiao et al., 2004), *C. canis* (dogs) (Fayer et al., 2001), *C. suis* (pigs) (Ryan et al., 2004) and *C. bovis* (cattle) (Fayer et al., 2005).

1.6 Biology of *Cryptosporidium*

Cryptosporidium species are coccidian (intestinal apicomplexans) protozoan parasites that grow and reproduce within epithelial cells of the digestive organs and the respiratory tract, infecting a wide range of vertebrate hosts, including humans. Transmission can occur via the direct faecal-oral route or ingestion of oocyst contaminated food or water. The pathogenicity of *Cryptosporidium* depends on the species of parasites involved and the type, age and immune status of the host. The *Cryptosporidium* life cycle is a multi-stage one consisting of six major stages:1) excystation (sporozoites are released from an oocyst); 2) merogony (asexual reproduction); 3) gametogony (a stage in the sexual cycle of sporozoans in which gametes are formed, often by schizogony which is asexual reproduction by multiple fission); 4) fertilization of the gamete by a microgamete with a formation of a zygote; 5) oocyst wall formation; and 6) sporogony which is sporozoite formation within the oocyst (Figure 2). Unlike "typical" coccidia (*Eimeria*, *Isospora*, *Sarcocystis* and *Toxoplasma*), the *Cryptosporidium* life cycle is completed within a single host, with the oocyst stage particularly important as it contains and releases the infective sporozoites (Fayer, 1997). *Cryptosporidium* oocysts are 4-6 μm in diameter, with one residual body and four sporozoites each measuring around 1 μm in diameter. Once ingested, viable oocysts pass through to the host's intestines and undergo excystation, releasing sporozoites into the intestinal lumen. The sporozoites then penetrate intestinal epithelial cells and undergo further developmental stages (Current and Garcia, 1991).

Figure 2. Life cycle of *Cryptosporidium parvum*

Life cycle of *C. parvum* as it occurs in the mucosal epithelium of an infected mammalian host. Living developmental stages of *C. parvum* correspond to those labeled a through l. After excysting from oocysts in the lumen of the intestine (a), sporozoites (b) penetrate into host cells and develop into trophozoites (= uninucleate meronts) (c) within parasitophorous vacuoles confined to the microvillous region of the mucosal epithelium. Trophozoites (uninucleate meronts) (c) undergo asexual division (merogony) (d and e) to form merozoites. After being released from type I meronts, the invasive merozoites enter adjacent host cells to form additional type I meronts (recycling of type I meronts) or to form type II meronts (f). Type II meronts do not recycle but enter host cells to form the sexual stages, microgamonts (g) and macrogamonts (h). Most (approximately 80%) of the zygotes (i) formed after fertilization of the microgamont by the microgametes (released from microgamont) develop into environmentally resistant, thick-walled oocysts (j) that undergo sporogony to form sporulated oocysts (k) containing four sporozoites. Sporulated oocysts released in faeces are the environmentally resistant life cycle forms that transmit the infection from one host to another. A smaller percentage of zygotes (approximately 20%) do not form a thick, two-layered oocyst wall; they only have a unit membrane surrounding the four sporozoites. These thin-walled oocysts (1) represent autoinfective life cycle forms that can maintain the parasite in the host without repeated oral exposure to the thick-walled oocysts present in the environment. The life cycle of *C. baileyi*, infecting chickens, differs from the one shown in that this parasite has an additional type (type III) of meront derived from type II merozoites.


Ingested
(Inhaled?)
Sporozoite
a
Type I Meront
Trophozoite
b
c
d
e
Exits Host
Auto-infection
Merozoite
Thick-walled oocyst
(sporulated)
k
Thin-walled oocyst
(sporulated)
l
Microgamont
g
Type II Meront
f
j
Zygote
i
Macrogamont
h
Merozoites

1.7 The Taxonomy of *Cryptosporidium* spp.

In mammals, the taxonomy of *Cryptosporidium* has been the subject of controversy since 1980, with only two species, *C. parvum*, the intestinal species and *C. muris*, the gastric species being identified. Ever since Tyzzer (1907) discovered *C. muris*, based only on morphology, *C. muris* or *C. muris*-like oocysts have been reported in the faeces of cattle in different parts of the world. For example, Pena et al. (1997) found *C. muris* oocysts in the faeces of calves (at least once during a 1-year period), but not adults. All calf infections were asymptomatic. Similarly, in Japan, a 1-year study of *Cryptosporidium* in adult cattle in a slaughterhouse was done, where *C. muris* was identified on the basis of morphological, pathological and histological investigations (Kaneta and Nakai, 1998). However, the authors did not use genetic or experimental techniques for species identification. Therefore, on this basis, these and other authors from U.S., Scotland, and Iran (Xiao et al., 2004), named these organisms *C. muris*-like. In support of this, experimental transmission studies showed *C. muris* to be capable of infecting a number of additional hosts.

In 2000, Lindsay et al. (2000) proposed that the large *C. muris*-like oocysts from cattle microvillous border of epithelial cells in the abomasum be considered as a separate species from *C. muris*. They named it *Cryptosporidium andersoni* and found its oocysts to be passed in faeces fully sporulated, ellipsoidal, and measuring 6.0-8.1 by 5.0-6.5 μm, with a length/width ratio of 1.35. *C. andersoni* was not infectious for outbred or inbred immunocompetent or immunodeficient mice, nor for chickens or goats. This coincided with the results from studies from the Czech Republic (Koudela et al., 1998) and Denmark (Enemark et al., 2002). However, two other studies reported experimental infection of mice and rats with large oocysts from rectal stools of adult cattle (Kaneta and Nakai, 1998), and

infection of severe combined immune deficiency (SCID) mice with large oocysts that resembled *C. andersoni* (Satoh et al., 2003). All this conflicting data and the lack of morphological differentiation of *C. andersoni* oocysts from *C. muris*, was resolved by molecular methods. Currently, using genetic-based confirmation, *C. andersoni* infection has only been seen in cattle, bactrian camels (*Camelus bactrianus*, two-humped camel of the cold deserts of central Asia) and a sheep (Xiao et al., 2004).

Cryptosporidium parvum is the most frequently reported species in mammals, and was first isolated from mice. It differs from *C. muris* in size (it is smaller than the latter) and in its location in the gastrointestinal tract (small intestine, as compared to gastric glands). *C. parvum* or *C. parvum*-like parasites have been reported in more than 150 species of mammals, mostly based on microscopy. However, the latest molecular studies have shown that many mammals have host-adapted *Cryptosporidium* genotypes. Morgan et al. (1999a) sequenced a 298-bp region of the *C. parvum* 18S rRNA gene and a 390-bp region of the acetyl coenzyme A synthetase gene from a range of *Cryptosporidium* isolates from wild house mice, a bat and cattle. They found that the *Cryptosporidium* "mouse" genotype is conserved across the world, and that mice could also be infected with the "cattle" genotype. Xiao et al. (2004) suggested that 'the name' *C. parvum* should only be used for *Cryptosporidium* isolates known as the bovine genotype, as they are known to infect mainly ruminants (cattle, sheep, goats and deer) and humans.

Cryptosporidium canis was identified using molecular data based on sequence data for the 18S rRNA and the heat-shock protein 70 (HSP-70) gene (Fayer et al., 2001). *C. canis* oocysts from a dog and an HIV-infected human were examined, based on their morphology, host specificity, and gene sequencing to see if these oocysts differed enough from known species of *Cryptosporidium* to be considered a new species. The oocysts of *C. canis* were

morphologically the same as those of the human and bovine genotypes of *C. parvum*, and also possessed the same surface antigens. Inoculated mice, even when immunosuppressed, were not infected by *C. canis*, and unlike the human genotype of *C. parvum*, *C. canis* was infectious for cattle and humans. Due to those characteristics and based on genetic differences from other *Cryptosporidium* spp., the parasite was named *C. canis.* This confirmed previous genetic and phylogenetic characterization of *Cryptosporidium* isolates obtained from Australia and U.S. dogs (Morgan et al., 2000a), as well as findings from the U.K. by Pedraza-Diaz et al. (2001a). Pieniazek et al. (1999) used DNA sequencing and phylogenetic analysis to identify *Cryptosporidium* genotypes in HIV-infected patients. They used the *Cryptosporidium* genus-specific primers CPBDIAGF and CPBDIAGR and identified a genotype 1 (human) and genotype 2 (bovine) *C. parvum*, a genotype identical to *C. felis,* and one identical to a *Cryptosporidium* sp. isolate from a dog. This was the first identification of human infection caused by the latter two genotypes (Pieniazek et al. 1999). Xiao et al. (2002) using multi-locus genetic characterization suggested that not all host-adapted genotypes should be counted as new species. For example, they found that a *Cryptosporidium* parasite recovered from a coyote and one of the two genotypes in foxes are genetically related to *C. canis*, but should be considered as a variant of *C. canis* (Xiao et al., 1999a; Xiao et al., 2002).

The isolation of *C. felis* was first reported by Iseki in 1979. He inoculated four cats, three ICR (Institute for Cancer Research) outbred mice, and three guinea pigs, with oocysts (5.0 x 4.5 μm) that were shed by cats (Xiao et al., 2004). Although the validity of *C. felis* as a new species has been questioned, molecular characterization of different loci has confirmed the existence of *C. felis* as a new species. Sulaiman et al. (2002) studied the evolutionary relationships of *Cryptosporidium* parasites at the actin locus, and found that isolates from

cats contained sequences that were different from other *Cryptosporidium* genotypes. Sulaiman et al. (2000), also characterized the nucleotide sequences of the HSP-70 genes of different *Cryptosporidium* species from various animals, and revealed the presence of several distinct species in the genus *Cryptosporidium*, with *C. felis* among them. Other loci that were used for molecular characterizations include the *Cryptosporidium* oocyst wall protein (COWP), the intergenic transcribed sequence (ITS-1) and 18S rRNA. During an outbreak of *Cryptosporidium* infection in cattle on a farm in northern Poland, Bornay-Llinares et al. (1999) reported on an infection caused by *C. felis*, in addition to infections with *C. muris* and *C. parvum*. Their identifications to the species level were based on the size of the oocysts (mean size, 4.3 ± 0.4 μm; range 3.5 to 5.0 μm) and on the analysis of the molecular sequence of the variable region of the 18S rRNA. In addition to this study, using a PCR-RFLP technique, Xiao et al. (2001) found *C. felis* along with other *Cryptosporidium* species in 132 stool samples from 80 Peruvian children. Direct sequencing of PCR products of the 18S rRNA and HSP-70 genes confirmed the identity of the *C. parvum* dog genotype, *C. meleagridis*, while the identification of *C. felis* was confirmed by RFLP analysis.

Cryptosporidium hominis was known for some time as either *C. parvum* human genotype, genotype I, or genotype H, and, largely due to molecular characterization, it was delineated as a separate species. *C. hominis* is very similar to *C. parvum*, and is considered non-infective for mice, rats, cats, dogs and cattle. Morgan-Ryan et al. (2002) described the structure and infectivity of the oocysts of a new species of *Cryptosporidium* that they isolated from the faeces of humans. The oocysts they had discovered were structurally the same as those from *C. parvum*, were passed in faeces fully sporulated, were not infectious for ARC Swiss mice, nude mice, Wistar rat pups, puppies, kittens or calves, but were infectious for neonatal gnotobiotic pigs. Pathogenicity studies showed differences in lesion

distribution and infection between *C. parvum* and this new species from humans. *In vitro* culture studies also showed some differences. Over the period from 1996 until 2002, scientists used multiple loci analysis (acetylCoA, actin gene, ß-tubulin, COWP, double-stranded RNA virus, dihydrofolate reductase thymidylate synthase gene (DHFR-TS), GP60/GP40/15 (glycoprotein) gene, HSP-70, 18S rRNA, ITS1, 5.8S, ITS2, microsatellite loci, poly-threonine gene, ribonucleotide reductase (RNR), thrombospondin-related adhesive proteins (TRAPC1 and TRAPC2)) and demonstrated a lack of recombination, providing further support for a new species. McLauchlin et al. (1999) genetically characterized *Cryptosporidium* strains from 218 patients with diarrhea. All samples were examined by light microscopy, followed by DNA extractions and PCR amplification of fragments of the COWP, TRAP-C1 and 18S rRNA genes. The sensitivity of the PCR method for the detection of the 18S rRNA, COWP, and TRAP-C1 gene fragments was 97, 91, and 66 %, respectively. Interestingly, they detected genotype 2 (*C. parvum*) in a greater proportion of the samples with small numbers of oocysts, and genotype 1 (*C. hominis*) in a greater proportion of samples with larger numbers of oocysts. Xiao et al. (2001), reported that in addition to infections with the *C. parvum* human and bovine genotypes, some children in their study were infected with *C. meleagridis*, *C. felis*, and the *C. parvum* dog genotype. They also noticed that oocyst shedding was more frequently associated with the 'novel' *C. parvum* human genotype. At a wide range of loci, genetic characterization of *C. parvum* and *C. hominis* has demonstrated differences between the two species. For example, Alves et al. (2001) studied the TRAP-C1 and COWP, 18S rRNA and a fragment of the DHFR gene, while Caccio et al. (2000) studied a locus containing a microsatellite (tandem repeats of the GAG trinucleotide). They used PCR and sequence analysis from 94 *C. parvum* isolates, which were collected from humans (immunocompetent and immunocompromised

individuals, outbreak and single cases) and from several animal hosts in three continents. Gasser et al. (2001) established a SSCP (single-strand conformation polymorphism) approach, using both the 18S rRNA and HSP-70 genes. Morgan et al. (2000b) studied a total of 22 *Cryptosporidium* isolates from HIV-infected patients from Kenya, Switzerland and the U.S. These isolates were examined at three genetic loci: the 18S rRNA, HSP-70 and acetyl coenzyme A synthetase genes. Four distinct *Cryptosporidium* genotypes were identified: the *C. parvum* "human" genotype, the *C. parvum* "cattle" genotype, *C. felis*, and *C. meleagridis*. This was the first report of *C. meleagridis* in a human host. In their other study, Morgan et al. (1999b) used a number of *Cryptosporidium* isolates to investigate the extent of sequence heterogeneity among human and cattle-derived isolates from different geographical locations, and also between isolates of *Cryptosporidium* from different hosts such as cats, pigs, mice and a koala. They used *Cryptosporidium* ITS1, 5.8S and ITS2 rDNA regions and found that calf-derived isolates from different continents were virtually identical, as were human-derived isolates from the U.K. and Australia. PCR-RFLP of the ITS1 region was undertaken in order to directly amplify and genotype *Cryptosporidium* isolates from different hosts. Another study by Morgan et al. (2001) used avian isolates of *Cryptosporidium* species, again from different geographic locations and addressed sequencing at two loci, the 18S rRNA gene and the HSP-70. Pedraza-Díaz et al. (2001b) developed a sensitive nested-PCR procedure for the COWP gene. Distinct differences between *C. parvum* and *C. hominis* at a wide range of loci were consistently demonstrated. There are also differences between *C. parvum* and *C. hominis* in ribosomal gene expression, with *C. parvum* expressing two types of rRNA genes (type A and type B), as demonstrated by Le Blancq et al. (1997). Today, there are close to 20 *Cryptosporidium* genotypes with uncertain species status in pigs (two

genotypes), sheep, horses, cattle, rabbits, marsupials, muskrats, squirrels, bear and deer (Xiao et al., 2004).

Cryptosporidium meleagridis, *C. baileyi*, and *C. galli*, are the three avian *Cryptosporidium* species that can infect a wide range of birds. *C. meleagridis* and *C. baileyi* can be found in the small and large intestines and bursa. They differ in oocyst size and also in the fact that *C. baileyi* can be found in the respiratory tissues. *C. galli* infects only the proventriculus. *C. meleagridis* was described by Slavin in 1955, during an outbreak in turkeys. Oocysts were indistinguishable from those of *C. parvum*, but no attempts were made at that time to try and transmit the parasite to other hosts (Xiao et al., 2004). Molecular analysis at the 18S rRNA, HSP-70 and actin loci (Sulaiman et al., 2000; Sulaiman et al., 2002), showed that *C. meleagridis* is a unique *Cryptosporidium* species. Other than turkeys, *C. meleagridis* infects other avian hosts. Next to *C. parvum* and *C. hominis* it is the third most common *Cryptosporidium* species isolated from humans (Xiao et al., 2001), as demonstrated by Pedraza-Diaz et al. (2000). Pedraza-Diaz et al. (2000) identified an unusual genotype of *Cryptosporidium* in the faeces of six human patients by PCR-RFLP analysis of the COWP gene. They also characterized those isolates by PCR-RFLP analysis of the TRAP-C1, and by DNA sequencing of the COWP and the TRAP-C1 gene fragments and of two regions of the 18S rRNA gene. Sequence analysis of the COWP, TRAP-C1, and 18S rRNA gene fragments confirmed that this genotype is genetically distinct from *C. parvum*; 18S rRNA gene sequences were found to be identical to those published for *C. meleagridis*.

Both host-parasite co-evolution and host adaptation have occurred in the genus *Cryptosporidium*. All gastric and intestinal *Cryptosporidium* spp. form their own morphological groups, based on their analysis at the 18S rRNA, HSP-70, and actin loci. Parasites of reptiles form the basal branches and mammalian parasites form later branches,

while the placement of *C. bailey* is still unclear as in the 18S rRNA and actin-based trees it groups with the intestinal parasites, while in the HSP-70-based trees it groups with the gastric parasites (Xiao et al., 2004). The taxonomy of *Cryptosporidium*, like the taxonomy of most organisms, will continue to evolve in the future.

1.8 Symptoms, diagnosis and treatment of cryptosporidiosis

The incubation period for cryptosporidiosis ranges from 2 to 10 days. Human infections are characterized by watery diarrhea, cramping, abdominal pain, weight loss, nausea, vomiting, fever and headache. In immunocompetent patients symptoms are self-limiting and usually last for 1-4 weeks. In contrast, in immunocompromised patients, cryptosporidiosis is far more severe. Infectious diarrhea can result in 10 watery stools per day accompanied by malabsorption and a 10% drop in body weight (Dixon, 2003).

As with *Giardia*, the detection of oocysts in multiple stool samples by microscopy is the method of diagnosis. Microscopy is done on either fresh or 10 % formalin, 2.5 % potassium dichromate, or polyvinyl alcohol preserved samples (Laberge et al., 1996). If the numbers of oocysts are low, concentration techniques such as Sheather's sucrose flotation (Sheather, 1923), or sedimentation in a solution of formalin-ethyl acetate or formalin-ether (Ritchie, 1948; Young et al., 1979) are often used. Wet mounts are commonly used with or without Lugol's iodine (*Giardia*), and also permanent stains such as acid-fast (*Cryptosporidium*). Recently, the use of fluorescein-labelled monoclonal antibodies specific to epitopes on the oocyst wall has provided better sensitivity and specificity than conventional staining methods (Dixon, 2003).

A large number of drugs (greater than 90) have been evaluated, without much success, in treating cryptosporidiosis. Some of these include paromomycin (used for treating amoebiasis, and has some activity against *Cryptosporidium*), spiramycin, and azithromycin, which has produced good results in children on chemotherapy and in AIDS patients (Dixon, 2003). As infections are most severe in immunocompromised patients, the need for effective treatment is still present. For immunocompetent individuals, the situation is different, as the disease is self-limiting, so proper fluid balance and nutrition accompanied by anti-diarrheal agents is often sufficient for restoring health.

1.9 Foodborne/waterborne outbreaks of *Giardia* and *Cryptosporidium*

Together with *Cyclospora* and *Toxoplasma*, over the past few decades, *Giardia* and *Cryptosporidium* have been the protozoan parasites of greatest concern in food and water production industries throughout the world (Dawson, 2005). *Giardia* was the first protozoan parasite connected with human disease, with many documented waterborne cases since the 1970s (Craun, 1986). *Cryptosporidium* followed in the 1980s as a threat to water supplies, especially in the United Kingdom (U.K.) (Jephcott et al., 1986) and in the United States (U.S.), with the Milwaukee, WI, outbreak in 1993, being the largest documented waterborne outbreak in the developed world (Mackenzie et al., 1994).

Due to their numerous reservoirs (human and animal), the ability of cysts/oocysts to stay infectious for long periods of time in cool, damp environments, and their great resistance to water chlorination, *Giardia* and *Cryptosporidium* pose a significant threat to humans via the food and water route. Their cysts and oocysts are often found in surface waters and shallow springs, as well as in agricultural runoffs and sewage effluent.

