INTRASPECIFIC VARIATION IN PATHOGENIC *CRYPTOSPORIDIUM PARVUM*

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Department:
Veterinary and Microbiological Sciences

March 2010

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

ABSTRACT

Herges, Grant Richard, M.S., Department of Veterinary and Microbiological Sciences, College of Agriculture, Food Systems, and Natural Resources, North Dakota State University, March 2010. Intraspecific Variation In Pathogenic *Cryptosporidium parvum*. Major Professor: Dr. John McEvoy.

Cryptosporidium causes cryptosporidiosis, an infectious diarrheal disease, which can become chronic and life-threatening in immunocompromised individuals. *Cryptosporidium parvum* and *C. hominis* are the primary causes of human cryptosporidiosis. Of these species, *C. hominis* only infects humans while *C. parvum* additionally infects ruminants, in particular neonatal calves. Therefore, understanding the transmission dynamics of *C. parvum*, particularly the specific contribution of zoonotic and anthroponotic transmission, is critical to the control of this pathogen. *Cryptosporidium parvum* genotypes have been identified which appear to be restricted to a single host, which suggests that this may be a heterogeneous species, with varying infection and transmission dynamics. The first objective to this thesis was to determine the population structure of pathogenic *C. parvum* in the upper Midwest United States. A total of 216 isolates from cases of human cryptosporidiosis in Minnesota and Wisconsin and 64 isolates from diarrheic calves in Minnesota, Wisconsin, and North Dakota were genotyped at 8 polymorphic loci. A total of 213 isolates, 167 from humans and 46 from calves, had complete multilocus types (MLT). There were 93 different MLTs and sixty of those MLTs were only represented by one isolate. Population analysis revealed a highly recombining, panmictic population that does not have any genetic, geographic, or host sub-structuring. The second objective to this thesis was to determine the variability in the *in vitro*

infectivity of different *C. parvum* strains, IOWA II and Moredun. A quantitative RT-PCR approach was used to quantify expression of target genes during infection of HCT-8 cells. Fluorescence microscopy was used to quantify life cycle stages during infection. Our data showed that the IOWA II reached its sexual stages earlier and had a greater number of trophozoites/meronts at 24 h p.i. than Moredun. The host used for propagation of *C. parvum* also affected subsequent infectivity of HCT-8 cells.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. John McEvoy, for being patient, respectful, and encouraging. Thank you for helping me to understand the difficult concepts and being willing to talk them through again when they didn't stick. I could not have completed this work without your academic guidance. Thank you to Catherine Giddings for taking care of all of the logistical details in the lab, and also for caring for each of your students like you do your own children. Your technical guidance and skills were invaluable, and you always went the extra mile to make sure things got finished. Thank you to my committee members, Dr. Neil Dyer, Dr. Margaret Khaitsa, and Dr. Mark Clark, for taking the time and effort to help me improve and complete my work. Thank you to all of the departmental staff and graduate students. You made my graduate experience rich with experiences that I will have for a lifetime. To my wife Becky, a mere thank you cannot start to explain how much you are a part of this work. Your support and love mean so much to me. To Gideon, I know you are not aware of the work that I have done in the paper, but you inspire me and I hope I make you proud. Thank you to my family for your encouragement and support even when you were not sure what my research was about. You gave me the gift of understanding the value of a hard day's work. Lastly, thank you Jesus for loving and saving me. I pray that my life will be a reflection of the great gift that you freely gave to me.

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LITERATURE REVIEW

Introduction

Cryptosporidium is a ubiquitous protozoan parasite that infects the epithelial cells of the gastrointestinal tract of many vertebrates including humans, domesticated (cattle, dogs, cats, poultry) and wild animals (O'Donoghue, 1995). Some species are pathogenic and cause cryptosporidiosis. Cryptosporidiosis is usually a self-limiting and acute diarrheal disease, but it can be chronic in immunocompromised persons. Severity and duration depends on the individual but the disease usually manifests itself in three ways: asymptomatic carriage, acute diarrhea (most common), and persistent diarrhea. Illness is mainly gastrointestinal with watery, voluminous diarrhea that usually lasts 6 to 14 days but it can be persistent in immunocompromised persons (Isaacs et al., 1985). Immunocompromised persons have also been shown to have more severe diarrhea, with up to 17 liters a day reported, which can lead to dehydration and death (Anonymous, 1982a; Anonymous, 1982b; Fayer and Ungar, 1986). Other symptoms that can accompany the severe diarrhea include malaise, vomiting, anorexia, abdominal cramps, fever, nausea, and headaches (Mac Kenzie et al., 1994). The primary health care concerns with *Cryptosporidium* are the lack of an effective drug treatment for the disease, particularly in immunocompromised persons, and the oocysts, which are the infective stage of the parasite, are highly resistant to common disinfection methods such as chlorination.

Historical Background on *Cryptosporidium*

In 1907, Ernest Edward Tyzzer identified *Cryptosporidium* in the gastric glands of common mice. He described the stages of the life cycle including its asexual and sexual phases and the oocysts being shed in the feces (Tyzzer, 1907). He proposed the genus name *Cryptosporidium* and named the specific organism he found, *Cryptosporidium muris* (Tyzzer, 1910). Tyzzer later identified *Cryptosporidium parvum* in the small intestine of experimentally infected mice (Tyzzer, 1912). There was not much interest in *Cryptosporidium* until the 1970's when researchers found that it was a cause of diarrhea in cattle and, therefore, an economic concern (Meuten et al., 1974; Panciera et al., 1971). Interest in *Cryptosporidium* increased in 1982 when the Centers for Disease Control and Prevention (CDC) published a report connecting it to severe protracted diarrhea in men with Acquired Immune Deficiency Syndrome (AIDS) (Anonymous, 1982b). An outbreak of approximately 403,000 persons in Milwaukee, Wisconsin in 1993 provided further impetus for research to understand this waterborne parasite (Mac Kenzie et al., 1994).

Life Cycle of *Cryptosporidium*

Combining *in vitro* and *in vivo* cultivation models with high resolution electron microscopy, researchers have been able to confirm and elaborate on the life cycles that Tyzzer had described in mouse intestines using a simple light microscope (Tyzzer, 1910). The life cycle has both asexual and sexual stages that occur within a single host and consist of endogenous and exogenous phases (Figure 1)(O'Donoghue, 1995).