Waterborne outbreaks usually affect a large number of people. In contrast, foodborne outbreaks are usually smaller, more difficult to detect, and are often related to fresh produce, especially those that are difficult to clean thoroughly or are consumed without cooking or additional processing. In the early 1970s, waterborne giardiasis surveillance started in the United States, as *Giardia* was the most frequently reported causative agent. The disease is also very common among travellers to the former Soviet Union and Eastern European countries. In order to estimate the prevalence and potential for human infectivity of *Giardia* cysts in Canadian drinking water supplies, several studies were done in Canada (Roach et al., 1993; Wallis et al., 1996). Foodborne outbreaks are usually related to food preparation by ill food handlers, contaminated imported produce, or due to contact with infected individuals, especially children. An example of the latter mode of transmission is a study of an outbreak which occurred during a family party for 25 people, when 9 individuals ate fruit salad and developed symptoms (Dawson, 2005). Giardiasis was spread from the food preparer who had a child in diapers and a pet rabbit at home, both of which were positive for *Giardia duodenalis* (Porter et al., 1990). In another outbreak among insurance company employees, there were 18 lab-confirmed illnesses and another 9 suspect cases. In this case, raw sliced vegetables were prepared and served by a *G. duodenalis* infected cafeteria employee (Mintz et al., 1993).

Cryptosporidium-related waterborne outbreaks were first documented in the U.S. and the U.K. in the mid-1980s. Beside the most widely recognized Milwaukee waterborne outbreak (Mackenzie et al., 1994), a number of outbreaks were related to different sources of water contamination. An outbreak of cryptosporidiosis in the summer of 1994, in a New Jersey state park, was the first reporting an association with recreational lake water. This outbreak affected 2,070 persons and was most likely due to contaminated runoff of rainwater

and infected bathers (Kramer et al., 1998). Between September 1994 and January 1995, laboratories in Sydney, Australia, reported 70 cases of cryptosporidiosis. *Cryptosporidium* oocysts were found in water from a swimming pool in January 1995, indicating that the outbreak was most likely due to ingestion of water from the indoor swimming pool contaminated by infected bathers (Lemmon et al., 1996). Another outbreak of 1060 laboratory-confirmed cryptosporidiosis cases was reported in New South Wales, Australia from December 1997 to April 1998 and was associated with swimming in public pools (Puech et al., 2001). In the U.K., in 1997, 345 people were infected due to a contaminated filtered borehole-derived water supply (Dawson, 2005). From April 2000 until April 2001, three drinking-water-related cryptosporidiosis outbreaks occurred in Northern Ireland, and all three were epidemiologically unrelated and originated from different geographic areas (Glaberman et al., 2002). Several waterborne outbreaks of cryptosporidiosis were reported in Canada as well. In 1996, the western Canadian province of British Columbia experienced four consecutive community outbreaks of cryptosporidiosis, three of which were confirmed by epidemiologic surveys to be caused by contaminated drinking water (Ong et al., 1999). In North Battleford, Saskatchewan, between late March and early May 2001, approximately 5,800 to 7,100 people were affected, and by May 2001, *C. parvum* infection was confirmed in 275 people (Stirling et al., 2001). Aside from waterborne cryptosporidiosis outbreaks, this protozoan infection is easily transmitted among children in daycare, patients in hospitals or other close social groups (Newman et al., 1994).

Foodborne illness outbreaks associated with *Cryptosporidium* are also common. Several foodborne outbreaks were reported by Centers for Disease Control and Prevention (CDC). One of them was due to *Cryptosporidium* contaminated apple cider in 1993 (From the Centers for Disease Control and Prevention, 1998), while in 1995 another foodborne

outbreak was associated with consumption of chicken salad that may have been contaminated by a food handler who most likely was the source of infection. In 1996, CDC reported another outbreak associated with drinking commercially produced unpasteurized apple cider, as apples were most likely washed with well water that had faecal contamination. In 1997, in Spokane, Washington, of 62 banquet attendees, 54 had illness probably due to contaminated green onions (From the Centers for Disease Control and Prevention, 1998).

1.10 Detection methods for *Giardia* and *Cryptosporidium*

Over the past decade, detection methods for *Giardia* and *Cryptosporidium* have greatly improved. These improvements were a result of the parasites' ubiquitous distribution in clinical specimens as well as in environmental samples, a need for relatively large volumes of samples, lengthy collection and processing times, and a lack of enrichment steps based on *in vitro* cultivation.

Detection of *Giardia* and *Cryptosporidium* from environmental samples (mostly water samples) and also from clinical samples can be done simultaneously. In the case of low-number (oo)cysts infections or few (oo)cysts per water sample, concentration techniques are necessary, as neither of the organisms can be easily cultured, though excystation and culturing procedures have been established for both *Giardia* and *Cryptosporidium*. When hydrochloric acid and trypsin are used, *Giardia* can be excysted and grown in TYI-S-33 (Trypticase, yeast extract, iron-serum) medium (Diamond et al., 1978; Rice and Schaefer, 1981), but the problem is usually a very low excystation rate for *G. duodenalis*. Excysted *C. parvum* oocysts are used for infecting bovine kidney cells *in vitro* (Upton et al., 1994). In this

cell culturing technique, problems arise due to the lengthy process and a need for a large number of oocysts. There are also animal models available including gerbils for *Giardia*, (Belosevic et al., 1983) or neonatal CD-1 mice for *Cryptosporidium* (Finch et al., 1993), but they are expensive and thus mostly used only in research laboratories.

Collected stool samples should always be sent fresh, preserved in 10 % buffered formalin, or suspended in aqueous potassium dichromate (2.5 % w/v, final concentration). As already mentioned, in asymptomatic infections, the number of (oo)cysts present in samples is usually very low and concentration techniques are often necessary. Some of them include Sheather's sucrose flotation, isopycnic or discontinuous Percoll, or cesium chloride gradient centrifugation, all of which are mostly used in research, while zinc sulfate flotation, saturated sodium chloride flotation, formalin-ether and formalin-ethyl acetate sedimentation are more popular in clinical laboratories (Fayer, 1997). Following flotation or filtration (used for environmental samples), the recovered material is centrifuged and the final pellet is examined microscopically.

As an alternative to density-gradient flotation procedures, immunomagnetic separation (IMS) is often used in water testing (McCuin et al., 2001; Rimhanen-Finne et al., 2001, 2002; Sturbaum et al., 2002; Ward et al., 2002), where usually 10-L samples are filtered, eluted with detergent and concentrated by centrifugation (Health Canada, 2004). After re-suspension in buffer, the concentrated material is mixed with specific monoclonal antibodies, attached to magnetized particles (immunomagnetic beads), and then the (oo)cysts are separated from the debris. This method is useful for improving specificity, and allows for better microscopic analysis, but is fairly expensive.

Microscopy is considered as the gold standard for *Giardia* and *Cryptosporidium* detection. Over the past decades, several different staining methods have been used, i.e., non-

invasive Giemsa staining (Tzipori et al., 1980), or the Ziehl-Nielsen acid fast staining technique which was launched in 1981 as a simple and effective method for identifying *Cryptosporidium* oocysts in stool samples (Fayer, 1997). The most commonly used method for *Giardia* cysts was staining with Lugol's iodine. There is also an array of alternatives to the brightfield microscopic acid-fast staining, including fluorescent stains (auramine O, auramine-rhodamine, auramine-carbol-fuchsin, acridine orange, mepacrine, and 4', 6-diamidino-2-phenylindole (DAPI) and propidium iodide) which have better sensitivity, but can yield false-positives or leave some (oo)cysts unstained (Fayer, 1997). In the mid-1980s, immunologic techniques were introduced for the detection of *Cryptosporidium* species in stool samples, and these showed increased sensitivity and specificity as compared to the conventional staining techniques. Immunofluorescent assays were used for detection of oocysts employing convalescent human serum (Casemore et al., 1985), oocyst-immunized rabbit antiserum (Stibbs and Ongerth, 1986), as well as oocyst-reactive monoclonal antibodies (Garcia et al., 1987; Arrowood and Sterling, 1989). The detection of *Giardia* cysts and *Cryptosporidium* oocysts in environmental and clinical samples has been greatly improved thanks to the use of monoclonal antibodies. However, the immunofluorescence detection method is time consuming, expensive and semi-quantitative, as it can only determine if the (oo)cysts are viable, but cannot ascertain if they are both viable and infective.

One of the increasingly popular methods being used for the detection of (oo)cysts following concentration and recovery, is flow cytometry (Dixon et al., 1997; Dixon et al., 2005). For flow cytometry analysis, different fluorochromes can be added to solutions as markers of viability, or other physiological conditions of the cell, or they can be simply conjugated to a monoclonal antibody specific for a particular component of the cell. Once the

fluorochrome is added, flow cytometry allows for the sample analysis according to non-fluorescing (non-stained) and fluorescing (stained) portions of the sample giving important information about the components of interest within the sample. It is also an expensive method that requires specialized equipment, but allows for particle separation and enumeration (Reynolds et al., 1999; Delaunay et al., 2000; Lindquist et al., 2001). A two-color flow cytometric assay using competing surface of mAbs (Ferrari et al., 2000), improved the detection of *Cryptosporidium* by decreasing the number of non-*Cryptosporidium* particles detected, while at the same time microscopic analysis times were simplified and also improved.

Molecular biology techniques have been used for some time in the detection of *Giardia* and *Cryptosporidium* (oo)cysts, with the most commonly used being the polymerase chain reaction (PCR), either alone or in combination with IMS (water testing). Due to its high specificity and sensitivity, when combined with other molecular biology techniques like restriction fragment length polymorphism (RFLP), PCR can be used for discrimination between species and strains. The major problem with PCR techniques is the presence of a number of inhibitors in both water (Jiang et al., 2005) and clinical samples. The latest PCR technique that is giving better and faster results is real-time PCR (qPCR). It is as specific and sensitive as PCR, and at the same time more sensitive and less time consuming than microscopy. A number of real-time PCR assays have been published for both *Giardia* (Amar et al., 2003; Verweij et al., 2003; Guy et al., 2004) and *Cryptosporidium* (Di Giovanni et al., 2005) using different fluorescent labeled probes or multiplex detection of different targets (Guy et al. 2003; Ng et al., 2005). This novel technique which not only enables detection, but also genotyping (Amar et al., 2003; Johnson et al., 2003; Guy et al., 2004), is making radical changes to routine parasitology methodology.

1.11 Hypothesis and Statement of Objectives

Due to the fact that among both *Giardia* and *Cryptosporidium* there is an Assemblage/genotype with zoonotic potential (Assemblage A for *Giardia*; *C. parvum* bovine genotype, *C. hominis* and *C. meleagridis* for *Cryptosporidium*), the hypothesis of this study is that **there is a potential for transmission of *Giardia* and *Cryptosporidium* between livestock and humans.** In a preliminary study on cattle stool samples from PEI, our findings showed the existence of zoonotic genotypes. This raised a question that similar genotypes might also be present among livestock in Ontario. To address this possibility, 84 animals (adults and calves) from a farm near Brockville, Ontario and 59 calves from Kemptville College, Ontario were sampled during the summer of 2005.

Overall Objective:

Develop a detection and characterization method for *Giardia* and *Cryptosporidium* spp. using molecular techniques in order to determine if transmission can occur between livestock and humans.

1. Develop a detection and characterization method for *Giardia* and *Cryptosporidium* using molecular techniques.

2. To assess the value of using the IMS method as a second concentration step in the detection of *Giardia* and *Cryptosporidium*.

3. To find "the best gene" among three genes for detection of both *Giardia* and *Cryptosporidium*.

4. To determine if there is a potential for the transmission of *Giardia* and *Cryptosporidium* between livestock and humans, based on genotyping of bovine samples from PEI and Ontario.

5. To conduct a survey on the prevalence of *Giardia* and *Cryptosporidium* in calf samples over time.

2 MATERIALS AND METHODS

2.1 Source and collection of specimens

Faeces were collected from 84 cattle, consisting of 46 adult cows and 38 calves (females and males, 2-24 months of age), at one dairy farm in Brockville, Ontario, and from 59 male calves from Kemptville College, Ontario. The farm in Brockville was visited once in May 2005, while Kemptville College animals were sampled twice, in May and July, 2005. All specimens were collected from animals housed in groups. At the farm in Brockville, animals were confined to large pens with cement floors fully covered by a roof, while Kemptville College calves were housed in hutches.

Samples from PEI were collected from 58 adult cows (>3 years old) from the Atlantic Veterinary College (AVC) bovine teaching herd, from September 2003 until April 2004; and 55 calves from 9 different farms throughout PEI, during 2002.

From July to October 2005, 29 male dairy calves were sampled six times each. These calves originated from different farms around Kemptville. Animals were purchased by Kemptville College for the summer students involved in different research projects. Animals were approximately 12-days old when they were separated from dams and transferred to individual hutches at Kemptville College. Most of the animals had one or more diarrheic episodes before weaning.

Faeces were collected directly from the rectum of each animal and transferred into a plastic cup. Cups were capped, labelled with the animal's ear tag number, and immediately placed into an insulated container packed with ice or cold packs. Specimens were transported

to the Parasitology Laboratory of the Microbiology Research Division of Health Canada, Ottawa, ON, and processed within 1-3 days of collection.

2.2 Cyst and oocyst concentration from faeces

The sucrose flotation method of O'Handley (1999) was used to concentrate *Giardia* cysts and *Cryptosporidium* oocysts from faecal samples. However, the method was slightly modified, in order to accommodate a sample size of 20 g for adults. To 5 g of faeces from calves or to 20 g of faeces from adults, 10 ml (35 ml for adult samples) of phosphate-buffered saline (PBS) was added and everything was thoroughly mixed. The suspension was passed through gauze (A.R. Medicom Ltd, Montreal, QC) and layered over 5 ml (15 ml for samples from adults) of 1 M sucrose (Sigma-Aldrich Canada Ltd, ON) solution (specific gravity 1.13) in a clean tube. The samples were centrifuged at 800 x g for 5 min. Following centrifugation, and using a disposable pipette, the interface and the upper layer of liquid was transferred to a clean cup, and re-centrifuged at 800 x g for 5 min. The supernatant was decanted, leaving a final pellet volume of 1 ml.

2.3 Determination of sensitivity of the PCR detection methods

To determine the sensitivity of the PCR methods used to detect *Giardia* cysts and *Cryptosporidium* oocysts in the present study, bovine faeces were obtained from a calf found to be negative for giardiasis and cryptosporidiosis. Fourteen replicate 5 g faecal specimens were each spiked with cysts of *G. duodenalis* and oocysts of *C. parvum* at the rate of 1, 10, 100, 1,000, 10,000, and 100,000 (oo)cysts per gram and subjected to the same methods of concentration and molecular detection as described below.

2.4 Immunofluorescence microscopy for *Cryptosporidium* and *Giardia*

After (oo)cyst isolation and concentration, a 20 µl sample of the concentrate was spotted onto a microscope slide (Fisher Scientific Co., Pittsburgh, PA). Fluorescein isothiocyanate (FITC)-labeled monoclonal antibody solutions (20 µl of each Giardi-a-glo and Crypt-o-glo, Waterborne Inc., New Orleans, LA) were applied to the slide, which was then incubated in a humid air chamber for 45 min. After incubation, the slide was briefly rinsed with PBS and sealed with a glass cover slip. Microscopic examinations were performed with a Nikon Eclipse E600 (Nikon, Japan) equipped with epifluorescence optics. *Giardia* cysts and *Cryptosporidium* oocysts were examined and enumerated under the epifluorescence microscope at 200 x magnification. The number of cysts and oocysts per gram of faeces was calculated using the following formula (O'Handley et al., 1999):

$$N=s/(vol \times wt \times pv)$$

Where N = number of cysts or oocysts per gram faeces, s = number of cysts or oocysts counted on the slide, vol = volume of sample examined (0.02 ml), wt = weight of faecal sample and pv = pellet volume (1 ml).

2.5 Flow cytometry

From the sucrose flotation cleaned samples, 200 µl was transferred into each of two 5 ml round-bottomed tubes (Falcon, Becton Dickinson). Fluorescein-labelled anti-*Giardia* monoclonal antibody (25 µl) (Giardi-a-Glo™, Waterborne, Inc., New Orleans, LA) and R-phycoerythrin-labelled anti-*Cryptosporidum* monoclonal antibody (50 µl) (Cryp-a-Glo™,

Waterborne, Inc.) were added to one tube, and 75 µl PBS (autofluorescence control) was added to the second tube. Tubes were then incubated for 45 min at room temperature in the dark. The contents of both tubes were washed with 1 ml PBS, and then centrifuged at 1,900 x g for 20 min at room temperature. Finally, the pellets were resuspended in 0.5 ml PBS, and kept in the dark at 4 ° C until flow cytometric analysis. Samples were analyzed on a FACSCalibur (Becton Dickinson, Mississauga, ON) equipped with an argon-ion laser operating at 488 nm and using CELLQuest software. The flow cytometer was calibrated using both CaliBRITE beads (Becton Dickinson) and FACSComp software, and QC3 beads according to the manufacturer's recommendations (Bangs Laboratories, Inc., Fishers, IN). As a positive control, the suspension of pure cysts and oocysts (Waterborne, Inc.) was used to set the analysis gate around the *Giardia* cysts and *Cryptosporidium* oocysts on a dual parameter dot plot (right angle light scatter vs. fluorescence). Gated on R1, a dual parameter dot plot (forward light scatter vs. right angle light scatter) was generated, and the analysis gate (R2) was set around the cysts. Using a double-anchor gating strategy (G6= R1 and R2), a dual dot plot (right angle light scatter vs. fluorescence) gated on G6 was generated, which displayed the number of *Giardia* cysts in the test sample. The same was done for *Cryptosporidium* oocysts. Gated on R3, a dual parameter dot plot (forward light scatter vs. right angle light scatter) was generated, and the analysis gate (R4) was set around the oocysts. Again using a double-anchor gating strategy (G7=R3 and R4) a dual dot plot (right angle light scatter vs. fluorescence) gated on G7 was generated which displayed the number of *Cryptosporidium* oocysts in the test sample. All samples were vortexed before and during analysis.

2.6 Immunomagnetic separation (IMS)

A Dynabeads GC-Combo kit (Dynal, Olso, Norway) was used for all IMS reactions, using Dynal's protocol, with the following modifications. Each IMS reaction (1ml) was composed of 200 µl of concentrated sample, 100 µl of 10X SL^{TM}- Buffer A, 100 µl of 10X SL^{TM}- Buffer B, 50 µl dynabeads anti-*Giardia*, 50 µl dynabeads anti-*Cryptosporidium*, and 500 µl double distilled water. Flat-top microtubes of 1.5 ml capacity containing the IMS components (DiaMed lab supplies Inc., Mississauga, ON) were then affixed to a rotating mixer (Dynal-MX1) and were rotated at 19 rpm for 1 h at room temperature. After rotation, tubes were placed in the magnetic particle concentrator (Dynal MPC-S) and gently rocked by tilting the cap-end and base-end of the tube up and down in turn for 2 min. The cap was then removed and all the supernatant from the tube held in the Dynal MPC-S was decanted. Tubes were then removed from the Dynal MPC-S and samples resuspended in 1 ml 1X SL^{TM}-Buffer A were mixed gently and again placed into the Dynal MPC-S and gently rocked for 1 min. This wash step was then repeated twice. The dissociation of dynabeads-cysts/oocysts was performed by removing the tubes from the Dynal MPC-S and adding 50 µl of 0.1N hydrochoric acid (HCl) to the microcentrifuge tube, followed by vortexing thoroughly for 10 seconds. Tubes were then placed in the Dynal MPC-S without the magnet for 10 min at room temperature, and then vortexed again for 10 seconds (before the magnetic strip was replaced) allowing the now dissociated beads to be removed from suspension. The supernatants were aspirated by using plugged Pasteur pipettes and were transferred into clean 1.5 ml microcentrifuge tubes containing 5 µl of 1N sodium hydroxide (NaOH) to neutralize the suspension.

2.7 DNA extraction

Total DNA was extracted from each sucrose flotation concentrated sample, as well as from each IMS concentrated sample using the DNeasyTissue Kit (Qiagen Inc., Mississauga, ON) using a slightly modified protocol. A total of 200 µl of sucrose flotation concentrated sample, or 50 µl of sample obtained from the final stage of IMS experiments, were added to 1.5 ml microcentrifuge tubes and lysed overnight at 56°C using 180 µl of lysis buffer and 20 µl of Proteinase K (20 mg/ml) supplied with the DNeasy Tissue Kit. The subsequent lysate was removed and the genomic DNA isolated using the DNeasy Tissue Kit following the manufacturer's instructions. At the end of the protocol, in order to increase the quantity of recovered DNA, the nucleic acid was eluted in 100 µl of elution buffer.