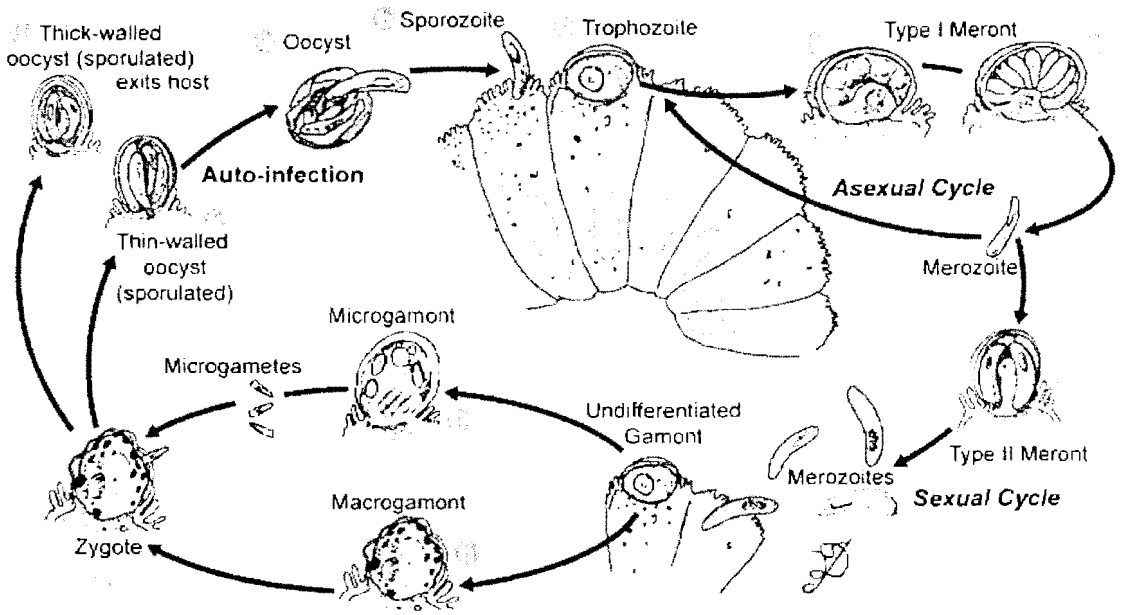

Figure 1. Outline of the *Cryptosporidium* life cycle (Centers for Disease Control and Prevention, 2005; Current and Garcia, 1991).

Ingestion and excystation

Cryptosporidium is passed as a sporulated oocyst form in the feces of an infected host. The oocyst is extremely robust which enables the parasite to survive for long periods in the environment before it is ingested by another susceptible host(Fayer and Ungar, 1986). Following ingestion, the oocyst excysts, releasing 4 sporozoites which invade intestinal epithelial cells (Figure 1a).

Attachment and invasion

Motile sporozoites target the apical surface of the host epithelial cells (Figure 1b) (Wetzel et al., 2005). The anterior ends of the sporozoites attach and change from a slender, crescent shape to an oval shape. Cytoplasmic vacuoles cluster at the anterior end and induce the formation of the pre-parasitophorous vacuole (Huang et al., 2004). As the pre-parasitophorous vacuole is forming, the vacuole membrane is pushed toward the

posterior portion of the parasite while the host cell microvilli membrane is being simultaneously pushed out. The membranes fuse together, and the host cell membrane encloses around the parasite and forms the parasitophorous vacuole. Sporozoites within the vacuole are intracellular but do not directly contact the host cell cytoplasm, thus they are extracytoplasmic (Morrissette and Sibley, 2002). An electron dense band at the base of the parasitophorous vacuole appears to connect the parasite with the host cell cytoplasm. While little is currently known about its function, the electron dense band appears to function as a feeder organelle (Huang et al., 2004). During process of internalization, the sporozoites develop into trophozoites, which are the feeding stage and are round in shape (Figure 1c).

Asexual and sexual development

Trophozoites develop into meronts when they undergo mitotic division to produce merozoites. This process is termed merogony. *Cryptosporidium parvum* has two types of meronts (O'Donoghue, 1995). Type I meronts develop six or eight merozoites, which are released from the parasitophorous vacuole when mature. Merozoites from type I meronts infect other host cells where they can become type I or type II meronts (Figure 1d-e). Type II meronts form four merozoites and, following their release, these merozoites infect host cells where they differentiate into macro- or microgamonts (sexual development)(Figure 1f-h). Microgamonts can produce 14-16 microgametes, which are similar to sperm cells (O'Donoghue, 1995). Macrogamonts have a single nucleus and are the ovum-like cell. Microgametes fertilize the macrogamonts and forming a zygote (Figure 1i). Zygotes, the only diploid stage in the life cycle, undergo meiosis (sporogony)

resulting in a sporulated oocyst that contains four (1N) sporozoites. Two types of oocysts can be produced, thin-walled and thick-walled. Thin-walled oocysts have been shown to release their sporozoites within the host and may contribute to autoinfection (Figure 1k). Thick-walled oocysts, which make up to approximately 80% of the total oocyst load, are shed in the feces and are fully infective (Figure 1j).

Methods for Propagating *Cryptosporidium*

There are significant limitations to propagating some clinically relevant *Cryptosporidium* species due to the lack of a suitable animal model. For example, *Cryptosporidium hominis* specifically infects humans in nature, but it is poorly infective for other host species. A gnotobiotic pig model has been described but it is not widely available (Widmer et al., 2000) Most *Cryptosporidium* research is carried out using *C. parvum* due to the effectiveness shown in previous studies, as it can be propagated in calves and mice with a high oocyst yield (O'Donoghue, 1995; Tzipori and Widmer, 2008). Furthermore, most research is carried out using just a few *C. parvum* strains, primarily the IOWA and Moredun isolates. This limits our understanding of intraspecific variation in *C. parvum*. This inability to continuously propagate many species of *Cryptosporidium* is a significant hurdle to furthering our understanding of this important genus (Fayer and Xiao, 2008).

In vivo amplification

The costs and resources to propagate *Cryptosporidium* strains using *in vivo* models are prohibitive for most labs. Also, long term storage of *Cryptosporidium* oocysts is not possible, necessitating regular passaging. As a result, most labs purchase their oocysts

from one of the few commercial suppliers. Many larger domestic animals including calves (Tzipori et al., 1983), lambs (Snodgrass et al., 1984; Tzipori et al., 1981a), goat kids (Tzipori et al., 1982) and piglets (Moon and Bemrick, 1981; Tzipori et al., 1981b) have been used to propagate *C. parvum* and produced large quantities of oocysts. Smaller animals such as mice are a less costly approach and are used in both research and commercial labs. Mice are most susceptible when they are <6 days old, with <100 oocysts required to establish an infection (Finch et al., 1993; Korich et al., 2000). Mice at 10 to 14 days old, shed oocysts but the infection is subclinical after which the mice recover and stop shedding (Tzipori and Widmer, 2008). Mice do not become infected with all species of *Cryptosporidium*, so the effectiveness of the model is limited (Meloni and Thompson, 1996).