2.8 Amplification of *Giardia* gene fragments by polymerase chain reaction

i) 18S rRNA gene

Fragments of the 18S rRNA (~292 bp) gene were amplified using modifications of previously described PCR protocols. A nested-PCR protocol was used with first round primers Gia2029 (5'-AAGTGTGGTGCAGACGGACTC-3') and Gia2150c (5'-CTGCTGCCGTCCTTGGATGT-3') amplifying a 497-bp product (Appelbee et al., 2003), and secondary primers RH11 (5'-CATCCGGTCGATCCTGCC-3'), and RH4 (5'-AGTCGAACCCTGATTCTCCGCCAGG-3') generating a 292-bp fragment (Hopkins et al., 1997). Primers were synthesized at the Oligonucletide Synthesis Service, Biotechnology Research Institute, University of Ottawa, Ottawa, ON. PCR reactions consisted of 1X PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 5 % v/v DMSO, 2 U Taq polymerase,

and 0.5 μM of each forward and reverse primer in a total reaction volume of 50 μl. Following an initial hot start at 96°C for 2 min, a total of 35 cycles, each consisting of 96°C for 45 seconds, 58°C for 30 seconds, and 72°C for 45 seconds were performed. A final extension step at 72°C for 4 min was also included. For the second round of PCR, the PCR mixtures were identical, as well as the number of cycles, temperatures and times, with the only exception being the annealing temperature which was decreased to 55°C for 30 seconds. PCR products were analyzed on 1 % w/v agarose gels and visualized by ethidium bromide (BioRad Laboratories Ltd., Mississauga, ON) staining.

ii) *β-giardin* gene

A two-step nested PCR protocol was used to amplify a 753-bp fragment of the *β-giardin* gene (Caccio et al., 2002) using the forward primer G7 (5'-AAGCCCGACGACCTCACCCGCAGTGC-3') and the reverse primer G759 (5'-GAGGCCGCCCTGGATCTTCGAGACGAC-3'). For the nested PCR, the forward primer G376 (5'-CATAACGACGCCATCGCGGCTCTCAGGAA-3') as described by Mahbubani et al. (1992), and the reverse primer G759, generated a 384-bp fragment. Primers were synthesized at the Oligonucletide Synthesis Service, Biotechnology Research Institute, University of Ottawa, Ottawa, ON. PCR mixtures consisted of 1X PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 U of ExTaq DNA polymerase (Takara Bio Inc., Japan) with 0.5 μM for each forward and reverse primer in a total of 50 μl. PCR was performed in a total of 35 cycles, each consisting of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, following an initial hot start at 94°C for 5 min, and a final extension step at 72°C for 10 min. The PCR mixtures were identical for the secondary PCR. A total of 35 cycles, each consisting of 94°C for 30 seconds, 66°C for 30 seconds, and 72°C for 30 seconds, were

performed; an initial hot start at 94°C for 5 min and final extension step at 72°C for 10 min were also included. PCR products were analyzed on 1 % w/v agarose gels and visualized by ethidium bromide (BioRad Laboratories Ltd.) staining.

iii) *gdh* gene

For the detection of the *Giardia* glutamate dehydrogenase *(gdh)* gene, novel primers were designed. From the NCBI database, sequence information was obtained for a representative isolate of each of the Assemblages AI, AII, BIII, BIV, C, D and E (GenBank accession numbers: L40509 (AI) (Monis et al., 1996), AF069059 (BIII) (Monis et al., 1999), L40510 (AII), L40508 (BIV) (Monis et al., 1996), U60984 (C), U60986 (D) (Monis et al., 1998) and U47632 (E) (Ey et al., 1997). Using Clustal W software (Thompson et al., 1994), the sequences were aligned and analysed for similar regions across all Assemblages. New primers were designed using KODON Total Genome and Sequence Analysis Software (Applied Maths, Inc. Austin, TX). To yield a 389-bp fragment of the *gdh* gene, the forward primer GiaTCF (5'-CGCTTCCACCCCTCTGTC-3') and the reverse primer GiaTCR (5'-TCCTTGCACATCTCCTC-3') were used. Primers were synthesized at the Oligonucletide Synthesis Service, Biotechnology Research Institute, University of Ottawa, Ottawa, ON. The PCR mix included 1X PCR buffer (1.5 mM $MgCl_2$), 0.2 mM each dNTP, 2.5 U of ExTaq DNA polymerase (Takara Bio Inc.) and 1 μM of each forward and reverse primer in a total of 50 μl reaction. A total of 35 cycles, each consisting of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds, were performed with an initial hot start at 95°C for 5 min and a final extension step at 72°C for 10 min. PCR products were analyzed on 1 % agarose gels and visualized by ethidium bromide (BioRad Laboratories Ltd.) staining.

2.9 Amplification of *Cryptosporidium* gene fragments by polymerase chain reaction

i) 18S rRNA gene

To amplify the 18S rRNA gene (~830 bp), a nested PCR protocol was followed, using the following primers previously described by Xiao et al. (1999b): 5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCTAATCCTTCGAAACAGGA -3' for primary PCR and 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA-3'for nested PCR. Primers were synthesized at the Oligonucletide Synthesis Service, Biotechnology Research Institute, University of Ottawa, Ottawa, ON. PCR amplification was performed in 50 µl volumes with 5 µl DNA in 1X PCR buffer, 3 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 U Taq, 2.5 µl fetal BSA (0.1g/10ml), and 1 µM for each forward and reverse primer. Samples were subjected to 35 cycles of 94°C for 45 seconds, 59°C for 45 seconds, and 72°C for 1 min, with an initial hot start at 94°C for 3 min, and a final extension step at 72°C for 7 min. The nested PCR mixture was identical except that 1 µl DNA and a concentration of 1.5 mM $MgCl_2$ were used. A total of 40 cycles, each consisting of 94°C for 30 seconds, 58°C for 90 seconds, and 72°C for 2 min, were performed with an initial hot start at 94°C for 3 min, and a final extension step at 72°C for 7 min. PCR products were analyzed on 1 % w/v agarose gels and visualized by ethidium bromide (BioRad Laboratories, Ltd.) staining.

ii) HSP-70 gene

A two-step nested PCR (Morgan et al., 2001) was used to amplify portions of the heat-shock protein 70 (HSP-70) gene, using the primers designed to amplify a 448-bp fragment of this gene (forward: 5'-GGTGGTGGTACTTTTGATGTATC-3'and reverse

primer: 5'- GCCTGAACCTTTGGAATACG-3'). In the secondary reaction, a 325-bp fragment of HSP-70 was amplified using forward primer 5'-GCTGATGATACTCACTTGGGTGG-3' and reverse primer 5'-CTCTTGTCCATACCAGCATCC-3'). Primers were synthesized at the Oligonucletide Synthesis Service, Biotechnology Research Institute, University of Ottawa, Ottawa, ON. PCR mixtures were the same as above for the 18S rRNA gene, while times and temperatures were different. A total of 35 cycles, each consisting of 94°C for 30 seconds, 61°C for 30 seconds, and 72°C for 30 seconds, were performed with an initial hot start at 94°C for 5 min, and a final extension step at 72°C for 10 min. The nested PCR annealing temperature was 70°C for 30 seconds, with the rest of the times and temperatures being the same as for the primary reaction.

iii) COWP gene

Another nested PCR procedure was used to amplify a fragment of the COWP gene. A 769-bp fragment of the COWP gene was amplified with forward primer 5'-ACCGCTTCTCAACAACCATCTTGTCCTC-3' and reverse primer 5'-CGCACCTGTTCCCACTCAATGTAAACCC-3' (Pedraza-Diaz et al., 2001b). The nested primer set included: Cry15 -- 5'-GTAGATAATGGAAGAGATTGTG-3' and Cry9 -- 5'-GGACTGAAATACAGGCATTATCTTG-3', which generated the 553-bp fragment (Spano et al., 1997). Primers were synthesized at the Oligonucletide Synthesis Service, Biotechnology Research Institute, University of Ottawa, Ottawa, ON. The primary PCR mixture consisted of 1X PCR buffer, with 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 U Taq, and 0.5 μM for each forward and reverse primer in a total reaction volume of 50 μl. A total of 35 cycles, each consisting of 94°C for 30 seconds, 61°C for 30 seconds, and 72°C for 30

seconds, were performed with an initial hot start at 94°C for 5 min, and a final extension step at 72°C for 10 min. The nested PCR mixture was identical to that used in the primary reaction. Again a total of 35 cycles, each consisting of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, were performed with an initial hot start at 94°C for 5 min and a final extension step at 72°C for 10 min. PCR products were analyzed on 1 % w/v agarose gels and visualized by ethidium bromide (BioRad Laboratories Ltd.) staining.

2.10 Sequence analysis

Sequencing was performed at the McGill University and the Genome Quebec Innovation Centre, where PCR products were purified and sequenced in both directions using the same PCR primers as for the original amplifications. The Genetic Computer Group sequence analysis package (version 10.3, Madison, Wisconsin) was used to assemble and analyse the DNA sequences. To determine the genotypes of *Giardia* and *Cryptosporidium*, each sequence was compared to the GenBank sequences of *Giardia* (accession numbers AY655701 and AY655700) and *Cryptosporidium* (accession numbers AY151416 and AY741306) genotypes, and the homology was determined.

2.11 Data analysis

All analyses were performed using the statistical software package Splus6.2 (Insightful Corporation, WA). A McNemar's test was performed to test marginal homogeneity of the counts with IMS and without IMS by gene site. For data analysis of infection over time, the method of estimation for the cumulative distribution function was used. A commonly used method of analysis for this type of attribute (time to infection) is the

analysis of waiting times (also referred to as survival analysis, when death is observed rather than infection). In such an analysis, the probability that infection occurs on or before a specified day is estimated. Mathematically, if t is a specified day, and T is the first time at which an infection is observed, the model estimates the cumulative probability function (CDF), denoted $\Pr\{T \le t\}$, of the time to infection data. Applying the commonly used Weibull distribution to the observed data yields the maximum likelihood estimate

$$\Pr\{T \le t\} = 1 - 10^{-(t/40)^3}.$$

For persistence of infection, a chi-square test for equality of proportions with four degrees of freedom was used.

3 METHOD DEVELOPMENT and PREVALENCE of *Giardia* and *Cryptosporidium* BASED on COMPARISON of MICROSCOPY, FLOW CYTOMETRY and PCR

3.1 Introduction

Many studies on detection methodologies for *Giardia* and *Cryptosporidium* have been published. However, the increasing interest in rapid diagnostic testing, as well as the increase in both food and waterborne outbreaks associated with protozoan parasites, have necessitated the development or optimization of methods that are acceptable in terms of sensitivity and specificity. The diagnosis of both *Giardia* and *Cryptosporidium* can be difficult, because they are not shed in the stool on a consistent basis and their numbers can vary from day-to-day (Nydam et al., 2001). Furthermore, neither *Giardia* nor *Cryptosporidium* can be easily cultured (Diamond et al. 1978; Rice and Schaefer 1981: Upton et al. 1994). This indicates a need for a concentration step as both parasites may be present in very low numbers in stool samples (Xiao and Herd, 1994).

Until recently, the diagnosis of giardiasis and cryptosporidiosis was done by faecal flotation, acid-fast staining (cryptosporidiosis) or Lugol's iodine staining (giardiasis), and direct immunofluorescent antibody detection methods. The disadvantage of these methods is the limit of detection of (oo)cysts per gram of faeces, as well as the inability of any microscopic method to genotype *Giardia* or *Cryptosporidium*. Although IMS has been used for some time as a secondary concentration step in processing (oo)cysts from water samples (McCuin et al., 2001; Greinert et al., 2004; Watanabe et al., 2005), it is not as commonly used in clinical sample analyses (Webster et al., 1996). IMS not only concentrates (oo)cysts but also "cleans" samples of particulate matter that can inhibit and interfere with detection

methods (PCR in particular) for *Giardia* and *Cryptosporidium* (Johnson et al., 1995; Hallier-Soullier and Guillot, 1999; Hallier-Soullier and Guillot, 2000; Feng et al., 2003).

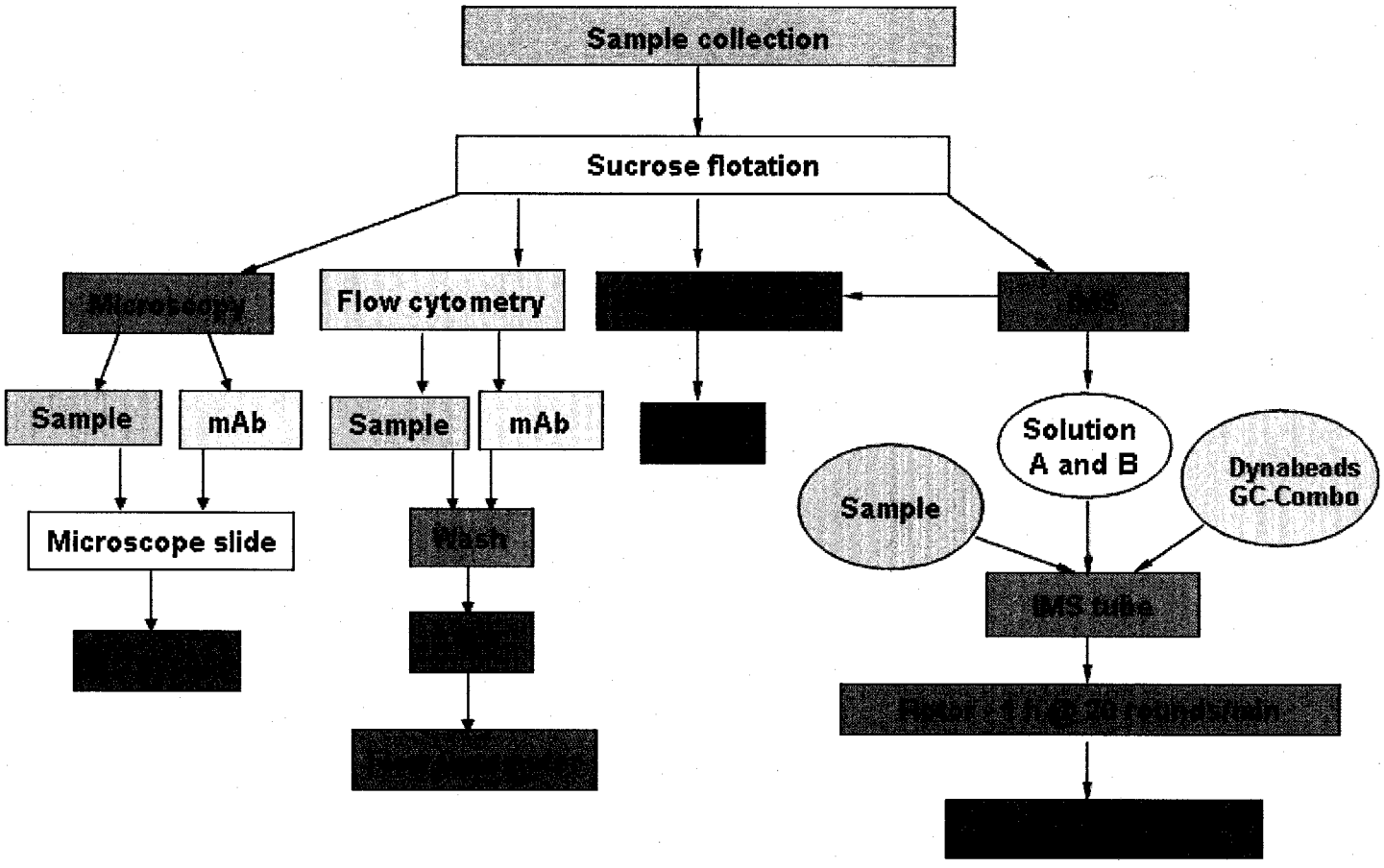
In light of these situations, one of the goals of this study was to evaluate the efficiency of IMS as a second concentration step in clinical sample analysis for *Giardia* and *Cryptosporidium*. A study of *Giardia duodenalis* and *Cryptosporidium* spp. in cattle stool samples collected from a farm near Brockville as well as from Kemptville College, Ontario, was undertaken for this project.

Many studies have reported on the molecular characterization of *G. duodenalis* and *Cryptosporidium* spp. by using amplification of fragments of different loci by PCR. The popularity of PCR in recent years has helped in the identification of additional host-adapted genotypes of *Cryptosporidium* spp., i.e., goose genotype, deer genotype, duck genotype, pig genotypes I and II, skunk genotype, ferret genotype, muskrat genotypes I and II, *C. canis* fox genotype, and bovine B genotype (Morgan et al., 1999c; Fayer et al., 2000a; Xiao et al., 2002, 2004; Enemark et al., 2003; Ryan et al., 2003a; Jellison et al., 2004). The other goal of this study was to compare conventional PCR methods, both with and without IMS. As *Giardia* and *Cryptosporidium* may be present in low numbers in stool samples (especially from adult animals due to their dilution in the large volumes of manure produced), an IMS-PCR assay was optimized for their detection. *Giardia duodenalis* fragments of 18S rRNA, *β-giardin* and *gdh*, as well as *Cryptosporidium* spp. fragments of 18S rRNA, HSP-70 and COWP loci were amplified by PCR from cattle stool samples, in order to determine the optimal gene for PCR detection.

In addition to molecular methods for the determination of the prevalence of both parasites among cattle, microscopy (gold standard) and flow cytometry were also evaluated and used for method comparison purposes (Figure 3). Flow cytometry was used not only due

Figure 3. Flow chart of the methods used for the detection of *Giardia* and *Cryptosporidium* in cattle faecal samples

(mAb = monoclonal antibodies)


Sample collection
Sucrose flotation
Microscopy
Flow cytometry
Sample
mAb
Sample
mAb
Microscope slide
Wash
Solution
A and B
Dynabeads
GC-Combo
Sample
IMS tube

to its increasing popularity, but also because it is a relatively simple and rapid method for analyzing cells in solution (Erlandsen et al., 1988; Vesey et al., 1993, 1994a, b; Arrowood et al., 1995).

3.2 Results

Of the 143 cattle faecal samples examined by immunofluorescence microscopy, 36 (25.2 %) and 30 (21 %) were positive for *Giardia* cysts and *Cryptosporidium* oocysts, respectively (Table 2). As mentioned, (oo)cysts were stained by fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies. *Giardia* cysts appeared as bright green oval-shaped bodies, easily detectable even in concentrated faecal debris. *Cryptosporidium* oocysts were visible as bright green round bodies, but due to their smaller sizes and overlapping debris, it is possible that some of them were missed (Figures 4 and 5).

The IMS-PCR assay was also compared to conventional PCR alone. To determine the sensitivity of the PCR methods used to detect *Giardia* cysts and *Cryptosporidium* oocysts, bovine faeces were obtained from an animal found to be negative for both parasites (Figures 6 and 7). Faecal specimens were each spiked with a known number of (oo)cysts that were counted using a hemocytometer, and subjected to sucrose flotation as described in Materials and Methods. The achieved detection sensitivity with primers specific for the 18S rRNA for both *Giardia* and *Cryptosporidium* was 100 (oo)cysts per gram of faeces (Figures 6 and 7). For *Giardia*, the use of IMS resulted in 64 (44.8 %) positive samples when using primers directed against 18S rRNA, while only 32 (22.4 %) samples were positive by the same PCR without IMS. The 18S rRNA primers were the most sensitive in the PCR assay, as the *β-giardin* IMS-PCR assay resulted in only 12 (8.4 %) positives and 11 (7.7 %) positives when using PCR alone. The third gene analyzed for *Giardia*, the *gdh* gene, gave 27 (18.9 %) positives in the IMS-PCR assay, and only 9 (6.3 %) positives with PCR only (Table 3).

Table 2. Presence of *Giardia duodenalis* cysts and *Cryptosporidium parvum* oocysts in cattle faecal samples[a] using three different methods

	Giardia *n* (%)	*Cryptosporidium* *n* (%)
Microscopy	36 (25.2)	30 (21)
PCR-IMS	64 (44.8)	60 (42)
Flow cytometry	11 (7.7)	36 (25.2)

[a] A total of 143 cattle faecal samples were examined.

Figure 4. Analysis of cattle faecal samples for *Giardia* cysts and *Cryptosporidium* oocysts by epifluorescence microscopy

Specimens were viewed with a Nikon Eclipse E600 (Nikon, Japan) using 200X magnification and stained with fluorescein isothiocyanate (FITC)-labeled monoclonal antibody solutions (Giardi-a-glo and Crypt-o-glo, Waterborne Inc.). *Cryptosporidium* oocysts (1) ranged from 4-6 μm in diameter, while *Giardia* cysts (2,3) ranged from 8-14 μm in length and 7-10 μm in width. Pictures were taken using a Nikon DS-5M-L1 digital camera and visualized with a 5-mega pixel color matrix CCD built-in image processing software.

Figure 5. Analysis of cattle faecal samples for *Giardia* cysts and *Cryptosporidium* oocysts by bright-field microscopy

Specimens were viewed with a Nikon Eclipse E600 (Nikon, Japan) using 200X magnification. *Cryptosporidium* oocysts (1) ranged from 4-6 μm in diameter, while *Giardia* cysts (2,3) ranged from 8-14 μm in length and 7-10 μm in width. Pictures were taken using a Nikon DS-5M-L1 digital camera and visualized with a 5-mega pixel color matrix CCD built-in image processing software.