In vitro development

The past twenty years have seen significant improvements in the *in vitro* culture of *Cryptosporidium*, and this approach is less expensive and easier to perform than *in vivo* propagation. In initial *in vitro* work, the asexual stages of the life cycle were obtained (Woodmansee and Pohlenz, 1984). However in 1984, human fetal lung cells (HFL), primary chicken kidney (PFK), and porcine kidney cells (PK-10) were all found to support the development of the entire *Cryptosporidium* life cycle (Current and Haynes, 1984). While cell culture can be used for culturing infectious stages, it cannot be used for propagation because the oocyst output is lower than the input. There have been numerous cell lines that have shown to support *Cryptosporidium* infection. Madin-Darby cattle kidney (MDBK) and Madin-Darby canine kidney (MDCK) cells were shown to be the

most effective for a *C. parvum* infection, with up to 90% of the cells becoming infected (Gut et al., 1991). Most infections will obtain peak growth around 48 to 72 hours with diminishing growth thereafter (Lawton et al., 1997; Tzipori, 1998). The inability to maintain high levels of infection is likely due to a lack of autoinfection, either from not producing thin-walled oocysts or a lack of Type I meront recycling (Current and Garcia, 1991). HCT-8 cells were the first cell line shown to maintain efficient and complete life cycles for an extended period of time for *C. parvum*, *C. hominis*, and *C. andersoni* (Hijjawi et al., 2001; Hijjawi et al., 2002). It was reported that regular changing of the media, every 2 to 3 days, as well as maintaining an optimal pH (7.2-7.6), and avoiding overgrowth of the cells were crucial to the success of an infection. Host cell-free development of *Cryptosporidium* has been reported (Hijjawi et al., 2004), but this remains controversial due to a failure to reproduce the findings in other labs.

Species and Genotypes of *Cryptosporidium*

Speciation of Cryptosporidium

Cryptosporidium is a member of the phylum apicomplexa, which includes over 300 genera and 4800 species with more to be named (Fayer and Xiao, 2008). Public health concerns in the 1980s (Anonymous, 1982b) due to secondary *Cryptosporidium* infections of AIDS patients created the need for a deeper understanding of the organism, particularly the identification of pathogenic and non-pathogenic species. Microscopic methods including acid fast staining and immunofluorescence assays were developed to detect *Cryptosporidium* oocysts in samples (Garcia et al., 1983; Kehl et al., 1995; Ma and Soave, 1983). These methods relied on measurements of oocyst morphology to identify

Cryptosporidium; however, similarities in oocyst size among many *Cryptosporidium* species prevented identification to the species level (Fall et al., 2003). It was initially assumed that *Cryptosporidium* was host specific, in line with findings from other coccidian parasites (Levine, 1980). However, this assumption proved to be at least partially false when it was shown that many species can infect other host species in addition to their primary host. Examples include *C. felis* (infects cats), *C. andersoni* (infects cattle), *C. canis* (infects dogs), and *C. suis* (infects pigs) have all been found to infect humans in addition to their primary hosts (Leoni et al., 2006). Species are characterized in a variety of ways including host adaption, oocyst morphology, and genotype. There are currently twenty-one recognized species of *Cryptosporidium* (Table 1).

Genotyping

Genotypes are more transitory and partial descriptors of *Cryptosporidium* when data are insufficient to merit speciation (Table 2) (Xiao and Ryan, 2008). Discovery and identification of genotypes began in the 1990s following the application of PCR and other molecular tools to the characterization of isolates. One of the most frequently used targets for species and genotyping is the 18S small subunit (SSU) rRNA gene(Spano et al., 1997b; Xiao et al., 1999). Other genes that are frequently used include *Cryptosporidium* oocyst wall protein (COWP) (McLauchlin et al., 1999; Patel et al., 1998), heat shock protein 70 (HSP70) (Gobet and Toze, 2001; Sulaiman et al., 2000), and the 60-kDa glycoprotein gene (GP60) (Leav et al., 2002; Muthusamy et al., 2006; Peng et al., 2003a) . Analysis of PCR amplicons using restriction fragment length polymorphism analysis (RFLP) and DNA sequencing have enabled genotyping (Spano et al., 1997b; Xiao et al., 1999).

RFLP is a technique that uses endonucleases to cut the amplified DNA at specific regions or recognition sites. Genetic polymorphisms within the DNA can result in a size difference in the cut fragments. Gel electrophoresis reveals the size of the DNA bands and allows the identification of unique banding patterns that allows for comparability among species and genotypes. DNA sequencing is more sensitive at detecting differences among isolates as the sequence of the entire amplicon is considered in the analysis; however, it is also considerably more expensive than RFLP.

Table 1. Valid named *Cryptosporidium* species.

Species	Major Host	Reference
C. parvum	Human, ruminant	Tyzzer, 1912
C. hominis	Human	Morgan-Ryan et al., 2002
C. andersoni	Cattle	Lindsay et al., 2000
C. bovis	Cattle	Fayer et al., 2005
C. meleagridis	Turkey	Slavin, 1955
C. muris	Mouse	Tyzzer, 1907
C. canis	Dog	Fayer et al., 2001
C. felis	Cat	Iseki, 1979
C. galli	Chicken	Ryan et al., 2003
C. baileyi	Chicken	Current et al., 1986
C. molnari	Fish	Alverez-Pellitero and Sitja-Bobadilla, 2002
C. scophthalmi	Fish	Alvarez-Pellitero et al., 2004
C. serpentis	Snake	Levine, 1980
C. suis	Pig	Ryan et al., 2004
C. varanii	Lizard	Pavlasek et al., 1995
C. wrairi	Guinea pig	Vetterling et al., 1971
C. macropodum	Grey kangaroo	Power and Ryan, 2008
C. fayeri	Red kangaroo	Ryan et al., 2008
C. xiaoi	Sheep	Fayer and Santin, 2009
C. ryanae	Cattle	Fayer et al., 2008
C. fragile	Black-spined toad	Jirku, et al., 2008

Table 2. Identified *Cryptosporidium* genotypes.

Bear	Goose I and II
Cervine I, II, and III	Horse
Deer	Marsupial II
Deer mice	Mongoose
Duck	Monkey
Ferret	Mouse
Fox and Fox II	Muskrat I and II
Ostrich	Opossum I and II
Ovine	Snake
Pig II	Caribou
Rabbit	Lizard
Raccoon	Tortoise
Seal I and II	Woodcock
Sheep novel genotype	
Skunk	

Transmission Dynamics

Cryptosporidium has a fecal-oral transmission route. The primary species of *Cryptosporidium* that infect humans are *C. parvum* and *C. hominis*. *C. parvum* has been shown to have a zoonotic transmission cycle, infecting a number of animals including neonatal ruminants. The primary risk factors for infection for *C. parvum* include direct contact with animals, drinking contaminated water, or foodborne transmission (Goh et al., 2004; Hunter et al., 2004; Roy et al., 2004). Visitors to petting farms and workers on farms or veterinary clinics are the most likely to come into direct contact with contaminated fecal material (Dawson et al., 1995; Mahdi and Ali, 2002). Neonatal cattle have been shown to be a significant host of *C. parvum* and contribute to waterborne contamination following fecal runoff into source waters (Graczyk et al., 2000).