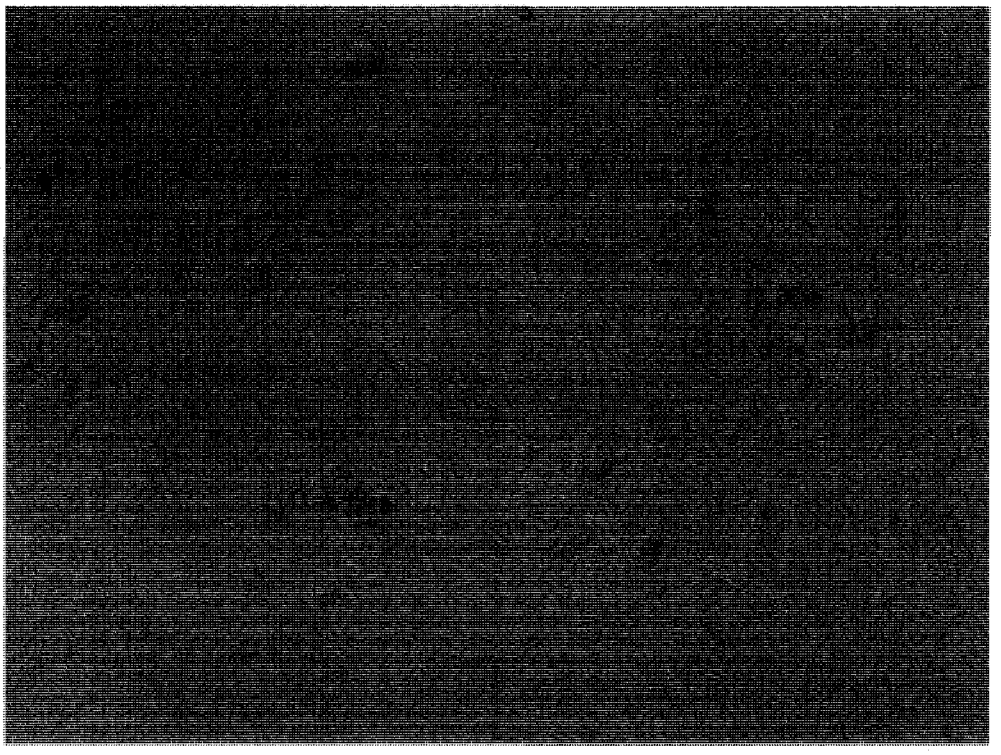
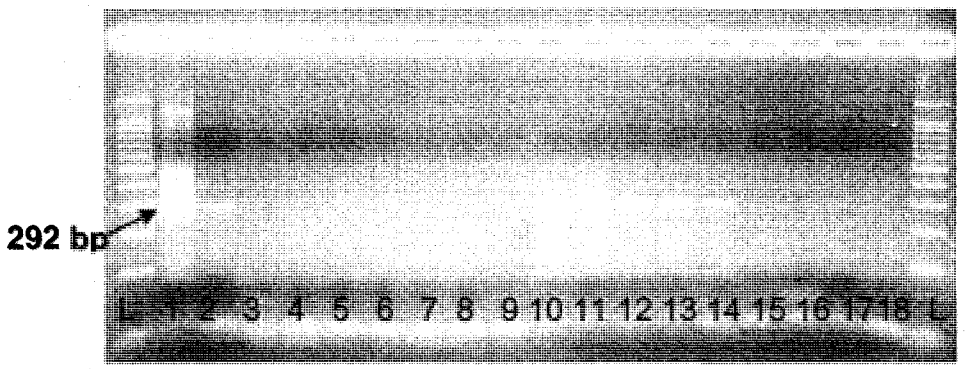


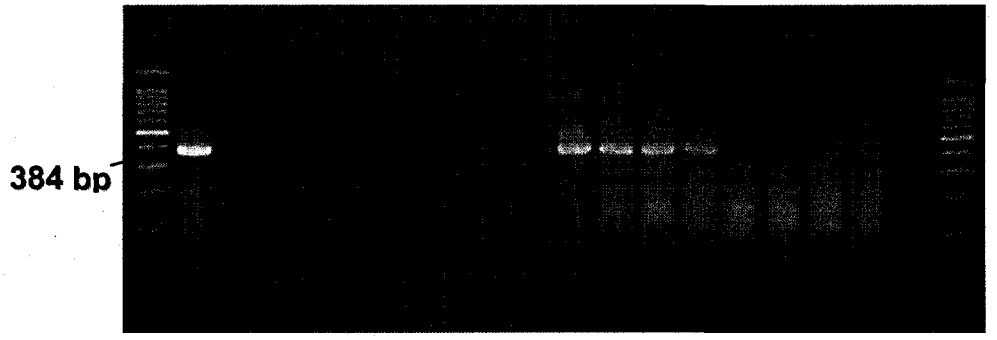
Figure 6. Tests done to determine the sensitivity of the PCR method with and without IMS for *Giardia*

Electrophoretic separation of 18S rRNA (A), *β-giardin* (B) and *gdh* (C) genes showing PCR amplification products from *Giardia*-negative stool samples spiked with known numbers of *Giardia* cysts. Lanes 2-9 no IMS, lanes 11-18 IMS. Lanes L, 100 bp ladder; lanes 1 and 10, positive control ($5x10^6$ cysts); lanes 2 and 11, $5x10^5$ cysts / 5g stool sample; lanes 3 and 12, $5x10^4$ cysts / 5g; lanes 4 and 13, $5x10^3$ cysts / 5g; lanes 5 and 14, $5x10^2$ cysts / 5g; lanes 6 and 15, $5x10^1$ cysts / 5g; lanes 7 and 16, $5x10^0$ cysts / 5g; lanes 8 and 17, negative control; lanes 9 and 18, internal negative controls (consisting of master mixes only). The Figure is representative of at least three independent experiments.

Panel A



Panel B



Panel C

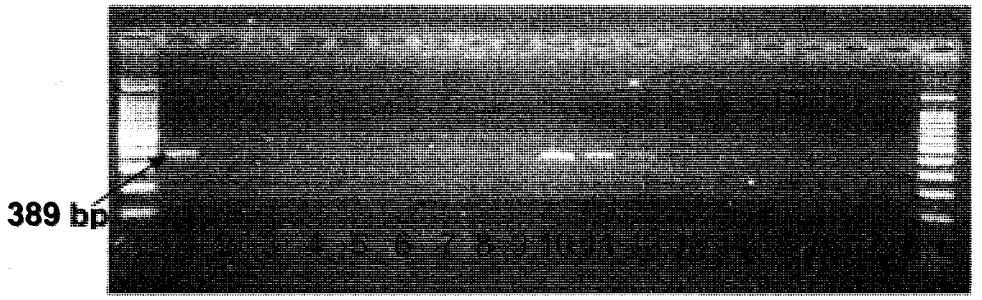
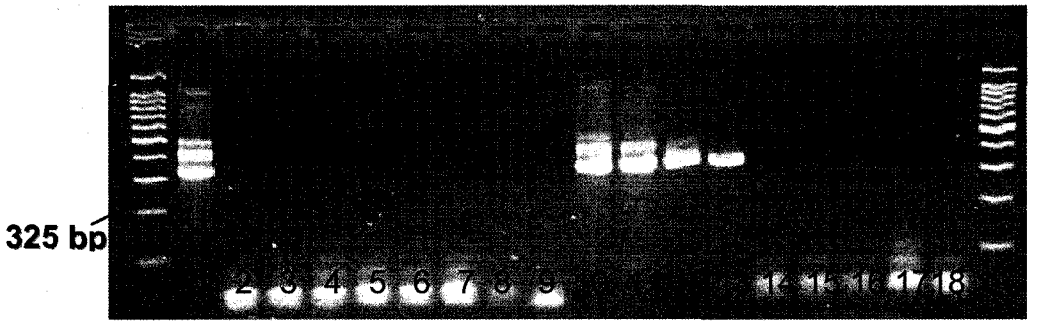


Figure 7. Tests done to determine the sensitivity of the PCR method with and without IMS for *Cryptosporidium*

Electrophoretic separation of COWP (A), HSP-70 (B), and 18S rRNA (C) genes showing PCR amplification products from *Cryptosporidium*-negative stool samples spiked with a known number of *Cryptosporidium* oocysts. Lanes 2-9 no IMS, lanes 11-18 IMS. Lanes L, 100 bp ladder; lanes 1 and 10, positive control ($5x10^6$ oocysts); lanes 2 and 11, $5x10^5$ oocysts / 5g stool sample; lanes 3 and 12, $5x10^4$ oocysts / 5g; lanes 4 and 13, $5x10^3$ oocysts / 5g; lanes 5 and 14, $5x10^2$ oocysts / 5g; lanes 6 and 15, $5x10^1$ oocysts / 5g; lanes 7 and 16, $5x10^0$ oocysts / 5g; lanes 8 and 17, negative control; lanes 9 and 18, internal negative controls (consisting of master mixes only). The Figure is representative of at least three independent experiments.

Panel A

Panel B



Panel C

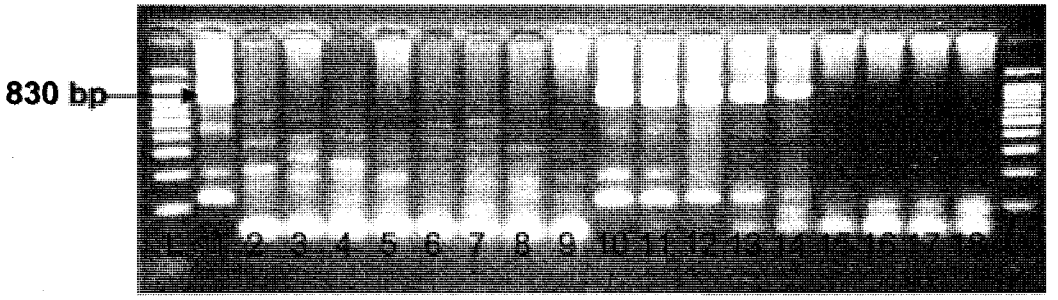


Table 3. The detection of *Giardia* cysts and *Cryptosporidium* oocysts in cattle faecal samples[a] by the use of a PCR method, with and without IMS

Giardia *n* (%)			*Cryptosporidium* *n* (%)		
18S rRNA[b]	IMS	64 (44.8)	18S rRNA	IMS	43 (30.1)
	No IMS	32 (22.4)		No IMS	42 (29.4)
β – giardin	IMS	12 (8.4)	HSP-70	IMS	60 (42)
	No IMS	11 (7.7)		No IMS	58 (40.6)
gdh[b]	IMS	27 (18.9)	COWP[b]	IMS	56 (39.2)
	No IMS	9 (6.3)		No IMS	45 (31.5)

[a] A total of 143 cattle faecal samples were examined.
[b] For these primers, significantly more positives were obtained when using IMS, as compared to not using IMS.

Using flow cytometry on those 143 samples, 11 (7.7 %) were positive for *Giardia* cysts, and 92 were negative. Out of the 143 samples, 40 results were considered equivocal, i.e, one could not ascertain whether they were positive or negative, due to the low numbers of *Giardia* cysts and *Cryptosporidium* oocyts. As a cut-off point by flow cytometry for *Giardia*, six events or more were considered a positive sample. In order to avoid false-positives, samples having only 1 to 5 events in the analysis gate were considered equivocal (Table 4). It should be noted that previous studies in our lab have found that only one event (one dot within the gated area) detected by flow cytometry could be considered as a positive if confirmed by microscopy. In the present study, 15 out of the 40 samples considered as equivocal by flow cytometry, were confirmed as positives by microscopy (data not shown).

In the case of *Cryptosporidium* oocysts, 36 (25.2 %) were positive by flow cytometry (Table 4). Samples having only 1 to 9 events were considered equivocal, again in order to avoid false-positives. Out of 143 samples, 51 were equivocal (Table 4), of which only five were confirmed by microscopy (data not shown). The dual parameter histograms for representative positive cattle samples for both *Cryptosporidium* and *Giardia*, along with their respective autofluorescence controls are shown in Figures 8 and 9. The fact that autofluorescent debris had no influence on the results, is indicated by the lack of particles in the pre-determined gate in the autofluorescence histograms (Figures 8 and 9).

McNemar's test was performed to test marginal homogeneity of the counts with IMS and without IMS by gene site. When the sum of the discordant counts were less than or equal to 10, an exact test based on the binomial distribution with $p=0.5$ was performed. A comparison of the PCR method for *Giardia* with and without IMS, showed a difference for the 18S rRNA ($p<0.01$) and *gdh* ($p<0.01$) sites.

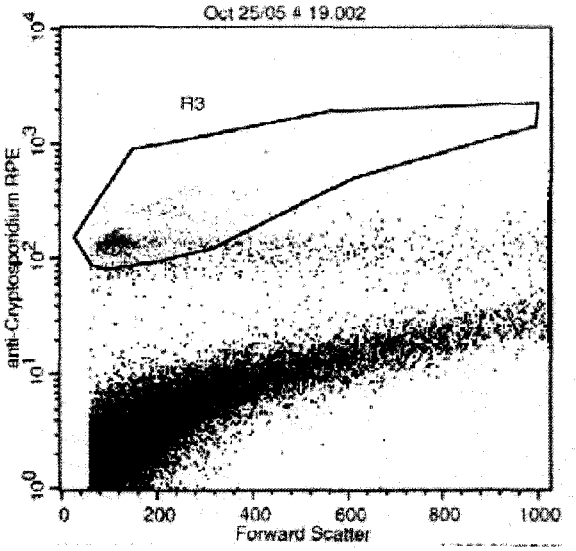
Table 4. Summary of flow cytometry results for *Giardia* cysts and *Cryptosporidium* oocysts in cattle faecal samples[a]

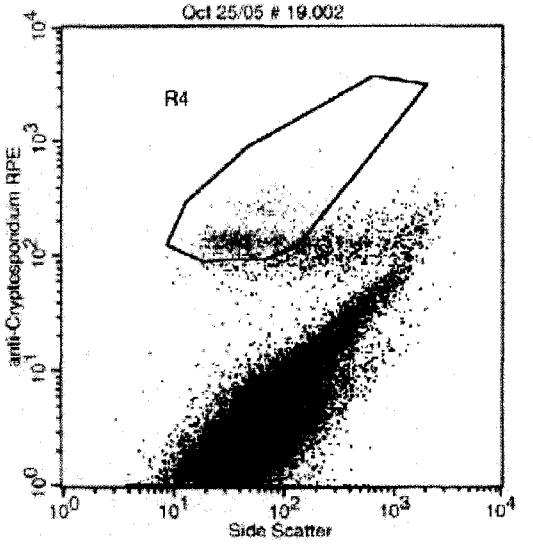
Number of gated events	*Giardia* *n* (%)	*Cryptosporidium* *n* (%)
0 (negative)	92 (64.3)	56 (39.2)
1-5, and 1-9 (equivocal)	40 (28)	51 (35.7)
≥ 6 or 10 (positive)	6 (4.2)	9 (6.3)
>100 (strong positive)	4 (2.8)	15 (10.5)
>1000 (very strong positive)	1 (0.7)	12 (8.4)
Total positive	11 (7.7)	36 (25.2)

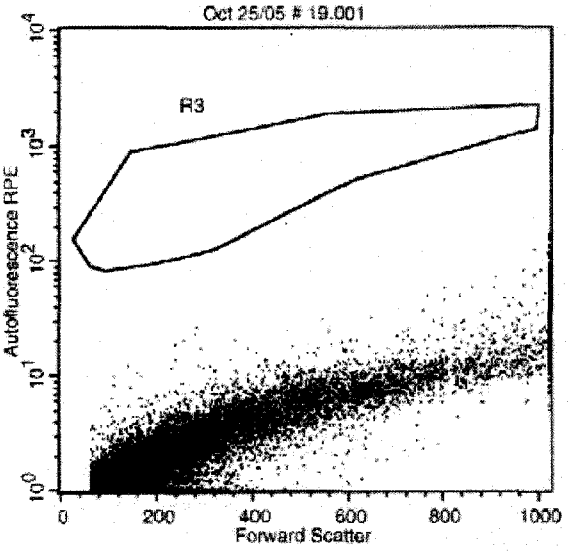
[a] A total of 143 samples were analyzed at least once at a dilution of 1:5; 100,000 total particles were analyzed per sample.

Figure 8. Dual parameter dot-plots of a representative cattle faecal suspension generated by immunofluorescence flow cytometry for *Cryptosporidium*

Positive cattle faecal sample was stained with anti-*Cryptosporidium* R-PE. Gated regions shown represent gates set for counting and sorting of particles (forward and side scatter) in positive samples (along with their representative autofluorescence controls).


Oct 25/05 # 19.002
R3
anti-Cryptosporidium RPE
Forward Scatter


Oct 25/05 # 19.002
R4
anti-Cryptosporidium RPE
Side Scatter


Oct 25/05 # 19.001
R3
Autofluorescence RPE
Forward Scatter

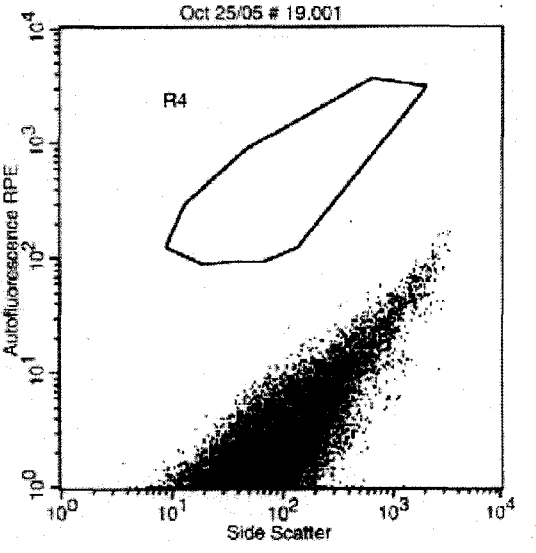
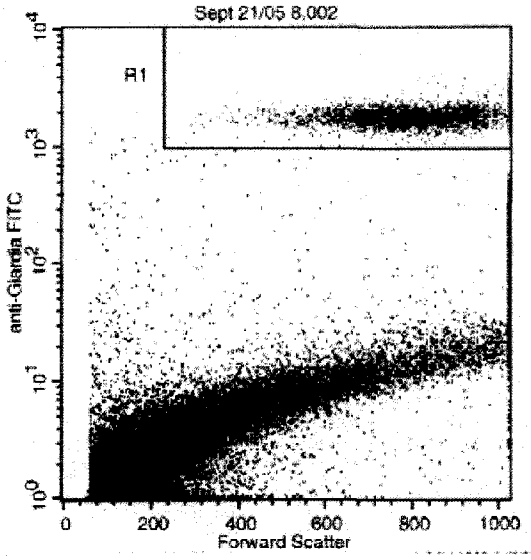
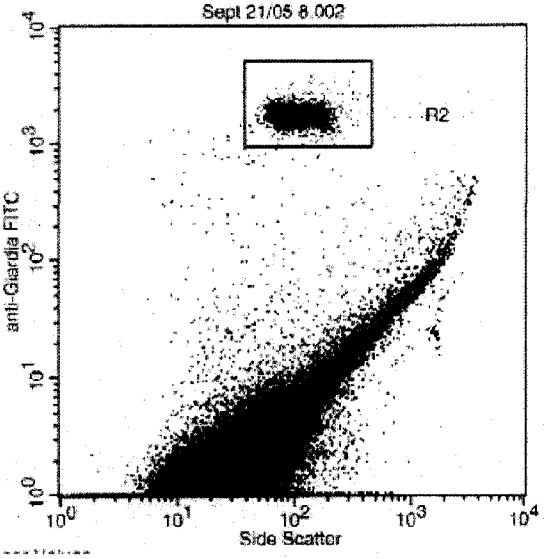
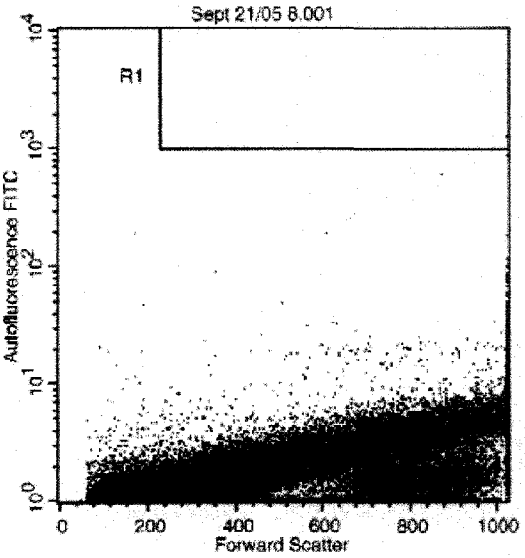

Oct 25/05 # 19.001
R4
Autofluorescence RPE
Side Scatter

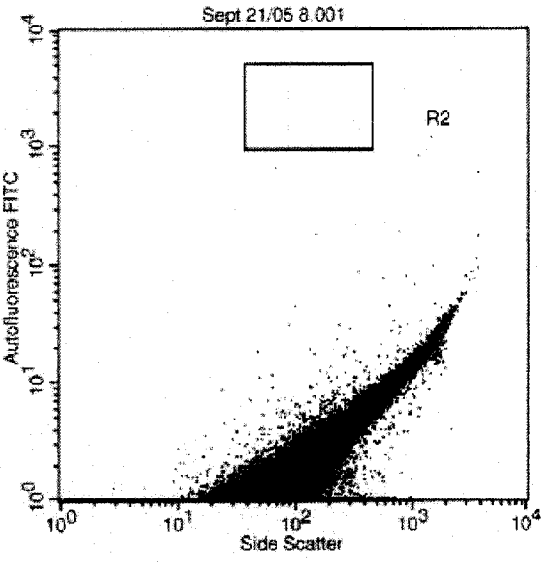
Figure 9. Dual parameter dot-plots of a representative cattle faecal suspension generated by immunofluorescence flow cytometry for *Giardia*

Positive cattle faecal sample was stained with anti-*Giardia* FITC. Gated regions shown represent gates set for counting and sorting of particles (forward and side scatter) in positive samples (along with their representative autofluorescence controls).