C. hominis has an anthroponotic transmission cycle. People can become infected with *C. hominis* by drinking contaminated water, swimming in a contaminated pool or

recreation area, changing diapers, direct contact, and eating contaminated food (Hunter et al., 2004; Hunter and Thompson, 2005; McLauchlin et al., 2000; Roy et al., 2004). Incidence of disease due to *C. hominis* increases in populated, impoverished regions of the world where improper sanitation and close living quarters are prevalent (Chacin-Bonilla et al., 2008).

Cryptosporidium Subgenotyping

The entire genomes of *C. parvum* and *C. hominis* have been sequenced and researchers have used the data to further understand their pathogenic characteristics (Abrahamsen et al., 2004; Xu et al., 2004). Regions in the genome that vary intraspecifically are necessary for subtyping of isolates. Microsatellite regions are loci that contain tandem repeated sequences of 1-4 base pairs, whereas minisatellite regions are usually 5-10 base pairs in length (Caccio et al., 2000; Caccio et al., 2001). Replication errors create variations in the number of tandem repeats, therefore subtyping is based on the fragment size of the PCR amplicon. Fragment size analysis discriminates based on the length of the amplified DNA and is sensitive enough to detect a single base difference in length. It also is cheaper than sequencing. Sequencing can detect single base pair changes, particularly point mutations; however it is expensive.

The 60-kDa glycoprotein (GP60) gene is regularly used in genotyping due to its microsatellite region as well as other variations in the nonrepeat regions. GP60 is a functional precursor protein that is found on the apical surface of sporozoites and merozoites and is important in the pathogenicity of *Cryptosporidium* (Strong et al., 2000). GP60 is currently recognized as the most polymorphic and the most widely targeted gene.

For *C. parvum* and *C. hominis*, GP60 is classified into subtypes based on the nonrepeat region variability (Xiao and Ryan, 2008). *C. parvum* has nine GP60 families (IIa-IIi) and *C. hominis* has six families (Ia, Ib, Id-Ig) (Strong et al., 2000; Sulaiman et al., 2005), and each family has subtypes. Subgenotyping is used to enhance understanding of *Cryptosporidium* epidemiology, transmission dynamics and population structure.

Population Genetics

Population genetics is the study of the change in frequencies and fitnesses of genotypes in natural populations (Gillespie, 2004). The population structure is the stratification of a population of organisms, and defines the general genetic diversity of a species (Gauthier and Tibayrenc, 2005). Population structures need to be measured with sufficient gene sampling, sampling a large representation of the natural population, and using a blind approach (Gauthier and Tibayrenc, 2005). Sufficient gene sampling requires multiple loci that represent the genetic variability of the species. A large number of samples need to be collected over multiple years to obtain a complete representation of the natural population. A blind approach limits bias from known biological information of a species. An example is *Toxoplasma gondii*, which has an obligatory sexual cycle but has a clonal population structure (Tibayrenc et al., 1991). There are three primary types of populations: clonal, epidemic, and panmictic. A clonal population structure has little to no genetic recombination, thus the progeny are genetically identical to the parents (Morrison et al., 2008; Tibayrenc and Ayala, 2002). The result is linkage disequilibrium, which is an association of genotypes occurring at independent loci that cannot be explained by random recombination (Smith et al., 1993). Epidemic population structures

are found in randomly mating populations that have free flowing genetic exchange, which superficially appear clonal due to the occasional expansion of temporary clones (Morrison et al., 2008; Smith et al., 1993). Panmictic population structures have random genetic exchange among individuals (Tibayrenc and Ayala, 2002). Panmixia does not refer to a mating system, but an overall distribution of genotypes throughout a population which is also described as linkage equilibrium (Gauthier and Tibayrenc, 2005).

Understanding the population structure of *Cryptosporidium* is important given its applicability in determining sources of infection, transmission routes, and epidemiology. Microsatellite and minisatellite regions have been used in studies of several other apicomplexan parasites (Anderson et al., 2000; MacLeod et al., 2000). Within the phylum, studies have shown significantly different population structures. *Toxoplasma gondii* is an apicomplexan that has been shown to have a clonal population structure (Howe and Sibley, 1995). Studies have shown that the apicomplexan *Theileria parva* has an epidemic population structure (Oura et al., 2005). *Plasmodium falciparum* has shown to be panmictic in high transmission regions (Anderson et al., 2000).

The study of the population structure of *Cryptosporidium* started in the late 1990's, when it was hypothesized that *C. parvum* had a clonal population structure based on similarities to other apicomplexan studies (wad-El-Kariem, 1999). A study in Scotland examined *C. parvum* in humans and cattle utilizing multilocus genotyping and found that the *C. parvum* in cattle had a panmictic population structure and *C. parvum* in humans had an epidemic population structure, which was confirmed with a later study (Mallon et al., 2003a; Mallon et al., 2003b). Multilocus genotyping means to target, via sequencing

or fragment size analysis, multiple loci that contain microsatellite or minisatellite regions within the genome, and the variation within each locus is assigned an allele which is then combined with the alleles from the other loci to provide a multilocus genotype. A study of isolates in Israel contrasted the Scotland study and found them to be clonal (Tanriverdi et al., 2006). In France, the population from humans was found to have a clonal structure, and the population from humans, cattle, and goats in Haiti was found to be panmictic or epidemically clonal (Ngouanesavanh et al., 2006). A more recent analysis from Scotland found two populations of *C. parvum* to be epidemic, Orkney and Thurso, and two to be panmictic, Dumfriesshire and Aberdeenshire, but the population of *C. parvum* was epidemic overall (Morrison et al., 2008). They also concluded that the movement of cattle allows *C. parvum* to circulate and spread throughout the region. A multi-country study found that *C. parvum* had a flexible reproductive strategy, which could not be classified as clonal or panmictic. Those authors concluded that the population structure is likely dependent upon ecological determinants of transmission (Tanriverdi et al., 2008). Determining the population structure of *C. parvum* is a vast and complex problem that has been shown to be variable throughout the world, and it will be addressed further in Chapter 1 of this thesis paper.

Thesis Objectives

- **Determine the population structure of *Cryptosporidium parvum* in the upper Midwest United States (Paper 1).**

Studies have shown that *C. parvum* has different population structures depending on the sampling location. We hypothesize that *C. parvum* has a panmictic population structure in the upper Midwest United States.