Sept 21/05 8.002
R1
anti-Giardia FITC
Forward Scatter
0
200
400
600
800
1000


Sept 21/05 8.002
R2
anti-Giardia FITC
Side Scatter


Sept 21/05 8.001
R1
Autofluorescence FITC
Forward Scatter
0
200
400
600
800
1000


Sept 21/05 8.001
R2
Autofluorescence FITC
Side Scatter

In the case of *Cryptosporidium*, the IMS-PCR and PCR method alone resulted in 43 (30.1 %) and 42 (29.4 %) positives, respectively, when using primers directed against the 18S rRNA gene (Table 3). The greatest number of positive results was obtained when using primers directed against the HSP-70 gene following IMS (60 or 42 % positive). PCR without IMS gave 58 (40.6 %) positive samples. COWP was the third gene analyzed for *Cryptosporidium*, with 56 (39.2 %) and 45 (31.5 %) positives in the IMS-PCR assay and PCR method alone, respectively (Table 3). When the McNemar's test was performed at p=0.5, the comparison of the PCR method for *Cryptosporidium* with IMS versus without IMS showed a difference for only the COWP site (p=0.02).

4 PREVALENCE of GENOTYPES of *Giardia* and *Cryptosporidium* FOUND in DAIRY CATTLE in ONTARIO and PRINCE EDWARD ISLAND

4.1 Introduction

A number of studies on the identification of *Giardia* and *Cryptosporidium* in cattle have been published based on (oo)cyst morphology (Quilez et al., 1996; Uga et al., 2000; Wade et al., 2000; Castro-Hermida et al., 2002a,b), as well as immunofluorescence microscopy (Xiao et al., 1993; Xiao and Herd, 1994; Olson et al., 1997a,b; O'Handley et al., 1999; Fayer et al., 2000b; Sischo et al., 2000; Ralston et al., 2003; Sturdee et al., 2003). All of them show evidence of *Giardia* and *Cryptosporidium* infection, but neither method can differentiate the species or genotypes of *Cryptosporidium*. In the case of *Giardia,* different species of *Giardia* cysts can be differentiated using microscopy.

In addition, these methods cannot confirm if a risk for human infections exists even though (oo)cysts are present (Fayer et al., 2000a; Egyed et al., 2003; Monis and Thompson, 2003). Molecular characterization techniques help not only in detecting but also in identifying *Giardia* and *Cryptosporidium* genotypes and species. In the case of *Giardia*, Assemblages A and B represent genotypes with the widest host range, i.e., humans and a number of animals (Table 1), including reports of Assemblage A being isolated from cattle (O'Handley et al., 2000; van Keulen et al., 2002; Appelbee et al., 2003). Assemblage B has been detected in humans and in a wide range of other mammalian hosts such as muskrat, beavers, rabbits and cattle (Sulaiman et al., 2003; Lalle et al., 2005). Assemblages C and D have been reported only in dogs, Assemblage E only in hoofed livestock, while Assemblages F and G have only been reported in cats and rats, respectively (Monis et al., 2003). Few studies have been done on examining the presence of *Giardia* in cattle. In western Canada,

two studies (O'Handley et al., 2000; Appelbee et al., 2003) found Assemblage E to be the predominant genotype in cattle, but the presence of Assemblage A was also noted. Assemblage A has also been reported from cattle in New York state (van Keulen et al., 2002). A larger study conducted in pre-weaned dairy calves in seven states in the eastern U.S. (Trout et al., 2004), demonstrated that while the majority of the calves were infected with Assemblage E, 7 out of 14 farms also contained Assemblage A. In another multi-state prevalence study done by the same group with post-weaned dairy calves (Trout et al., 2005), very similar findings were reported. Both Assemblage A and E were again present.

Cryptosporidium bovis, a recently named species, formerly known as *Cryptosporidium* genotype bovine B (Fayer et al., 2005), is one of three species of *Cryptosporidium* that infect cattle (Table 5). The other two are *Cryptosporidium parvum* and *Cryptosporidium andersoni* (formerly known as *Cryptosporidium muris*; Lindsay et al., 2000). The small intestine of pre-weaned calves as well as humans and other animals is the preferred site for *C. parvum*, which often causes diarrheal disease (Morgan et al., 1999c; Fayer et al., 2000a; Santin et al., 2004). On the other hand, *C. andersoni* usually causes asymptomatic infections, but its presence in cattle is characterized by decreased milk production (Olson et al., 1997a; Lindsay et al., 2000). Its site of infection is the abomasum of juvenile and mature cattle. *C. bovis* was also found to cause asymptomatic infections, mostly among 2 to 11 month-old dairy calves (Santin et al., 2004). Finally, a fourth unknown species of *Cryptosporidium*, known as the deer-like genotype, has been isolated from both pre-weaned and 2 to 11 month-old calves, and appears to cause only asymptomatic infections (Santin et al., 2004).

Table 5. Current *Cryptosporidium* species with their major hosts

Species	Major host	Reference
C. hominis	Humans, monkeys	Morgan-Ryan et al., 2002
C. parvum	Cattle, other ruminants, humans	Tyzzer, 1912
C. bovis	Cattle	Fayer et al., 2005
C. andersoni	Cattle	Lindsay et al., 2000
C. muris	Rodents	Tyzzer, 1907
C. suis	Pigs	Ryan et al., 2004
C. felis	Cats	Iseki, 1979
C. canis	Dogs	Fayer et al., 2001
C. wrairi	Guinea pigs	Vetterling et al., 1971
C. bailey	Poultry	Current et al., 1986
C. meleagridis	Turkeys, humans	Slavin, 1955
C. galli	Finches, chicken	Revised by Ryan et al., 2003b
C. serpentis	Reptiles	Levine, 1980
C. saurophilum	Lizard	Koudela and Modry, 1998
C. molnari	Fish	Alvarez-Pellitero and Sitja-Bobadilla, 2002

The genotypes/Assemblages with zoonotic potential from cattle include Assemblages A and B for *Giardia*, and *C. parvum* for *Cryptosporidium* infections. Thus, the extent to which those two parasites infect cattle could provide valuable information regarding potential risks for human infections. If cattle can harbor *G. duodenalis* Assemblage A and *C. parvum*, they must be considered as a potential source of human infective (oo)cysts in the environment. Therefore, this study was undertaken in order to identify the prevalence of the species and genotypes of *Giardia* and *Cryptosporidium* in calves and adult cattle on farms in Ontario and PEI.

4.2 Results

4.2.1 Prevalence and molecular characterization of *Giardia duodenalis* in cattle in Ontario and PEI

PCR positive results for the 18S rRNA gene of *Giardia* were obtained on two farms in Ontario, one farm at the AVC and 9 farms in PEI (Tables 6 and 7). The percentage of positive samples for adult dairy cattle was 28.3 % in Ontario and 27.5 % in PEI. The average prevalence for AVC and Brockville farm was 27.8 %. *G. duodenalis* Assemblage A and Assemblage E were found on both farms. Assemblage A was found in 12.0 % and 21.7 % of the samples in PEI and Ontario, respectively, while Assemblage E was recovered from 15.5 % and 6.5 % of farm samples in PEI and Ontario, respectively. PCR-positive results were obtained for 29 (27.8 %) of the 104 adult faecal specimens (Table 6). Of these 29 specimens, 12 had 100 % homology with Assemblage E (GenBank accession number: AY655701), 14 had 99 % and 3 specimens 98 % homology with Assemblage A (GenBank accession number: AY655700).

Among the dairy calves, the percentage of faecal samples that were positive for *G. duodenalis* was 32.7 % in PEI, 42.1 % in Brockville and 57.6 % in Kemptville, Ontario. The average prevalence for all the farms was 44.7 %. The percentage of strains that fell into Assemblage A positives were 26.3 % for the farm in Brockville, 30.5 % for the Kemptville College in Ontario, and 10.9 % for the PEI farms. Assemblage E isolates comprised 15.8 % and 27.1 % of the total in Ontario, and 21.8 % of the total in PEI. Out of a total of 152 calf samples analyzed by PCR, 68 (44.7 %) samples were positive (Table 7). Of these 68 samples, 33 showed 100 %, and only one sample showed 99 % homology with Assemblage E (GenBank accession number: AY655701). As for Assemblage A, only one sample had 100

% homology. Thirty samples showed 99 %, one sample had 98 %, and two samples had 97 % homology with Assemblage A (GenBank accession number: AY655700).

Table 6. The prevalence of *Giardia duodenalis* genotypes designated based on 18S rRNA sequencing in adult dairy cattle from Ontario and Prince Edward Island

Province	Farm	Number of samples	*Giardia*	*Giardia (Assemblage A)*	*Giardia (Assemblage E)*
ON	BV[a]	46	13 (28.3)[c]	10 (21.7)	3 (6.5)
PEI	AVC[b]	58	16 (27.5)	7 (12)	9 (15.5)
Total		104	29 (27.8)	17 (16.3)	12 (11.5)

[a] BV; Brockville.
[b] AVC; Atlantic Veterinary College.
[c] Number of positive samples (%).

Table 7. The prevalence of *Giardia duodenalis* genotypes designated based on 18S rRNA sequencing in dairy calves from Ontario and Prince Edward Island

Province	Farm	Number of samples	*Giardia*	*Giardia (Assemblage A)*	*Giardia (Assemblage E)*
ON	BV[a]	38	16 (42.1)[d]	10 (26.3)	6 (15.8)
ON	KV[b]	59	34 (57.6)	18 (30.5)	16 (27.1)
PEI	VF[c]	55	18 (32.7)	6 (10.9)	12 (21.8)
Total		152	68 (44.7)	34 (22.4)	34 (22.4)

[a] BV; Brockville.
[b] KV; Kemptville.
[c] VF; Various Farms in PEI (9 in total).
[d] Number of positive samples (%).

4.2.2 Prevalence and molecular characterization of *Cryptosporidium* in cattle in Ontario and PEI

PCR-positive results based on the HSP-70 gene of *Cryptosporidium* were obtained from 39 animals from two farms in Ontario, and from 8 animals from 9 farms in PEI (Table 8). The percentage of positive specimens ranged from 14.5 % in PEI to 45.8 % at the Kemptville College farm in Ontario. The average prevalence for the 11 farms was 23.7 %. *C. parvum* was found in Ontario and in PEI, while *C. bovis* was found only in Ontario. PCR positive results were obtained for 47 of 198 calf faecal specimens. Of these 47 specimens, the following from Ontario had 100 % homology with genotypes listed in GenBank: two *C. bovis* (GenBank accession number: AY741306) and 31 *C. parvum* (GenBank accession number: AY151416). Of 47 samples from PEI, seven had 100 % homology with the genotype listed in GenBank (accession number AY151416) and one sample had 99 % homology with the same accession number (AY151416).

Table 8. The prevalence of *Cryptosporidium* spp. by PCR based on HSP-70 gene sequencing in dairy cattle from Ontario and Prince Edward Island

Province	Farm	Number of samples	*Cryptosporidium*	*C. parvum*	*C. bovis*
ON	BV[a]	84	12 (14.3)[d]	11 (13.1)	1 (1.2)
ON	KV[b]	59	27 (45.8)	26 (44.1)	1 (1.7)
PEI	VF[c]	55	8 (14.5)	8 (14.5)	0
Total		198	47 (23.7)	45 (22.7)	2 (1)

[a] BV; Brockville.
[b] KV; Kemptville.
[c] VF; Various Farms in PEI (9 in total).
[d] Number of positive samples (%)

5 TEMPORAL STUDY OF THE PREVALENCE OF *GIARDIA* AND *CRYPTOSPORIDIUM* IN CALVES FROM KEMPTVILLE COLLEGE, ONTARIO

5.1 Introduction

Giardia and *Cryptosporidium* are known causative agents of diarrhea in young animals. *Giardia* infections have been reported in calves as young as 4 days of age, although they are most commonly present in calves between 5 and 10 weeks of age (Xiao and Herd, 1994; O'Handley 1999). *Cryptosporidium* infections have also been reported in calves as young as 4 days of age (Xiao and Herd, 1994), as well as in calves 7-days old (Trotz-Williams et al., 2005). *Giardia*'s high prevalence among calves has been reported in several studies in North America. In a study on two dairy farms in Ohio, the prevalence of *Giardia* among diarrheic calves ranged from 82.4 % in April 1992 to 40 % in August 1992, and positive calves ranged from 11 to 164 days in age (Xiao et al., 1993). Another study on *Giardia* and *Cryptosporidium* prevalence in calves demonstrated 100 % infection rates for both parasites (Xiao and Herd, 1994). Several studies have been done in Canada to examine the prevalence of *Giardia* and *Cryptosporidium* in dairy and beef cattle. One of them included a prevalence study for *Giardia* and *Cryptosporidium* in cattle, sheep, pigs and horses from 8 different provinces and one territory. In cattle, the overall prevalence for *Giardia* and *Cryptosporidium* was greater in calves (31 % and 15 %) as compared to (11 % and 9 %) adults (Olson et al., 1997a). O'Handley et al. (1999) in their study of dairy calves in Alberta, Canada, reported 100 % infection rates for *Giardia duodenalis* and *Cryptosporidium parvum*. In another study, faecal samples were collected from a number of beef cows on 39 farms in Ontario, and from a number of calves from 10 farms in British Columbia. The overall prevalence of *G. duodenalis*, *Cryptosporidium andersoni* (previously known as *Cryptosporidium muris*), and *C. parvum* in Ontario cows was 8.7, 10.6, and 18.4

%, respectively. In British Columbia, the overall prevalence of *G. duodenalis* and *Cryptosporidium* spp. in calves was 36 and 13 %, respectively (McAllister et al., 2005). In another recent study in southwestern Ontario, 500 dairy calves from 51 farms were sampled to determine the prevalence of *C. parvum*. The authors found *C. parvum* in 40.6 % of dairy calves aged 7 to 21 days (Trotz-Williams et al., 2005). Naturally-acquired giardiasis and cryptosporidiosis in 20 ranch-raised beef calves and their dams from birth to weaning were studied in Alberta. The peak of *Giardia* infection (i.e., 85 %) occurred at 5 weeks of age, and then decreased to 21 % at 25-27 weeks of age. In contrast, only one calf (5 %) shed *Cryptosporidium* oocysts during this study (Ralston et al., 2003).

In light of these findings, the purpose of this study was to determine the prevalence and pattern of shedding of *Giardia* and *Cryptosporidium* in dairy calves housed in Kemptville College, Ontario.

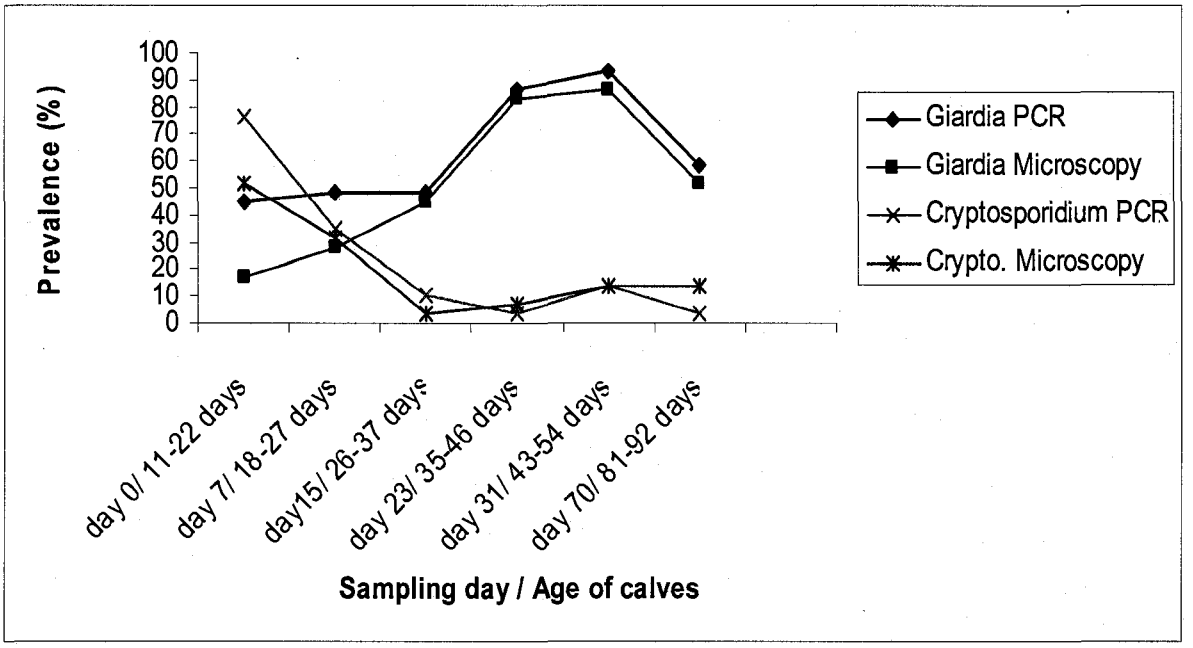
5.2 Results

All 29 calves shed *Giardia* cysts and *Cryptosporidium* oocysts at some time during the study. The age of the calves at which *Giardia* cysts and *Cryptosporidium* oocysts were first detected was 12 days. The calves entered the study at various ages ranging from 11-22 days. More specifically, the entry times into the study were: 1x11, 3x12, 2x13, 1x15, 9x16, 1x18, 3x19, 5x20, 1x21, and 2 at 22 days of age.

For *Giardia,* during sampling on day zero (calves ranged from 11-22 days old), 13 of the 29 calves (44.8 %) were positive based on PCR of a fragment of the 18S rRNA gene, and five (17.2 %) were positive by microscopy (Figure 10). The number of *Cryptosporidium*

Figure 10. The prevalence of calves shedding *Giardia* cysts and *Cryptosporidium* oocysts in relation to sampling day

Results were obtained using microscopy and PCR of a fragment of the 18S rRNA gene of *Giardia* and *Cryptosporidium*.


Prevalence (%)
100
90
80
70
60
50
40
30
20
10
0
day 0/ 11-22 days
day 7/ 18-27 days
day15/ 26-37 days
day 23/ 35-46 days
day 31/ 43-54 days
day 70/ 81-92 days
Sampling day / Age of calves
Giardia PCR
Giardia Microscopy
Cryptosporidium PCR
Crypto. Microscopy

positives was 22 (76 %) based on PCR of a fragment of the 18S rRNA gene, and 15 (51.7 %) samples were positive by microscopy (Figure 10).

The percentage of calves shedding *Giardia* cysts rose steadily with time and reached 93.1 % on sampling day 31 (Figure 10). After 43 to 54 days of age, the prevalence of *Giardia* cysts in dairy calves began to decrease, but they were still shedding cysts at the end of the study (day 70). The prevalence of *Cryptosporidium* oocysts in faeces was highest when the calves were between 11 and 22 days of age, and then decreased and remained low for the duration of the study (Figure 10).

The results of the (oo)cyst enumerations indicated that individually, animals shed from zero to more than 1000 *Giardia* cysts and *Cryptosporidium* oocysts per gram of faecal matter during this temporal study (Table 9). On sampling day zero, only two (6.9 %) calves shed more than 1000 *Giardia* cysts, while in the case of *Cryptosporidium* 14 animals (48.3 %) shed more than 1000 oocysts per gram of faecal matter (Figures 11 and 12 and Appendix III). Out of 29 calves, four (13.8 %) shed both *Giardia* and *Cryptosporidium* on sampling day zero. During sampling days 7 to 31, it was noted that the number of *Giardia* cysts shed per gram of faeces had increased, while the numbers of *Cryptosporidium* oocysts shed per gram of faeces decreased (Figures 11 and 12 Appendix III).

With respect to repeated measurements of the presence/concentration of *Giardia* and *Cryptosporidium* in the stool samples, statistical methods describing the onset of infection (Weibull distribution) and persistence of infection (Pearson chi-square test) were used. The Weibull distribution estimated that on average in the present study, on or before 40 days, 90 % of calves will start shedding (oo)cysts, i.e., a calf has a 90 % chance of starting to shed (oo)cysts on or before 40 days of age. As sampling times were at fixed intervals, no data on

Table 9. The presence and numbers of *Giardia* cysts and *Cryptosporidium* oocysts detected in 174 faecal samples collected from 29 calves on six different sampling days at Kemptville College, Ontario

Number of cysts and oocysts (detected per gram of faeces)	*Giardia* *n (%)*	*Cryptosporidium* *n (%)*
0	84 (48.3)	142 (81.6)
1-50	12 (6.9)	13 (7.5)
50-300	21 (12.1)	1 (0.6)
300-1000	14 (8)	2 (1.1)
> 1000[a]	42 (24.1)	16 (9.2)

[a] Includes animals that shed 100,000 *Cryptosporidium* oocysts and 25,890 *Giardia* cysts per gram of faeces

Figure 11. The numbers of *Giardia* cysts shed by dairy cattle

On six different sampling days 174 faecal samples were collected from 29 calves at Kemptville College, Ontario

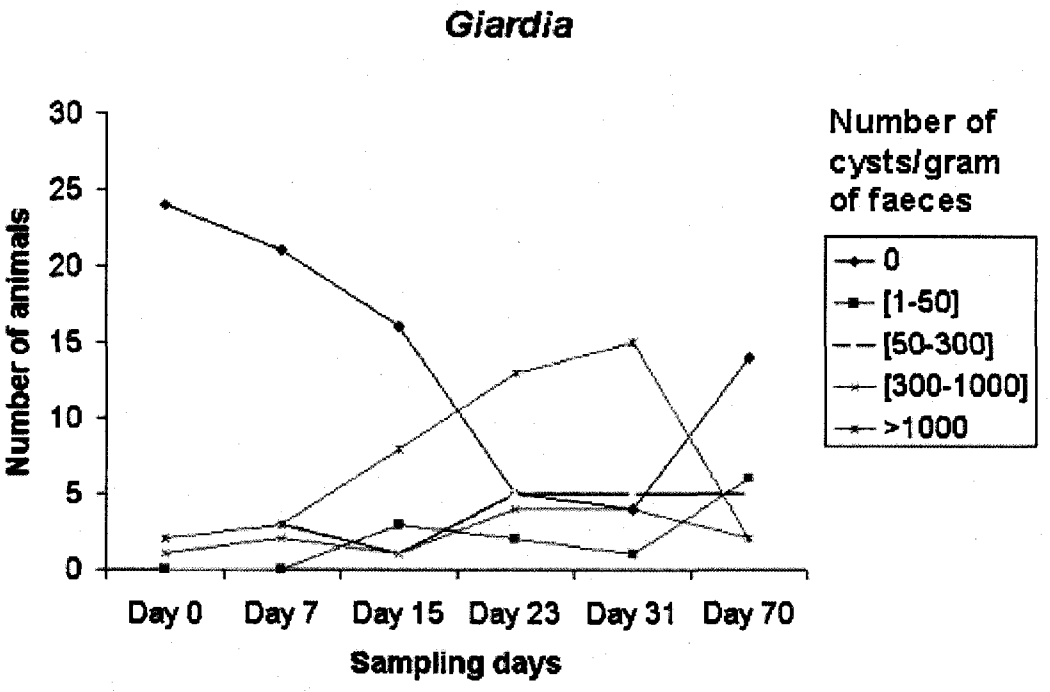
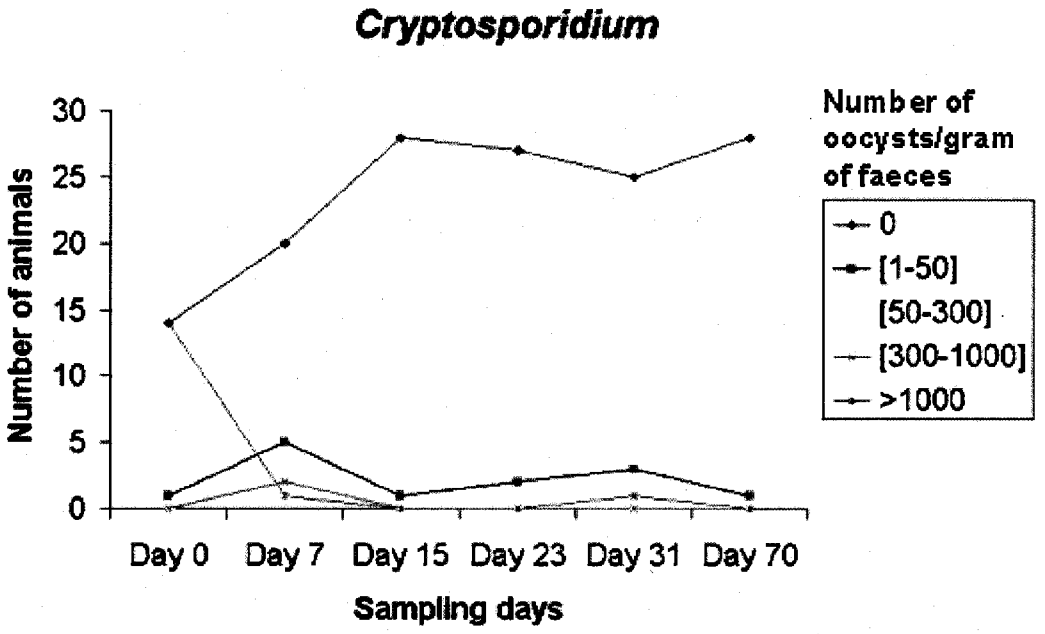

Giardia
Number of animals
30
25
20
15
10
5
0
Day 0
Day 7
Day 15
Day 23
Day 31
Day 70
Sampling days
Number of cysts/gram of faeces
0
[1-50]
[50-300]
[300-1000]
>1000

Figure 12. The numbers of *Cryptosporidium* oocysts shed by dairy cattle

On six different sampling days 174 faecal samples were collected from 29 calves at Kemptville College, Ontario


Cryptosporidium
Number of animals
30
25
20
15
10
5
0
Day 0
Day 7
Day 15
Day 23
Day 31
Day 70
Sampling days
Number of oocysts/gram of faeces
0
[1-50]
[50-300]
[300-1000]
>1000

the presence of *Giardia* cysts or *Cryptosporidium* oocysts in calves were available between collection days, and the estimation procedure took this into account. This is illustrated in Figure 13, which gives the upper and lower cumulative distribution functions (CDF) corresponding to the upper and lower intervals for the data, along with an estimated curve. The Table which accompanies the graph shows the cumulative probability that infection will be indicated at one week intervals (Table 10). As mentioned, the chi-square test was used to measure the "steady-state" persistence of *Giardia* in calves by the proportion of infected calves on the final sampling day, which was 52 % (Table 11). In the case of *Cryptosporidium*, after about 3 weeks, cyst shedding was rare and sporadic. When all measurements were combined (before and after 22 days), estimated infection rates for *Cryptosporidium* were $p_{before} = 0.543$ (19/35), and $p_{after} = 0.094$ (13/139). A 95 % confidence interval for the difference in proportions is (0.259, 0.6390) which does not contain 0, and therefore indicates rejection of the null hypothesis of equality.

Figure 13. Statistical analysis of the onset of *Giardia* infection

The estimation procedure was done using the Weibull distribution, which gives the upper and lower empirical cumulative distribution functions (CDF) corresponding to the upper and lower intervals for the data, along with the estimated curve.

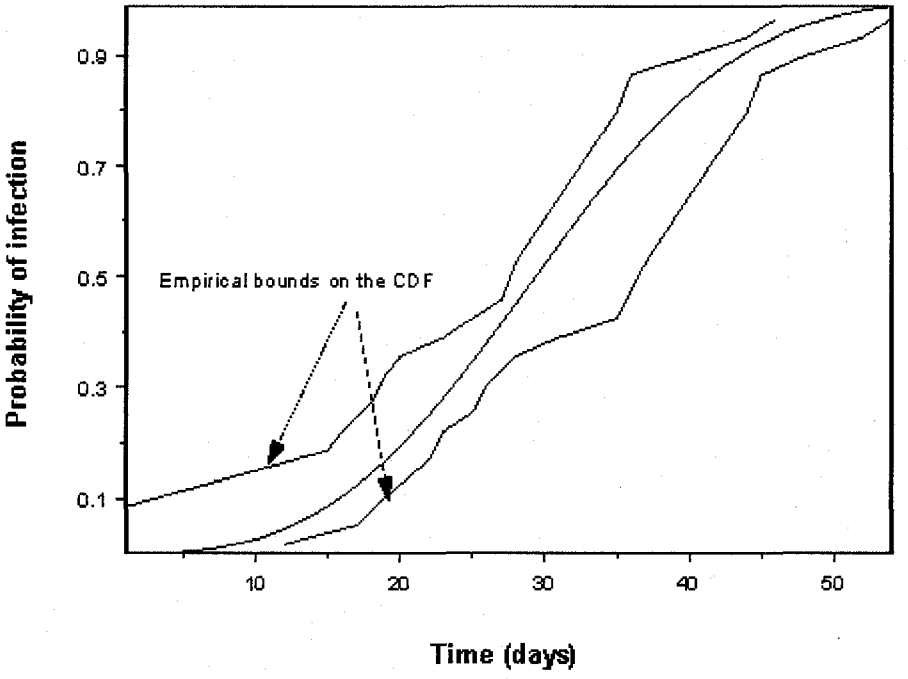

Probability of infection
0.9
0.7
0.5
0.3
0.1
Empirical bounds on the CDF
10
20
30
40
50
Time (days)

Table 10. Probability that *Giardia* cysts will occur on or before a given day, based on the maximum likelihood estimate

Time (days) until cysts will occur/age of animals	7	14	28	31	35	42	49
Probability	0.01	0.09	0.28	0.55	0.79	0.93	0.99

Table 11. Five-sample test for equality of proportions without continuity correction[a]

Difference (in days) between the last sampling day and initial detection of *Giardia* cysts	38[b]	46	55	63	70[c]	Overall
Total number of positive animals in each measurement group	3	13	4	4	5	29
Number of animals shedding cysts on the final sampling day	3	5	2	2	3	15
% of animals shedding cysts on the final sampling day	100	38.5	50	50	60	52

[a] Statistical analysis was based on the chi-square test which was used in order to measure the "steady state" persistence of *Giardia* in calves by the proportion of calves shedding cysts on the final sampling day. (X-square = 3.8624, df = 4, p-value = 0.4249)
[b] 38 represents time difference (in days) in between sampling day 70 and sampling day 31.
[c] 70 represents time difference (in days) in between sampling day 70 and sampling day 0.

6 DISCUSSION

6.1 Method development and prevalence of *Giardia* and *Cryptosporidium* based on a comparison of microscopy, flow cytometry and PCR

In this study, following sucrose flotation, the first (oo)cyst concentration step, a combination of IMS, as a second concentration step, followed by PCR was compared with conventional PCR for the detection of *Giardia* and *Cryptosporidium* in cattle faecal samples. In addition, a comparison of three different detection methods (microscopy, flow cytometry, and PCR), was done in terms of their ability to detect the presence of *Giardia* and *Cryptospordium* in dairy cattle stool samples.

The detection of (oo)cysts by microscopy was done using immunofluorescence staining with fluorescein-labeled anti-*Giardia* and anti-*Cryptosporidium* antibodies. This made (oo)cyst identification among faecal debris easier, even though this technique was still time-consuming, especially if only a few or no (oo)cysts were present on the slide. On the other hand, the use of flow cytometry was rapid and effective, providing analysis of many more samples in a day, and allowing more consistent and reliable results as viewer fatigue was not a factor. Overall, whenever a large number of (oo)cysts was present, they were not difficult to detect, no matter what method was used.

In our preliminary study on cattle stool samples from PEI, it was recognized that in order to maximize the sensitivity of any molecular detection method, a second concentration step (IMS) would be helpful prior to PCR, because *Giardia* or *Cryptosporidium* may be present in low numbers in adults or intermittently shed in the faeces. The results obtained in the present study showed that the sensitivity of PCR in detecting (oo)cysts was significantly higher when IMS was used as a second concentration step. For *Giardia*, comparisons of the

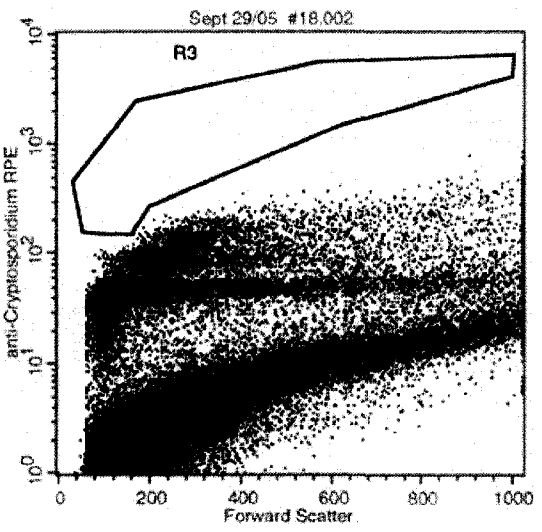
PCR method with IMS versus PCR without IMS (using McNemar's test with p=0.5) showed a significant difference for the fragments of the 18S rRNA ($p<0.01$) and *gdh* ($p<0.01$) genes, while for *Cryptosporidium* a difference was observed only for the fragment of the COWP gene ($p=0.02$). The use of IMS resulted in improved detection of both *Cryptosporidium* oocysts and *Giardia* cysts per gram of faecal matter when tests were done to determine the sensitivity of the PCR method (Figures 6 and 7). This was most likely due to the removal by IMS of PCR inhibitors in the faecal debris.

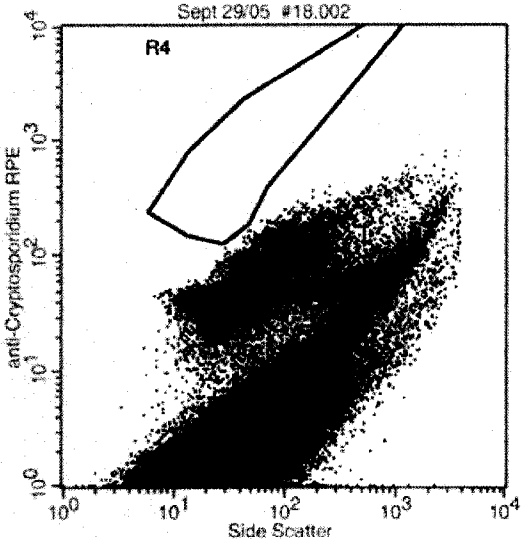
For the detection of *Cryptosporidium*, it is apparent from the results that flow cytometry resulted in more positives as compared to microscopy, as 36 samples (25.2 %) were found positive by flow cytometry, while 30 (21 %) were positive by microscopy. Samples having only 1 to 9 events in the analysis gate were considered equivocal in terms of the presence or absence of *Cryptosporidium* oocysts. It is possible that some of those samples were positive as well. The degree of confidence in reporting positive results is related to the stringency used in the number of gated events required for a positive result.

When calf samples from Kemptville College were analyzed, flow cytometer dot plots of *Cryptosporidium* oocysts showed a cluster of "probable" oocysts outside the gated area (Figure 14). On the other hand, microscopy results of the same samples were showing large numbers of oocysts. This discrepancy could have been due to i) the low pH value of the stool sample which was interfering with R-phycoerythrin-labelled (R-PE) anti-*Cryptosporidium* monoclonal antibody; or ii) due to insufficient monoclonal antibodies to bind oocysts, causing a shifting of the cluster outside the gated area. When efforts were made to try different concentrations of R-PE monoclonal antibody, as well as different dilutions of the original stool samples, the oocysts became properly aligned within the gate. Different

Figure 14. Flow cytometry dot plots of *Cryptosporidium parvum* oocysts

The cluster of probable oocysts is outside the gated area due to the thickness of the sample (i.e., sample had too much debris).


Sept 29/05 #18.002
R3
anti-Cryptosporidium RPE
Forward Scatter
0
200
400
600
800
1000


Sept 29/05 #18.002
R4
anti-Cryptosporidium RPE
Side Scatter

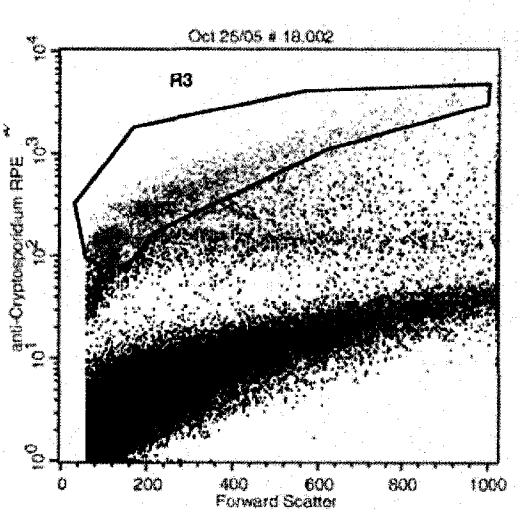
concentrations of R-PE monoclonal antibody did not affect the results, while a 1:10 dilution of the samples used for flow analysis was optimal (Figure 15).

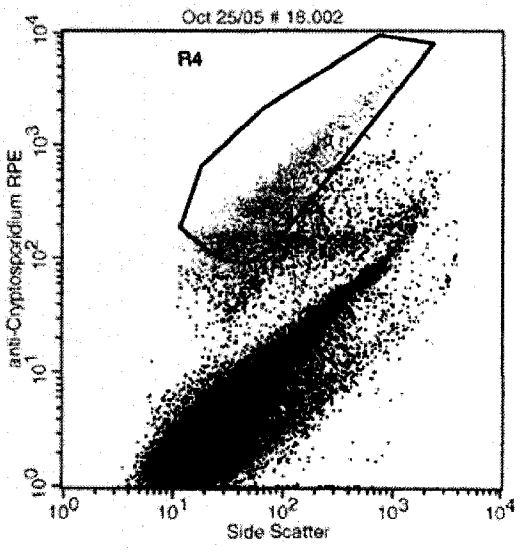
Surprisingly, the number of *Giardia* positive samples detected by flow cytometry was lower than that obtained when using microscopy (Table 4). Only 11 samples (7.7 %) were positive by flow cytometry, while 36 (25.2 %) samples were positive by microscopy. Equivocal results were obtained for 40 (28 %) samples, of which 15 were confirmed by microscopy. The other 25 samples that were considered equivocal by flow cytometry most likely contained *Giardia* cysts. However, as previously mentioned, in order to avoid false-positive results, we opted for a more conservative approach.

A number of studies have demonstrated the advantages of using flow cytometry and cell sorting in conjunction with immunofluorescence for the detection and enumeration of *Giardia* cysts and *Cryptosporidium* oocysts in water (Vesey et al., 1993; Vesey et. al., 1994a, b; Ferrari et al., 2006) and faecal samples (Arrowood et al., 1995; Dixon et al., 1997; Valdez et al., 1997; Cole et al., 1999; Moss and Arrowood, 2001; Power et al., 2003), and *G. duodenalis* cyst suspensions (Erlandsen et al., 1988). A flow cytometric method for the quantification of oocysts in stool specimens from SCID mice demonstrated that the flow cytometry method was approximately 10 times more sensitive than conventional immunofluorescence assays (Arrowood et al., 1995). Valdez et al. (1997), used human stool samples seeded with known concentrations of oocysts, which were collected and passaged in calves, for flow cytometry analysis. Their findings demonstrated that flow cytometry provides a reproducible and sensitive method for *C. parvum* oocyst detection. Another study compared acid-fast staining, an immunofluorescent antibody technique and flow cytometry; flow cytometry was found to be the most sensitive method for oocyst detection (Cole et al., 1999).

Figure 15. Flow cytometry dot plots of *Cryptosporidium parvum* oocysts following sample dilution

The original sample from Figure 14 was used for flow analysis. After making a 1:10 sample dilution, the cluster of *C. parvum* oocysts is within the gated area.