- **Determine the extent to which two strains of *Cryptosporidium* behave differently during an infection (Paper 2).**

Commercially produced strains of *C. parvum* are used in most research labs due to the difficulty and expense of propagating *C. parvum*. The two most common strains used are the IOWA II and Moredun. We hypothesize that different strains of *C. parvum* will act differently in cell culture.

CHAPTER 1. THE POPULATION STRUCTURE OF *CRYPTOSPORIDIUM PARVUM* IN THE UPPER MIDWEST UNITED STATES

Abstract

Cryptosporidium parvum is a zoonotic parasite that causes cryptosporidiosis, an infectious diarrheal disease, which can become chronic and life-threatening in immunocompromised individuals. *Cryptosporidium parvum* primarily infects humans and ruminants, in particular neonatal calves. Understanding the transmission dynamics of *C. parvum*, particularly the specific contribution of zoonotic and anthroponotic transmission, is critical to the control of this pathogen. *Cryptosporidium parvum* genotypes have been identified which appear to be restricted to a single host, which suggests that this may be a heterogeneous species, with varying infection and transmission dynamics. The aim of this study was to determine the population structure of *C. parvum* in the upper Midwest United States. A total of 216 isolates from cases of human cryptosporidiosis in Minnesota and Wisconsin and 64 isolates from diarrheic calves in Minnesota, Wisconsin, and North Dakota were genotyped at 8 polymorphic loci. A total of 213 isolates, 167 from humans and 46 from calves, had complete multilocus types (MLT). There were 93 different MLTs and sixty of those MLTs were only represented by one isolate. MLT 2 was represented by 20% of the isolates. Population analysis revealed a highly recombining, panmictic population that does not have any genetic, geographic, or host sub-structuring.

Introduction

Cryptosporidium parvum is a zoonotic parasite that causes cryptosporidiosis, a self-limiting diarrheal disease that can be life-threatening in immunocompromised

persons. It has no effective treatment. Neonatal ruminants can become infected with *C. parvum* and shed large numbers of oocysts (>10^{10} per gram of feces) into the environment (Fayer et al., 1998). *Cryptosporidium parvum* can be transmitted directly from human to human or ruminant to human or indirectly by food or water. Waterborne transmission is a particular problem as oocysts can survive for long periods in water and are resistant to many disinfectants including chlorination. A large waterborne outbreak in Milwaukee in 1993 affected over 400,000 people (Mac Kenzie et al., 1994). Retrospective analysis of isolates from the Milwaukee outbreak identified the anthroponotic species, *C. hominis*; however, *C. parvum* was also identified and therefore cattle could not be discounted as a source. Identifying the source of *C. parvum* in an outbreak is complicated by the zoonotic nature of this species. However, certain *C. parvum* subgenotypes have been identified that appear to be species restricted. For example, GP60 subtype IIc has only been identified in humans suggesting an anthroponotic transmission cycle. This raises the question that *C. parvum* may be a heterogeneous species with host restricted subtypes. This question can be addressed using a number of tools, the most powerful of which is population structure analysis.

Cryptosporidium parvum has been previously described as having a clonal, panmictic or epidemically clonal population structure. A clonal population structure has very little recombination, therefore the progeny are identical to the parents (Morrison et al., 2008; Tibayrenc and Ayala, 2002). Panmictic and epidemic clonal populations are randomly mating, but the epidemic clonal population has a temporary expansion of identical parasites and appears clonal (Smith et al., 1993; Tibayrenc and Ayala, 2002). An

early study of *C. parvum* in humans and cattle in Scotland revealed a panmictic population structure (Mallon et al., 2003a; wad-El-Kariem, 1999). However, in a more substantial follow-up study, the population structure was described as being epidemically clonal (Mallon et al., 2003a; Morrison et al., 2008; wad-El-Kariem, 1999). A study in Israel reported a clonal population structure among isolates from cattle (Tanriverdi et al., 2006). Ngouanesavanh and others found that *C. parvum* had a clonal population structure among humans in France while it had a panmictic or epidemically clonal population structure among humans, cattle, and goats in Haiti (Ngouanesavanh et al., 2006). A global study by Tanriverdi and others concluded that *C. parvum* has a varying reproductive strategy that is possibly influenced by transmission factors specific to the region (Tanriverdi et al., 2008).

Our study aims to determine the population structure of *C. parvum* in the upper Midwest United States. From 2004-2009, we collected human fecal samples from the Minnesota Department of Health (MDH), Wisconsin State Department of Hygiene (WDH), and cattle fecal samples from the North Dakota Veterinary Diagnostic Laboratory and the University of Wisconsin-Eau Claire. Among the *C. parvum* isolated, we used sequencing and fragment size analysis to genotype 8 polymorphic loci. Our findings indicate that the *C. parvum* population is panmictic in the upper Midwest.

Materials and Methods

Parasite samples

Two hundred and eighty total isolates, 216 from humans and 64 from cattle, were initially typed using PCR-RFLP analysis of the 18S rRNA gene, which distinguishes *C.*

parvum from other *Cryptosporidium* species. One hundred and ninety-nine of the 216 human samples, 92%, were identified as *C. parvum*. Human isolates were obtained from separate cases of human cryptosporidiosis in Minnesota and Wisconsin. Fifty-five of the 64 cattle samples, 86%, were typed as *C. parvum*. Cattle isolates were obtained from separate cases of calf cryptosporidiosis in North Dakota, Minnesota, and Wisconsin. All isolates were from clinical cases.

DNA extraction

An alkaline digestion and phenol-chloroform extraction was used to extract DNA from 0.2 g fecal samples and purified by the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) as previously described (Peng et al., 2003b). A 0.5 g aliquot of stool was added to a 1.5 mL centrifuge tube and spun at 14000 x g (IEC Micromax centrifuge) for 5 minutes. The supernatant was removed and 0.5 mL of water was added to the tube, which was centrifuged at 14000 x g. The washing step was repeated three times and the final stool was resuspended in 0.2 mL of water. A 66.6 µL aliquot of 1 M KOH and 18.6 µL of 1 M DTT (Dithiothreitol) was subsequently added, mixed into the stool suspension, and incubated at 65°C for 15 minutes. After incubation, the stool mixture was neutralized with 8.6 µL of 25% HCl, buffered with 160 µL of 2 M Tris-HCl (pH 8.3), and centrifuged at 3300 x g for 5 minutes. DNA was extracted with 250 µL of phenol: chloroform:isoamyl alcohol (Invitrogen, Carlsbad, CA) and centrifugation at 3300 x g for 5 minutes. The supernatant was transferred to a 2.0 mL Eppendorf tube, which contained 1.0 mL of buffer ASL from QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA). Further purification steps followed the manufacturer's instructions and the samples were stored at -20°C.