Oct 25/05 # 18.002
R3
anti-Cryptosporidium RPE
Forward Scatter
0
200
400
600
800
1000


Oct 25/05 # 18.002
R4
anti-Cryptosporidium RPE
Side Scatter

Power et al. (2003) did an evaluation study of a combined immunomagnetic separation/flow cytometry technique and formol-ether concentration for detection of *Cryptosporidium* oocysts in domestic and native Australian animals. They used different faecal types, including bovine, spiked with *Cryptosporidium* oocysts to test the IMS/flow cytometry recovery rates. Their findings again demonstrated flow cytometry to be more sensitive than formol-ether concentration in detecting oocysts.

Our study is in agreement with the published literature on the sensitivity of flow cytometry for the detection of *C. parvum* oocysts. The difference in literature results for the detection of *Giardia* cysts by flow cytometry, as compared to previous study done by our laboratory (Dixon et al., 1997), could be due to the fact that different types of faecal samples were analyzed in the various studies. Elimination of large debris particles that might clog the instruments is very important in flow cytometry (Valdez et al., 1997). Therefore, it can be speculated that the high fiber content of bovine faecal samples may have limited the sensitivity of flow cytometry, since temporary disruptions of flow by large faecal particles can decrease the number of events observed. Our conservative approach resulted in lower numbers of *Giardia* positive samples by flow cytometry. It is important to note that microscopy, as mentioned, was done only once per sample, except in cases where results obtained by flow cytometry were equivocal. In the cases where large numbers of samples showed equivocal results for *Giardia* cysts, microscopy was repeated. As a result, the number of positive samples increased, suggesting that if we did microscopy more than once on flow cytometry samples, we would probably have found even more positives. Finally, a study on flow cytometry analysis of faecal samples from beavers (Dixon et al., 1997) is the only one published in the scientific literature on *Giardia* cysts in faecal samples. More

research needs to be done in this area, as it is likely that faecal samples from different animal species would give variable results when analyzed by flow cytometry.

Using molecular methods, we were able to detect the presence of *Giardia* and *Cryptosporidium* in dairy cattle from both Brockville and Kemptville sites. Approximately 45 % (using PCR of the 18S rRNA gene) and 39.2 % (when targeting the COWP gene) of the total cattle faecal samples analyzed were positive for *Giardia* and *Cryptosporidium*, respectively, by the IMS-PCR assay. Three different genes were used for the detection of *Cryptosporidium* oocysts (18S rRNA, HSP-70 and COWP) and *Giardia* cysts (18S rRNA, *β-giardin* and *gdh*) in cattle samples. In addition to highly conserved target genes, such as 18S rRNA and HSP-70, which have been widely used as diagnostic markers, we also selected highly variable genes or genes that were unique to *Giardia* (the gene encoding giardin and GDH), and *Cryptosporidium* (COWP). As genetic markers differ in their information content, to detect species, highly conserved coding regions should be analyzed. On the other hand, identification of genotypes and subtypes requires more discriminatory fingerprinting techniques (Caccio et al., 2005). This may help to explain why some primers directed against certain gene loci are more sensitive than others in the detection of *Giardia* and *Cryptosporidium*. The objective of this part of the study where we used three different gene loci for *Giardia* and *Cryptosporidium* detection was to determine which PCR target would be the most sensitive and give us the greatest number of positive results. As demonstrated in this study, the use of multiple genetic markers, both conserved and variable, increased the sensitivity of detection.

Significantly, the use of the PCR-IMS assay with primers directed against the COWP gene was more sensitive than the PCR-IMS with the other primers used for *Cryptosporidium*

detection. For *Giardia*, after using IMS, the most sensitive PCR assay was the one using primers directed against the 18S rRNA gene. The use of primers directed against the *gdh* gene in the PCR assay following IMS incorporation was also significantly greater in sensitivity, as compared to methodology using primers against *β-giardin*. The present study demonstrated that IMS improved the results obtained by PCR alone, especially for the detection of *Giardia*. IMS was not only capable of concentrating more cysts, thus likely allowing for more DNA to be extracted, but it probably also removed PCR inhibitors from the samples. Overall, most animals harbouring *Cryptosporidium* were very young. The presence of *Cryptosporidium* oocysts among calves is very common (Bednarska et al., 1998; de la Fuente et al., 1999; Becher et al., 2004; Santin et al., 2004; Fayer et al., 2006; Geurden et al., 2006; Hamnes et al., 2006; Kvac et al., 2006; Singh et al., 2006), and present usually in high numbers. This is likely the reason why the PCR method alone was effective, even without using IMS. In fact, the actual number of cattle infected with both *Giardia* and *Cryptosporidium* was most likely underestimated, due to the fact that the animals were sampled only once. If a specimen was found to be negative during a period when an animal was intermittently shedding (oo)cysts, the animal would be considered negative. It is known that both *Giardia* and *Cryptosporidium* are shed intermittently in stool samples and in some instances because of intermittent or low levels of shedding, it is necessary to examine more than three stool samples (Gardner and Hill, 2001).

In conclusion, in the present study, the comparison of three different methods for *Giardia* and *Cryptosporidium* detection demonstrated that the PCR assay was much more sensitive than microscopy or flow cytometry. The difference between the microscopy and PCR results could have been due to the fact that with few exceptions, samples were only examined once by microscopy. If a sample was found to be positive both by PCR and flow

cytometry, microscopy was repeated; microscopy was important in confirming positive results. Faecal shedding of *Giardia* and *Cryptosporidium* spp. is often sporadic and in low numbers, which can make microscopy difficult (Elliot et al., 1999). However, the major disadvantage of microscopy is that it is time consuming because only small amounts of sample can be analyzed per slide. Viewer fatigue also plays an important role in reporting either false-negatives, or just simply missing the organisms of interest (Dixon et al., 1997).

In this study, flow cytometry showed a lower sensitivity for *Giardia* cysts, as compared to a previous study done in our laboratory (Dixon et al., 1997). This could be due to the fact that previous studies were done on other animals (beavers), or that we opted for a more conservative approach and raised the number of cysts and oocysts needed for a sample to be considered positive. As mentioned, microscopy was generally done only once per sample, so not all samples reported as equivocal could be confirmed by microscopy. Therefore, these might be considered a presumptive positive only. However, flow cytometry allowed more samples to be analyzed in a day. A disadvantage of the latter method is that the equipment is expensive.

Overall, PCR proved to be the best method for detecting cysts/oocysts because it was the most sensitive, specific and easiest to use. The use of the IMS method most likely removed inhibitors present in the faecal matter, such as bilirubin, bile salts, and complex polysaccharides (Widjojoatmodjo et al., 1992; Monteiro et al., 1997). In contrast to an earlier study where immunomagnetic separation was done on seeded faeces from a limited number of cattle (Webster et al., 1996), the PCR-IMS method was used on a larger number of samples. The main disadvantage of the PCR-IMS method is that it is costly and time-consuming.

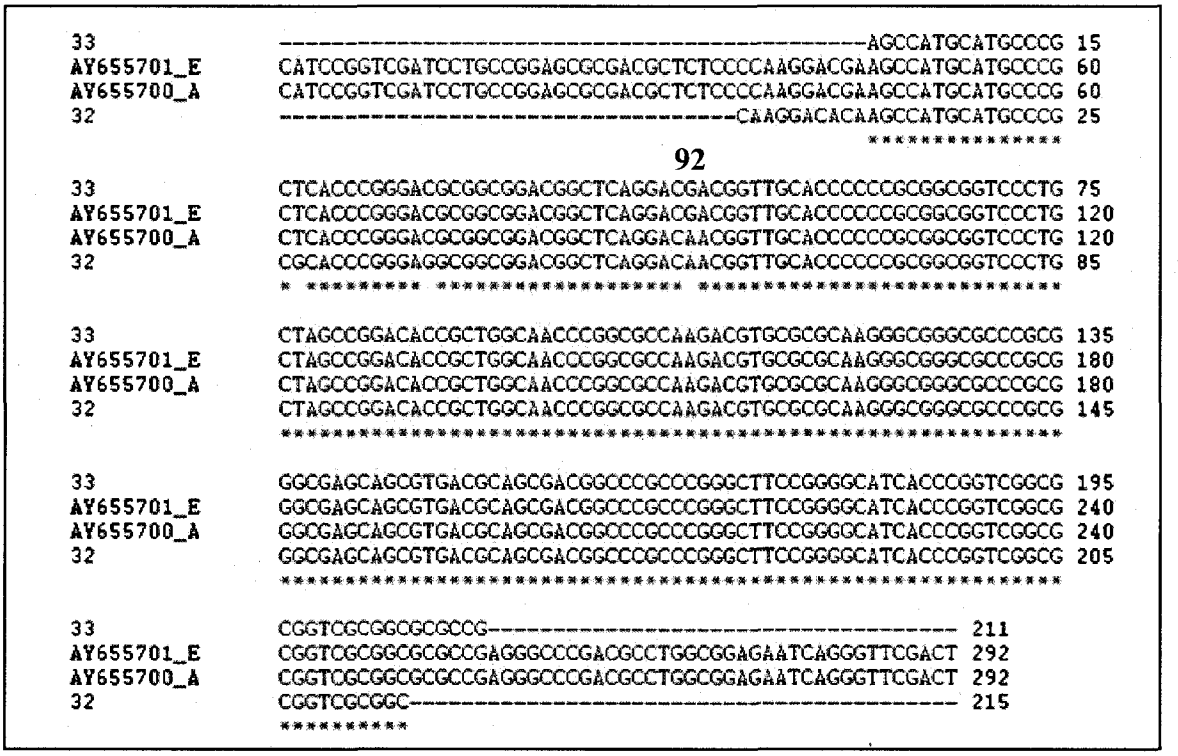
6.2 Prevalence of genotypes of *Giardia* and *Cryptosporidium* found in dairy cattle in Ontario and PEI

Giardia and *Cryptosporidium* were found on all the farms tested in Ontario and PEI. Approximately 38 % of all specimens analyzed by PCR were found to be positive for *Giardia*, while 24 % were positive for *Cryptosporidium*.

Giardia cysts were found both in calves and in adult cattle in Ontario and PEI. Sequence analysis based on the 18S rRNA gene (Figure 16) demonstrated the presence of both Assemblages A and E in dairy cattle samples. PCR targets (18S rRNA gene) were chosen based on the fact that i) they have been successfully used in epidemiological and taxonomic studies and ii) the use of these sequences have been validated in a multi-centre study (Smith et al., 2006). When adult cattle samples were analyzed, the average prevalence was approximately 28 %. Only a few studies have been done so far on faecal samples from adult cattle (Fayer et al., 2000b; Gow and Waldner, 2006), but no molecular characterization was done in these studies. A prevalence study of *Cryptosporidium* and *Giardia* infections was done in post-weaned and adult cattle on three farms in Maryland, U.S. (Fayer et al., 2000b). Out of a total of 24 cows analyzed at a research dairy farm, three were positive for *Cryptosporidium andersoni*, while no *Giardia* cysts were found. On the second, commercial dairy farm, from 19 cows examined, *C. parvum* and *G. duodenalis* were both found in two, cows. On the third, research farm, of the specimens examined from 118 beef cattle, *C. parvum* and *G. duodenalis* were found in 34 and 44 animals, respectively. The other study on adult cattle faecal samples was done in western Canada (Gow and Waldner, 2006). The authors reported collection of fresh faecal samples from 560 beef cows from 59 farms. Only 1.1 % of the cows were positive for *Cryptosporidium* spp., while *Giardia* spp were detected

Figure 16. Multiple alignment of the reference and two representative sample sequences

Reference sequences and two representative sample (32 and 33) sequences, showing one nucleotide difference between Assemblage A and Assemblage E at base-pair position 92 of the 18S rRNA gene of *Giardia duodenalis*. CLUSTAL W (1.82) software was used for sequence alignment.


33 ----------AGCCATGCATGCCCG 15
AY655701_E CATCCGGTCGATCCTGCCGGAGCGCGACGCTCTCCCCAAGGACGAAGCCATGCATGCCCG 60
AY655700_A CATCCGGTCGATCCTGCCGGAGCGCGACGCTCTCCCCAAGGACGAAGCCATGCATGCCCG 60
32 ----------CAAGGACACAAGCCATGCATGCCCG 25
****
92
33 CTCACCCGGGACGCGGCGGACGGCTCAGGACGACGGTTGCACCCCCCGCGGCGGTCCCTG 75
AY655701_E CTCACCCGGGACGCGGCGGACGGCTCAGGACGACGGTTGCACCCCCCGCGGCGGTCCCTG 120
AY655700_A CTCACCCGGGACGCGGCGGACGGCTCAGGACAACGGTTGCACCCCCCGCGGCGGTCCCTG 120
32 CGCACCCGGGAGGCGGCGGACGGCTCAGGACAACGGTTGCACCCCCCGCGGCGGTCCCTG 85
* ********* ********** **********
33 CTAGCCGGACACCGCTGGCAACCCGGCGCCAAGACGTGCGCGCAAGGGCGGGCGCCCGCG 135
AY655701_E CTAGCCGGACACCGCTGGCAACCCGGCGCCAAGACGTGCGCGCAAGGGCGGGCGCCCGCG 180
AY655700_A CTAGCCGGACACCGCTGGCAACCCGGCGCCAAGACGTGCGCGCAAGGGCGGGCGCCCGCG 180
32 CTAGCCGGACACCGCTGGCAACCCGGCGCCAAGACGTGCGCGCAAGGGCGGGCGCCCGCG 145
****
33 GGCGAGCAGCGTGACGCAGCGACGGCCCGCCCGGGCTTCCGGGGCATCACCCGGTCGGCG 195
AY655701_E GGCGAGCAGCGTGACGCAGCGACGGCCCGCCCGGGCTTCCGGGGCATCACCCGGTCGGCG 240
AY655700_A GGCGAGCAGCGTGACGCAGCGACGGCCCGCCCGGGCTTCCGGGGCATCACCCGGTCGGCG 240
32 GGCGAGCAGCGTGACGCAGCGACGGCCCGCCCGGGCTTCCGGGGCATCACCCGGTCGGCG 205
****
33 CGGTCGCGGCGCGCCG---------- 211
AY655701_E CGGTCGCGGCGCGCCGAGGGCCCGACGCCTGGCGGAGAATCAGGGTTCGACT 292
AY655700_A CGGTCGCGGCGCGCCGAGGGCCCGACGCCTGGCGGAGAATCAGGGTTCGACT 292
32 CGGTCGCGGC---------- 215
****

in 17 % of the cow faecal samples.

Many prevalence studies of *Giardia* infection in cattle have reported a significant percentage of *Giardia*-infected animals (Xiao and Herd, 1994; Olson et al, 1997a,b; Ruest et al., 1998; O'Handley et al., 1999; Ralston et al., 2003). Genotyping studies demonstrated that Assemblage E was a dominant genotype in Australian dairy cattle and Canadian dairy and beef cattle (O'Handley et al., 2000; Appelbee et al., 2003). However, Assemblage A was found in a smaller percentage in Canada, Australia and the Netherlands (O'Handley et al., 2000; Huetnik et al., 2001; Appelbee et al., 2003). In the U.S., an analysis of three bovine specimens from New York demonstrated the presence of Assemblage A in cattle (van Keulen et al., 2002). Both Assemblages A and E were found in two multi-state studies, on pre-weaned, less than two months old , and post-weaned calves, two to 12-month old calves in the eastern U.S. (Trout et al., 2004, 2005). The prevalence of Assemblage E ranged from 85 to 87 %, of pre- and post-weaned calves, respectively, while Assemblage A was present in 15 and 13 % of pre- and post-weaned calves, respectively (Trout et al., 2004, 2005). The authors reported significant farm-to-farm variation in *Giardia* Assemblages, with Assemblage E being present in animals on all farms, but not Assemblage A. The percentage of isolates belonging to Assemblage A ranged from 0 % on 7 of 14 farms, to 45 % on a farm in New York, in pre-weaned calves (Trout et al., 2004). In post-weaned calves, Assemblage A ranged from not being present on five of 14 farms to a high of 67 % on a Maryland farm (Trout et al., 2005). In a recent study on the prevalence and genotypes of *G. duodenalis* in 1 to 2 year-old dairy cattle the prevalence of Assemblage A and E varied greatly from farm-to-farm, with four farms having exclusively Assemblage E *Giardia*. However, 1 to 2 year-old heifers on 10 of 14 farms did have Assemblage A *Giardia*, with one farm in North Carolina

having 50 % Assemblage A and 50 % Assemblage E isolates, respectively (Trout et al., 2006).

In the current study, the zoonotic Assemblage A and livestock Assemblage E, were both present in Ontario and PEI (Tables 6 and 7). According to the results it can be concluded that adult cattle in Ontario, as well as in PEI, could pose a risk to humans. The data presented here for PEI is part of the study done by our laboratory and the AVC collaborators in PEI (Uehlinger et al., 2006).

There was a higher prevalence of *Giardia* Assemblage A (28.9 %) in Ontario as compared to PEI (10.9 %), while the prevalence of Assemblage E was almost the same in both regions. In Ontario, the prevalence of Assemblage A was higher in both adults and calves as compared to Assemblage E. However, in PEI a higher prevalence of Assemblage E in both adults and calves as compared to Assemblage A was found (Tables 6 and 7). These results are somewhat different from published results in the literature. In a preliminary study done on adult cattle at the AVC by our collaborators in PEI, Assemblage A was found to be more prevalent than Assemblage E (data not shown). Therefore, findings in our study are comparable to what has been reported elsewhere. The present study on cattle faecal samples is the first study on the prevalence of *Giardia* genotypes in Ontario. The prevalence of *Giardia* and *Cryptosporidium* in beef cows in southern Ontario is the only study published so far in the literature, but in that study, molecular characterization was not done (McAllister et al., 2005). Based on the results published in the literature so far, and on the results obtained in the current study, it can be speculated that zoonotic *Giardia* Assemblage A is present in calves, as well as in adult cattle, and should be considered as a potential source of human infectious *Giardia* cysts in the environment. In the multi-state studies examining the presence of *Giardia* cysts in cattle in the U.S., it was found that Assemblage A can be

present on a particular farm one year and not the next or, not present one year and then present the next year (Trout et al., 2004, 2005). In the present study, only 5 (3.5 %) of the animals from Kemptville College had both *Giardia* and *Cryptosporidium* infections. Sequence analysis based on the HSP-70 gene demonstrated that no animals were infected with more than one species of *Cryptosporidium*. The same criterium as for *Giardia* was followed for *Cryptosporidium* sequencing. PCR targets (HSP-70 gene) were chosen based on the fact that they were successfully used in epidemiological or taxonomic studies and the use of the sequences were validated in a multi-centre study (Smith et al., 2006). *C. bovis* was only found in Ontario dairy cattle and in only 2 (1 %) of the 198 animals examined. *C. bovis* has recently been added to a growing list of *Cryptosporidium* species (Fayer et al., 2005). Its presence has been documented in the eastern U.S. where a multi-state study was done on 1 to 2 year-old dairy cattle (Fayer et al., 2006). The majority of the specimens analyzed in the present study had *C. parvum* infection, which is known to be zoonotic (Morgan et al., 1999c). *C. parvum* was detected in 46 (23.2 %) of the 198 calves examined. This was almost the only species found in calves 2 weeks of age and it constituted all positive specimens associated with all pre-weaned calves.

It should be noted that at the farm in Brockville, animals were confined to large barns with cement floors fully covered by a roof. Removal of the faecal material from the barn where cows were housed was automated. Calves were housed in individual pens with hay bedding. A similar situation was observed at the AVC in PEI, where cows were also confined to large pens with cement floors and with automated faecal removal. On one working farm that we visited in PEI, cows were housed in a barn with cement floor and hay bedding. All faecal removal was done manually. On this farm, calves were housed on the upper floor of the barn, on wooden floors covered with hay bedding. The wooden floors had

gaps. Thus, there was an increased potential for loose watery stools from calves to pass through the hay bedding to the lower floor of the barn, and, in this manner, enhance the spread of *Giardia* cysts and *Cryptosporidium* oocysts. At the Kemptville College, calves were housed on the ground in hutches that have been used for many years. Hence, it is possible that (oo)cysts were still present in the environment and may have been transferred by shedding to other calves, via personnel and students who were working with the calves.

In conclusion, we found that the prevalence of both zoonotic genotypes for *Giardia* and *Cryptosporidium* is high among dairy cattle in both Ontario and PEI. Therefore, livestock may pose a risk to animal handlers, as they may come into contact with *Giardia* cysts and *Cryptosporidium* oocysts via direct handling. Animal handlers and livestock can also be vectors in the contamination of the food and water supply. In order to determine the importance of cattle in the transmission of *G. duodenalis* and *C. parvum*, further work is warranted. Collection of faecal samples from staff and students at Kemptville College and AVC, or animal handlers at the farms, would be beneficial in providing better insight on possible transmission dynamics of *Giardia* and *Cryptosporidium*.