Species identification

Species and genotypes were identified by sequencing or RFLP analysis when the PCR products were unable to be sequenced. A portion of the 18S rRNA gene was amplified using a previously described nested PCR protocol (Santin et al., 2004). A 1300-bp fragment was amplified in the primary step of the 18S PCR reaction with the primers 18SF1 5'-TTCTAGAGCTAATAC ATGCG-3' and 18SR1 5'- CCCATTTCCTTCGAAACAGGA-3'. Primary PCR reactions were performed with three different volumes of template DNA: 2 µL, 1 µL, and 0.5 µL. The master mix included 0.5X PCR buffer (Promega, Madison, WI), 6 mM $MgCl_2$, 0.2mM of each dNTP, 10 pmol of each primer, 2.5 U of *Taq* DNA polymerase, and 600 ng/µL of non-acetylated cattle serum albumin (10 mg/mL) (New England Biolabs, Beverly, MA) in a 100 µL reaction volume. PCR conditions for the primary step were an initial denaturation of 94°C for 3 minutes, followed by 35 cycles of 94°C for 45 seconds, 45°C for 45 seconds, and 72°C for 1 minute, and a final extension of 72°C for 7 minutes. An 830-bp fragment within the primary product was amplified in the secondary reaction using primers 18SF2 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 18SR2 5'-AAGGAGTAAGGAACAACCTCCA-3'. The secondary PCR conditions were the same as stated for the primary step except that 2 µL of primary PCR product was used as the template and the $MgCl_2$ concentration was 3 mM. The cycling conditions were the same for both the primary and secondary steps. Secondary PCR product of expected size were digested using *AseI* and *SspI* (Xiao et al., 1999) (New England Biolabs, Beverly, MA) at 37°C. Digested DNA was subsequently separated by electrophoresis on a 2% agarose gel,

stained with either EZ-Vision™ intercalating DNA dye (Amresco Inc, Solen, OH) or ethidium bromide, and examined under ultraviolet transillumination.

To identify the species, the known RFLP profiles from literature were compared to the RFLP profiles provided by the electrophoresis (Feng et al., 2007). The secondary PCR product was purified from excised gel bands or the PCR reaction product using the Wizard SV gel and PCR product clean-up kit (Promega, Madison, WI). For every 10 mg of gel slice, 10 µL of membrane binding solution was added, vortexed, and incubated in a 1.5 mL microcentrifuge tube at 50-65°C until the gel slice is dissolved. If the PCR reaction was used, an equal volume of membrane binding solution was added to the PCR reaction in the tube. The resulting mixture was added to a collection tube that contained a SV minicolumn and then incubated for 1 minute at room temperature. The tube was centrifuged for 1 minute at 16,000 x g and the flowthrough was discarded. We added an additional 30 seconds to our centrifugation time to allow for ramping time. Then 700 µL of membrane wash solution was added to the minicolumn and centrifuged for 1 minute at 16,000 x g. The flowthrough was discarded and the step was repeated with 500 µL of membrane wash solution, centrifuged for 7 minutes at 16,000 x g, and the flowthrough was discarded. To allow for evaporation of any remaining ethanol in the column, the tube was centrifuged without the microcentrifuge lid on for 3 minutes at 16,000 x g. Minicolumns were transferred to new 1.5 mL microcentrifuge tubes. The column was washed with 50 µL of nuclease-free water, incubated at room temperature for 1 minute, and centrifuged for 1 minute at 16,000 x g. All DNA was stored at -4°C. The DNA was sequenced bi-directionally on an ABI 3730XL DNA Analyzer (Macrogen, Rockville, MD).

The data was then assembled and edited using SeqMan (DnaStar, Madison, WI) and compared to non-redundant DNA sequences using the BLAST algorithm in GenBank™.

Multilocus genotyping

An approximately 830-bp region of the gp60 gene was amplified using nested PCR (Glaberman et al., 2001). The primers used for the primary reaction were AL3531F 5'-ATAGTCTCCGCTGTATTC and AL3535R 5'-GGAAGGAACGATGTATCT. For the secondary reaction, the primers were AL3532F 5'-TCCGCTGTATTCTCAGCC and AL3534R 5'-GAGATA TATCTTGGTGCG. The PCR mastermix contained 1X buffer, 3 mM $MgCl_2$, 400 ng/µl nonacetylated cattle serum albumin, 0.2 mM dNTP, 10 pmol of each primer, and 2.5 U Taq polymerase (Promega, WI). 1 µl of DNA template was added for primary reaction and 2 µl of primary PCR product was added for the secondary reaction. PCR cycling conditions for both reactions had an initial denaturation at 95°C for 3 minutes, 34 cycles of 94°C for 45 seconds, an annealing temperature at 50°C for 45 seconds, an extension of 72°C for 1 minute, and a final extension at 72°C for 10 minutes. A 1.5% agarose gel was used to separate the secondary product and it was visualized under UV transillumination following EZ-Vision™ (Amresco, OH) or ethidium bromide staining.

Appropriate sized bands of secondary PCR DNA were cut out from the gel and purified using the Wizard SV gel clean-up system according to the manufacturer's directions (Promega, Madison, WI). The amplified product was sequenced in both directions using the secondary primers on an ABI 3730XL DNA Analyzer (Macrogen, Rockville, MD). Sequences were assembled and edited using SeqMan (DnaStar, Madison, WI), aligned using the Clustal W algorithm(Thompson et al., 1997), and compared to

sequences using the BLAST algorithm in GenBank™ to determine the allelic family assignment. The sequences were further analyzed for the number of TCA, TCG, and ACATCA repeats in the microsatellite region. TCA repeats were assigned as an A allele, TCG was assigned by a G, and ACATCA was assigned by a R. An example of this typing nomenclature would be a sequence that contains 16 TCA repeats, 2 TCG repeats, and 1 ACATCA repeat, which would then be assigned the type A16G2R1.

Seven additional loci were examined in this study in addition to the GP60 locus. These loci included mini- and microsatellite regions that were previously described in other research studies. One of the loci GRH, a predicted secreted signal peptide, was located using CryptoDB (www.cryptodb.org) and the primers were designed using CLC Main Workbench 4 (CLC bio, Aarhus, Denmark). The mastermix reagent concentrations, primary and secondary primer set sequences, and the PCR thermocycler conditions for all seven of the loci are found in Tables 3-5. All reaction volumes were 50 µL and each forward primer for the secondary reaction was labeled on the 5' end with FAM which is a fluorescent dye. Secondary PCR product was separated via electrophoresis on a 1.5% agarose gel, stained with EZ-Vision™ intercalating dye, and examined using UV transillumination. PCR secondary products of the expected size were diluted 1:200 and 1.5 µL were placed in a half-skirted 96-well plate to be separated with a Prism 3100 DNA Analyzer (Applied Biosystems) with a ROX-size standard. Target fragment sizes were assigned using Peak Scanner v1.0 (Applied Biosystems). If more than one peak appeared for a sample, the highest peak was used to assign the allele.

Table 3. Mastermix components for each targeted loci.

	Buffer	MgCl2	dNTP	Primers (primary)	Primers (secondary)	Taq Polymerase	Cattle Serum Albumin
TP14	1X	1.5 mM	0.2 mM	50 pmol	50 pmol	2.5 U	Not included
CP47	1X	3 mM	0.2 mM	12.5 pmol	25 pmol	1.25 U	400 ng/μL
MS5	1X	1.5 mM	0.2 mM	25 pmol	25 pmol	1.25 U	Not included
MS9	1X	1.5 mM	0.2 mM	50 pmol	50 pmol	2. 5 U	Not included
MSC 6-7	1X	1.5 mM	0.2 mM	12.5 pmol	25 pmol	1.25 U	400 ng/μL (1° only)
DZ-HRGP	1X	1.5 mM	0.2 mM	5 pmol	10 pmol	1.25 U	400 ng/μL
GRH	1X	1.5 mM	0.2 mM	25 pmol	25 pmol	1.25 U	Not included

Table 4. Primary and secondary PCR primer sets for each targeted loci.

Loci		Primers (5'-3')	Chromosome	Source
TP14	F1	CTAACGTTCACAGCCAACAGTACC	8	Mallon et al. (2003a)
	R1	GTACAGCTCCTGTTCCTGTTG		
	F2	GTTCACAGCCAACAGT		
	R2	CATTTTGATTTTGGGAGT		
CP47	F1	GCTTAGATTCTGATATGGATCTAT	6	Gatei et al. (2006a)
	R1	AGCTTACTGGTCCTGTATCAGTT		
	F2	ACCCCAGAAGGCGGACCAAGGTT		
	R2	GTATCGTGGCGTTCTGAATTATCAA		
MS5	F1	GCTTCCTTACTATCATTTCC	8	Mallon et al. (2003a)
	R1	GCTTCAGGCTATGACAAA		
	F2	ATGTAGTCGTATCCGGAA		
	R2	GTATGCTGGGTGAATATAG		
MS9	F1	GGACTAGAAATAGAGCTTTGGCTGG	5	Mallon et al. (2003b)
	R1	GTCTGAGACAGAATCTAGGATCTAC		
	F2	ACCTGGAGTGTGATTTGG		
	R2	GTTCTTGTTCAAAGTCA		
MSC 6-7	F1	ATTGAACAAACGCCGCAAATGTACA	6	Gatei et al. (2006a)
	R1	CGATTATCTCAATATTGGCTGTTATTGC		
	F2	GCTATTTGCTATCGTCTCACATAACT		
	R2	CTACTGAATCTGATCTTGCATCAAGT		
DZ-HRGP	F1	TGGTTGAGGTTGAAGGCCCAT	6	Cama et al. (2006b)
	R1	CATTTCAGCTATTTTAGCTCAACC		
	F2	CATTAATCTTTTAGCAAGAGTAGCTGA		
	R2	AATGCGTTAAGCCTTAAAGCTGG		
GRH	F1	AGATCTTCAGGTGGCCATCATCCT	1	This study
	R1	TTGTCTCCTTTATCGTCACCGCCT		
	F2	TCAGGTGGCCATCATCCTCTTGAA		
	R2	TGTGACGGAAGGTATGGCAGCAAA		

Table 5. Primary and secondary PCR conditions for each targeted loci

	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	# of Cycles
Primary PCR						
TP14	95°C for 3 min	95°C for 50 s	61.8°C for 50 s	70°C for 1 min	70°C for 10 min	35
CP47	94°C for 5 min	94°C for 45 s	43°C for 45 s	72°C for 1 min	72°C for 10 min	35
MS5	94°C for 3 min	94°C for 30 s	53°C for 50 s	72°C for 40 s	72°C for 7 min	35
MS9	95°C for 3 min	95°C for 50 s	57°C for 50 s	70°C for 1 min	70°C for 10 min	35
MSC 6-7	94°C for 5 min	94°C for 45 s	55°C for 45 s	72°C for 1 min	72°C for 10 min	35
DZ-HRGP	94°C for 2.5 min	94°C for 30 s	55°C for 1 min	72°C for 40 s	72°C for 10 min	40
GRH	94°C for 2 min	94°C for 30 s	60°C for 45 s	72°C for 1 min	72°C for 7 min	40
Secondary PCR						
	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	# of Cycles
TP14	95°C for 3 min	95°C for 50 s	50°C for 30 s	72°C for 30 s	72°C for 10 min	45
CP47	94°C for 5 min	94°C for 45 s	55°C for 45 s	72°C for 1 min	72°C for 10 min	35
MS5	94°C for 3 min	94°C for 30 s	53°C for 50 s	72°C for 40 s	72°C for 7 min	35
MS9	95°C for 3 min	95°C for 50 s	50°C for 30 s	72°C for 30 s	72°C for 10 min	44
MSC 6-7	94°C for 5 min	94°C for 45 s	55°C for 45 s	72°C for 1 min	72°C for 10 min	35
DZ-HRGP	94°C for 2.5 min	94°C for 30 s	55°C for 1 min	72°C for 40 s	72°C for 10 min	40
GRH	94°C for 2 min	94°C for 30 s	60°C for 45 s	72°C for 1 min	72°C for 7 min	40

Data analysis

Isolates were grouped into three different populations for analysis: human and cattle combined, human only, and cattle only. Populations were also analyzed on two levels. The first analysis included all of the isolates and the second analysis removed duplicate MLTs and counted them as one data point. Removing the duplicates in the second analysis tested whether or not the population structure was epidemic (Morrison et al., 2008). Duplicate cattle isolates from the same farm were removed from analysis to eliminate redundancy. Isolates from humans were analyzed as a single group and separated by state. For Wisconsin isolates, it was assumed that those reported to the WLH were from the state. Further information was obtained from the MDH on whether the isolates were obtained from Minnesota residents and whether they were associated with out-of-state travel. If previous travel was unknown, if they were not a Minnesota resident, or if they did travel out of state, the data was excluded in that analysis.

Linkage disequilibrium for all of the loci was assessed using the standardized index of association (sIa) (Haubold and Hudson, 2000). The index is derived from the Maynard Smith index of association and it fluctuates randomly around zero in complete panmixia, but as linkage disequilibrium increases the number moves further from zero. LIAN software (http://adenine.biz.fh-weihenstephan.de/cgi-bin/lian/lian.cgi.pl) was used to analyze the index and its probability under a null hypothesis of complete panmixia with 1,000 allele randomizations. The Monte Carlo simulation was used, which scrambles the inputted data set by resampling the loci without replacement. A probability (P) value was

calculated with respect to how well the observed pairwise allele frequencies fit a random mating simulation (Morrison et al., 2008).