6.3 Temporal study of the prevalence of *Giardia* and *Cryptosporidium* in calves from Kemptville College, Ontario

During this temporal study, both *Giardia* and *Cryptosporidium* were detected in Kemptville College calves. When the study started, the youngest animal was 11 days old, and was free of both parasites. Both *Giardia* and *Cryptosporidium* have been found in calves as young as 4 days old (Xiao and Herd, 1994; Joachim et al., 2003; Sturdee et al., 2003). In the present study during sampling on day zero, a 12 day-old calf started shedding both *Giardia* cysts and *Cryptosporidium* oocysts. The percentage of calves shedding *Giardia* cysts increased as they grew older. The highest prevalence of *Giardia* infections among calves was seen on day 31 of sampling (calves were 43 to 54 days old). When the calves were 90 days-old, (sampling day 70), the prevalence of *Giardia* cysts began to decrease, but 15 out of 29 (51.7 %) calves were still shedding cysts. These findings are similar to previously published studies that found *Giardia* infections in calves to be at their highest prevalence between five and 10 weeks of age (Xiao et al., 1993; Xiao and Herd, 1994; O'Handley et al., 1999). In our study, *Giardia* infections were present beyond 10 weeks of age among Kemptville calves. Other studies have reported the highest prevalence of *Giardia* infection in calves between three and four months of age (Olson et al., 1997b; Huetnik et al., 2001; Trout et al., 2005; Hamnes et al., 2006).

In the present study, the highest *Cryptosporidium* oocyst counts in calf faecal samples was recorded on sampling day 0, when animals were 11 to 22 days old. This is in agreement with the findings of Olson et al. (1997b), who found the highest *Cryptosporidium* oocyst counts between zero and two weeks of age in animals in Canada. In another study in North America, *C. parvum* was recovered from calves younger than 30 days of age (Wade et al., 2000). A similar situation has been reported in other parts of the world. In the Netherlands,

Huetnik et al. (2001) found the highest oocysts counts in animals who were between 9 and 29-days old. In France, a survey was done which involved two age groups of calves, i.e., four to 12-day-old and four to 21-day-old calves. Both age groups tested positive for *Cryptosporidium* oocysts (Lefay et al., 2000). A study conducted on calves in three areas in Norway, found *Cryptosporidium* oocysts to be present in the age group of two to three months (Hamnes et al., 2006). A prevalence study of *Cryptosporidium* oocysts in dairy calves in India demonstrated that oocysts peaked between zero and 30 days of age (Singh et al., 2006). Similarly, in Japan, Uga et al. (2000) reported the greatest number of positive calves at 15 days of age, followed by a declining trend to a rate of approximately 10 % on day 24. The intensity of *Cryptosporidium* infection in calves appeared to decline with age in the Norway study as well, but the trend was not statistically significant (Hamnes et al., 2006). This is in agreement with our study, where the prevalence of *Cryptosporidium* declined during sampling days 15 and 23, and then, after a small increase on day 31, a drop in the number of positive calves occurred again on sampling day 70, when calves were 81 to 92 days old (Figure 10).

The high infection rate in the present study suggests that both parasites are easily transmitted among calves in this region. Figures 11 and 12 show a positive correlation between (oo)cysts per gram of faeces and prevalence, i.e., for *Giardia*, as the prevalence increases, the number of cysts per gram of faeces increases. Similar results were observed for *Cryptosporidium* (Figure 10). There is a negative correlation between the animals with zero cysts per gram and those with more than 1000 cysts per gram of faeces, while the moderate shedders (1-50, 50-300, 300-1000) are fairly stable over time.

Calves entered the College after they received colostrum from their dams. In a few cases, colostrum came with the calf and it was given upon arrival. However, measurement of

total serum solids, as an assessment of passive immunity (IgG) transfer, was done at the College and it showed that many calves (more than 50 %) did not receive enough colostrum, received poor quality colostrum or did not receive colostrum early enough. While receiving the first milk from their mothers, the calves could have possibly contracted *Giardia* and *Cryptosporidium* from the dams' udders and/or the surroundings. One of the important aspects in future studies would be a follow up of the *Giardia* and *Cryptosporidium* (oo)cyst status in dams in the maternity pens. Fayer et al. (1998) reported a pre-patent period (the interval between infection of a host by a parasitic organism and the first ability to detect from that host a diagnostic stage of the organism) for *C. parvum* infection of 8 days. In the present study, if the dams were infected and were a routine source of infection, a different pattern of infection might have been seen with many more calves being positive in the first few days of their lives. Naciri et al. (1999) tested calves for *Cryptosporidium* from suckling herds (calves who stay with their mothers) and dairy herds (calves removed shortly after birth), and reported that 90 % of calves from suckling herds demonstrated infection by five days of age. A high prevalence of 59 % occurred at a later age in calves from dairy herds, supporting the idea of contamination from sources other than, or in addition to, the dams (Naciri et al., 1999). Even though calves were housed in individual hutches at Kemptville College, and did not have direct contact with neighbouring animals, parasites could have been transmitted to the calves as a result of the transmission of infective cysts and oocysts from animal-to-animal, by dairy workers and students. Some other mechanical vectors involved in the transmission of *Giardia* cysts and *Cryptosporidium* oocysts can include wild mice (Klesius et al., 1986) or in the summer, house flies (Graczyk et al., 1999). It is also possible that the (oo)cysts could have been spread from one hutch to another during runoff following a heavy rainfall in the summer. The chances of spreading infections to the calves through the water or

feed were also high, as all the water and milk containers were washed together without any sterilization or thorough cleaning. The hutches may have also been contaminated by previous calves, despite cleaning efforts. The hutch area of the Kemptville College has been used for many years, and is often populated with different animals. As many as 10^4 cysts or 10^5 oocysts were shed per gram of faeces (Table 9), and *Giardia* cysts and *Cryptosporidium* oocysts are extremely resistant to the environmental conditions and are capable of remaining infective for weeks after being passed in faeces. Research done by Olson et al. (1999) examining the susceptibility of *Giardia* cysts and *Cryptosporidium* oocysts to environmental stresses, showed that temperatures as low as -4°C can inactivate *Giardia* cysts in water, while *Cryptosporidium* oocysts can remain viable for more than 12 weeks at these temperatures. *Giardia* cysts and *Cryptosporidium* oocysts were infective for 11 weeks and greater than 12 weeks, respectively, in water at 4°C. At 25°C, in water, *Giardia* cysts were infective for two and *Cryptosporidium* oocysts for up to 10 weeks (Olson et al., 1999). The same study reported that *Giardia* cysts were non-infective after 7 days at -4°C, but that *Cryptosporidium* survived for more than 12 weeks in soil. Again, at 25°C, *Giardia* cysts lasted for one week in soil while *Cryptosporidium* oocysts were infective for 4 weeks. In manure, *Giardia* cysts in cattle faeces were non-infective within one week at -4°C and were infective for only one week at 4 and 25°C, while *Cryptosporidium* oocysts were infective for more than 12 weeks at -4°C, 8 weeks at 4°C, and four weeks at 25°C (Olson et al., 1999).

It can be speculated that all of the above mentioned factors provide ideal conditions for the transmission of *Giardia* and *Cryptosporidium* between calves, and to humans and other animals. During the final sampling (day 70), calves were housed in the barns in groups of four animals. The calves were in close confinement, and transmission of *Giardia* and *Cryptosporidium* was probably facilitated even more at the time, but a change in prevalence

was not immediately seen. In fact, faecal shedding of both *Giardia* and *Cryptosporidium* was in decline, but as all the calves were destined for the market, it was impossible to follow them up for a longer period of time.

On six sampling days, measurements of the presence/concentration of *Giardia* cysts and *Cryptosporidium* oocysts, showed two clear trends in the *Giardia* data which may be useful in describing the infection dynamics. Firstly, the time at which infection was first detected varied considerably. However, by 54 days of age, all calves were shedding *Giardia* cysts at least once. Secondly, *Giardia* cysts persisted in all 29 calves at the end of the study period (day 70), but at a lower rate. Data were recorded at fixed intervals and no information was collected between those collection times. As a result, if a sample was collected at 54 days of age and was positive, but the previous sample collected at 46 days was not, we cannot say with certainty what happened in between days 46 and 54. Such data are referred to as interval censored. The fact that a sample was negative on day 46 and positive on day 54 tells us that *Giardia* was present sometime in the interval, but not exactly when. The estimation procedure took this into account, and, on average, estimated that on or before day 40, 90 % of calves will be shedding *Giardia* cysts, i.e., a calf has a 90 % chance of becoming infected on or before 40 days of age. The statistical analysis for the persistence of *Giardia* cysts showed that the proportion of infected calves on the final sampling day was 52 %. Since the initial time of detection varied among calves, it was useful to test if the time of infection influenced the probability that a calf would shed *Giardia* cysts at the end of the study. To test this, the difference (in days) between the time of the last measurement and the time at which the first *Giardia* cysts were observed was calculated. There were only five distinct time differences, 38 (difference in days between sampling day 70 and sampling day 31), 46 (between sampling day 70 and 23), 55 (between sampling day 70 and 15), 63

(between sampling day 70 and 7), and 70 days (between sampling day 70 and 0). The statistical analysis using the chi-square test showed that there were no statistically significant differences between the five groups. It is interesting to note that the three calves were still shedding *Giardia* cysts after 38 days (Table 11). It was, in fact, difficult to establish a trend or prediction for the timing and duration of faecal shedding in the calves in our study.

In the case of *Cryptosporidium*, the pattern of presence or absence of oocysts was different from that of *Giardia*, and, after about three weeks, infection was rare and sporadic. The result appeared somewhat dependent on the cut-off day, and, after initial infection of the calves at an early age, *Cryptosporidium* oocysts in faecal samples were found only sporadically and did not persist.

Our results are similar to those of other investigators, who found that calves usually become infected with *Cryptosporidium* between one and four weeks of age. In contrast, the infection pattern for *Giardia* is different. *Giardia* cysts are often first observed at the same time as *Cryptosporidium* oocysts, but while *Cryptosporidium* infections are in decline, *Giardia* infections usually continue for greater than six months (O'Handley et al., 1999).

Overall, our results suggest that both *Giardia* and *Cryptosporidium* shed by calves can probably infect humans, through direct or indirect contact. Thorough cleaning of hutches and barns as well as a low density of animals, would likely reduce the level of (oo)cysts in the environment. As suggested by Ruest et al. (1998), based on their experience at the Veterinary School Hospital in Saint-Hyacinthe, Quebec, flame sterilizing the premises is the only totally effective means of eliminating these protozoans. Therefore, to protect themselves from infection and from becoming vectors of infection, it is strongly recommended that adequate care be taken by individuals involved with the husbandry of calves.

7 CONCLUSIONS

The present study demonstrated that as compared with microscopy and flow cytometry, PCR combined with IMS was the most sensitive method for detecting *Giardia* and *Cryptosporidium*. This method has a good potential to be used for the detection of *Giardia* and *Cryptosporidium* (oo)cysts in food and stool samples, in which both *Giardia* and *Cryptosporidium* are usually present in very low numbers, or PCR inhibitors are numerous.

A temporal study of prevalence of *Giardia* and *Cryptosporidium* demonstrated that both *Giardia* and *Cryptosporidium* are present in young cattle. These findings are significant in that they point to this young group of animals as being a major source of two water and foodborne pathogens, namely, *Giardia* and *Cryptosporidium*.

Furthermore, this study demonstrated the presence of zoonotic *Giardia* Assemblage A and *Cryptosporidium parvum* among both adult dairy cattle and calves.

The presence of zoonotic *Giardia* and *Cryptosporidium* indicates that more analytical studies are needed to identify the factors leading to the transmission of these protozoans, to humans. It is well known that animals shedding *Giardia* cysts and *Cryptosporidium* oocysts, pastured or housed close to human drinking water supplies could be a source of giardiasis and cryptosporidiosis, as water is generally considered to be a major vector for cryptosporidiosis (Fayer, 2004). Increased contamination of surface waters by agricultural animal waste might be expected during periods of heavy rainfall or snow melt down. Contamination of surface waters contributes to the contamination of food such as fruits and vegetables irrigated with these waters (Figure 17). The use of (oo)cyst-contaminated faeces, farmyard manure and slurry as a fertilizer for crop cultivation are other possible sources of

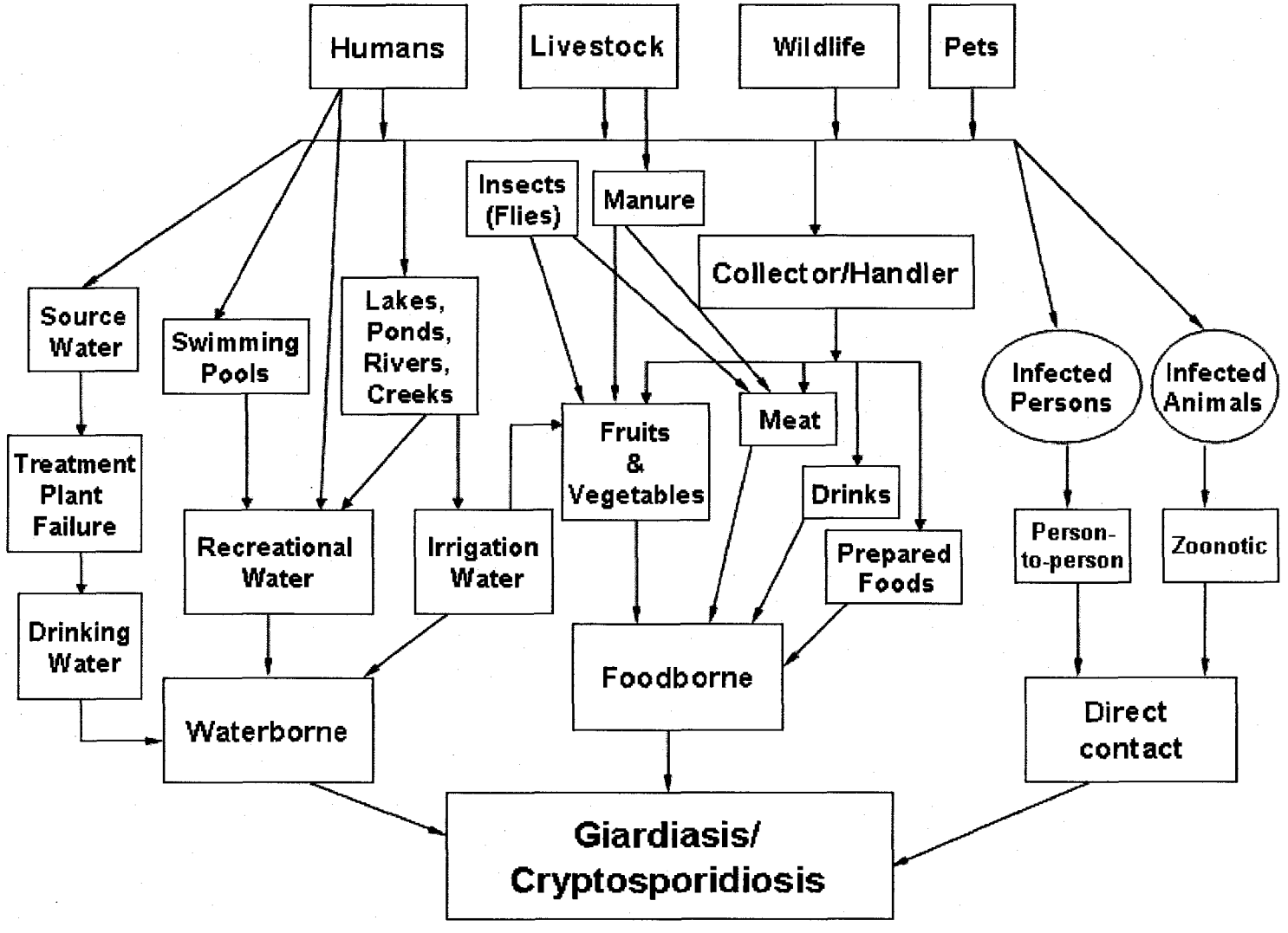
food contamination with *Giardia* cysts and *Cryptosporidium* oocysts (Smith et al., 2006). Additional sources of food contamination could include coprophagous transport hosts such as house flies, (Graczyk et al., 1999), washing fruits and vegetables in contaminated waters, or contamination and cross-contamination of meat from the gut contents of animals during slaughter (Kaneta and Nakai, 1998).

Aside from waterborne and foodborne *Giardia* and *Cryptosporidium* infections, some other possible transmission routes include direct contact with farm animals, close contact between humans, poor personal hygiene of food handlers, human activities such as discharging sewage effluent into rivers and lakes, close contact with companion animals which have long been considered as potential sources for both parasites, as well as the presence of *Giardia* and *Cryptosporidium* in wildlife (Figure 17).

Given the numerous transmission routes and the low infectious doses for humans of *Giardia* and *Cryptosporidium*, studies of this kind are important in clarifying potential routes of transmission, and may be useful in estimating the risk to human health associated with these pathogens. Further studies such as geographical location including data on temperature, rainfall and soil type, herd size and management practices related to husbandry are all important in trying to further understand the epidemiology of *Giardia* and *Cryptosporidium*, and are necessary to give us a better understanding of the impact of (oo)cyst contamination on public health.

Figure 17. Model illustrating possible transmission pathways of *Giardia* cysts and *Cryptosporidium* oocysts.

In red, possible modes of *Giardia* cyst and *Cryptosporidium* oocyst transmission as partially implied or demonstrated in the present study.


Humans
Livestock
Wildlife
Pets
Insects (Flies)
Manure
Collector/Handler
Source Water
Swimming Pools
Lakes, Ponds, Rivers, Creeks
Infected Persons
Infected Animals
Fruits & Vegetables
Meat
Drinks
Treatment Plant Failure
Recreational Water
Irrigation Water
Prepared Foods
Person-to-person
Zoonotic
Drinking Water
Foodborne
Waterborne
Direct contact
Giardiasis/ Cryptosporidiosis

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APPENDIX I

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Fig 1 from Ortega YR, Adam RD, "Giardia: overview and update" CLIN INFECT DIS 1997;25(3):545-550.

To appear in: a Master's thesis by Tatjana Coklin for a degree at the University of Ottawa, Canada.

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APPENDIX II

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The proposed title for my thesis is Detection and molecular characterization of Giardia and Cryptosporidium in livestock.

It will be published by University of Ottawa, Ottawa, Canada.

The number of copies of the thesis is five (one for the University's Library, one for the Department, two for my two supervisors and one for me).

APPENDIX III

The presence and numbers of *Giardia* cysts and *Cryptosporidium* oocysts in 174 faecal samples collected from 29 calves on six different sampling days at Kemptville College, Ontario

Sampling day 0

Number of cysts and oocysts (detected per gram of faeces)	*Giardia* *n (%)*	*Cryptosporidium* *n (%)*
0	24 (82.7%)	14 (48.3%)
1-50	0	1 (3.5%)
50-300	2 (6.9%)	0
300-1000	1 (3.5%)	0
> 1000	2 (6.9%)	14 (48.3%)

Sampling day 7

Number of cysts and oocysts (detected per gram of faeces)	*Giardia* *n (%)*	*Cryptosporidium* *n (%)*
0	21 (72.4%)	20 (69.0%)
1-50	0	5 (17.2%)
50-300	3 (10.4%)	1 (3.5%)
300-1000	2 (6.9%)	2 (6.9%)
> 1000	3 (10.4%)	1 (3.5%)

Sampling day 15

Number of cysts and oocysts (detected per gram of faeces)	*Giardia* *n (%)*	*Cryptosporidium* *n (%)*
0	16 (55.2%)	28 (96.6%)
1-50	3 (10.4%)	1 (3.5%)
50-300	1 (3.5%)	0
300-1000	1 (3.5%)	0
> 1000	8 (27.6%)	0

Sampling day 23

Number of cysts and oocysts (detected per gram of faeces)	*Giardia* *n (%)*	*Cryptosporidium* *n (%)*
0	5 (17.2%)	27 (93.1%)
1-50	2 (6.9%)	2 (6.9%)
50-300	5 (17.2%)	0
300-1000	4 (13.8%)	0
> 1000	13 (44.8%)	0

Sampling day 31

Number of cysts and oocysts (detected per gram of faeces)	*Giardia* *n (%)*	*Cryptosporidium* *n (%)*
0	4 (13.8%)	25 (86.2%)
1-50	1 (3.5%)	3 (10.4%)
50-300	5 (17.2%)	0
300-1000	4 (13.8%)	0
> 1000	15 (51.7%)	1 (3.5%)

Sampling day 70

Number of cysts and oocysts (detected per gram of faeces)	*Giardia* *n (%)*	*Cryptosporidium* *n (%)*
0	14 (48.3%)	28 (96.6%)
1-50	6 (20.7%)	1 (3.5%)
50-300	5 (17.2%)	0
300-1000	2 (6.9%)	0
> 1000	2 (6.9%)	0