The population structure was visualized using eBURST software (Feil et al., 2004). A diagram was created that represents multilocus genotypes (MLT) as dots, which were linked to their single locus variants by lines. The diameter of the dot is proportional to the frequency of the MLT, meaning that the most represented MLT would have the largest dot. A founding genotype was selected based on the MLT that had the highest number of MLTs that are different by one locus (single locus variants). Founding genotypes were represented by a blue dot and were not dependent upon the frequency of the MLT. Subgroups occurred if there was a SLV from the original founder that diversified to have multiple SLVs of its own. Subgroup founders were represented by yellow dots.

For the subpopulation analysis, Nei's genetic distance and a variation of F_{ST} were calculated using GenAlEx 6.2 (Peakall and Smouse, 2006). Nei's genetic distance measures the genetic relatedness of samples of populations (Nei and Roychoudhury, 1974). Wright's fixation index (F_{ST}) is a measure of population subdivision (Morrison et al., 2008). F_{ST} was calculated using the Analysis for Molecular Variance (AMOVA) in GenAlEx 6.2, with 999 permutations in each analysis. An F_{ST} of zero means that there is no subdivision of the population and random mating is occurring.

Results

Multilocus genotyping

Two-hundred and thirteen of the isolates, 167 human and 46 cattle, had a complete multilocus type (MLT), with no missing data points over eight markers, i.e. all

eight polymorphic loci were typeable (Table 6 and 7). GP60 had 23 alleles and was the most variable. GRH had 10 alleles, MS9 had 8, DZ-HRGP had 6, and TP14, MSC 6-7, CP47, and MS5 all had 3 alleles. GP60 was the most variable locus in human isolates, and the fourth most variable locus in cattle isolates. MS9 was the most variable locus in cattle isolates, and GRH was the second most variable locus in both human and cattle isolates.

Table 6. Allele sizes and assigned number for each of the seven micro- and mini-satellite regions.

Marker	Allele (bp)	Allele no.
TP14	212	1
	189	2
	221	3
MS9	192	1
	186	2
	198	3
	180	4
	174	5
	189	6
	204	7
	222	8
CP47	414	1
	417	2
	411	3
MS5	326	1
	276	2
	349	3
GRH	342	1
	353	2
	359	3
	348	4
	336	5
	412	6
	418	7
	424	8
	388	9
	420	10
DZ-HRGP	572	1
	566	2
	597	3
	579	4
	501	5
	591	6
MSC 6-7	554	1
	524	2
	570	3

Ninety-three unique MLTs were identified (Table 8). Seventy-five MLTs were exclusive to human isolates and 9 MLTs were unique cattle isolates. Nine MLTs were common to both humans and cattle isolates. Those 9 MLTs comprised 43% of all MLTs. MLT 2 was the most common MLT in isolates from both humans and cattle occurring in 20% (42 out of 213) of isolates. MLT 1 was represented in 14 of the isolates and it was the second most common MLT. Sixty of the MLTs were represented by one isolate.

Population analysis

If a population fits the null hypothesis of panmixia, the variance of pairwise differences (V_D) is less than the 95% critical value of V_D (L). V_D was not greater than L for any of the populations tested supporting the null hypothesis of panmixia (Table 8). All of the V_D values were less than their respective L values, the sIa values were also close to zero (0.007-0.0209), and the P-values were greater than 0.05 which are all support for a panmictic population. When all of the MLTs with multiples were counted as one, the sIa values became negative and remained close to zero. V_D was less than L for all three populations and the P-values increased in value. These data support a panmictic population structure. Panmixia was also supported when human *C. parvum* populations were analyzed separately by state. The Wisconsin population showed stronger evidence for panmixia with a negative sIa and higher P-value. Excluding out-of-state travel from the Minnesota population had no effect on the population structure.

Nei's Genetic Distance (D) and F_{ST} values strongly support a panmictic population with little to no subdivision (Table 9). For the comparison of the human and cattle populations, D was 0.03 and F_{ST} was 0.067. When the human isolates were divided by

state, D was 0.012 and F_{ST} was 0.017. When F_{ST} is zero or close to zero, there is no subdivision of the populations and the population is considered panmictic.

All five of the eBURST diagrams had a singular straggly arrangement, which is indicative of a population that has high level of recombination and little mutation. MLT 2 was the founding genotype of the diagrams for both the combined and human diagrams. MLT 1 was the founding genotype for the cattle diagram. There were 10 eBURST subgroups for the combined population. The total isolates from humans also had 10 subgroups, while the isolates from the MDH had 7, isolates from WLH had 4, and the isolates from cattle only had one subgroup. There were also 11 singletons for the overall combined population. A singleton occurred when the MLT does not share 7 loci (out of 8) with another MLT.

Table 7. GP60 types and assigned allele number. Allele number 21 was not present in a complete multilocus type.

GP60 Type	Allele number
IIaA15G2R1	1
IIaA15G2R2	2
IIaA14G1R2	3
IIaA13G1R1	4
IIaA17G2R1	5
IIaA16G2R3	6
IIaA16G3R2	7
IIaA17G3R1	8
IIaA16G1R1	9
IIaA14G2R1	10
IIaA17G2R2	11
IIaA16G2R1	12
IIaA18G2R1	13
IIaA17G4R2	14
IIaA12G1R2	15
IIaA16G3R1	16
IIaA19G2R1	17
IIaA19G4R2	18
IIaA14G1R1	19
IIaA16G2R2	20
IIaA13G1R2	21
IIaA13G2R1	22
IIaA18G3R2	23

Table 8. Linkage analysis of human and cattle isolates

Population	n	Standardized IA	P-value	VD>L	LE or LD
Human and Cattle	213	0.007	0.161	N	LE
Human	167	0.0019	0.39	N	LE
Cattle	46	0.0209	0.114	N	LE
Human and Cattle*	93	-0.0358	1	N	LE
Human*	84	-0.0328	0.99	N	LE
Cattle*	18	-0.0054	0.55	N	LE
$Human^{W}$	73	-0.0025	0.544	N	LE
$Human^{M}$	94	0.0092	0.195	N	LE
$Human^{MD}$	59	0.0097	0.207	N	LE

(Abbreviations: n, number of isolates with complete MLT; Standardized I_A, standardized index of association; V_D, variance of pairwise differences; L, 95% critical value for V_D; LE, Linkage equilibrium; LD, Linkage disequilibrium). The populations marked with an asterisk include all of their respective MLTs, but the MLTs that have multiples are only represented once. The W represents the isolates from humans acquired from the Wisconsin State Lab of Hygiene. The M represents the isolates from humans acquired from the Minnesota State Department of Health. The MD represents the isolates from humans acquired from the MDH that did not report travelling out of state.

Table 9. Nei's Genetic Distance (D) and F_{ST} analysis. Populations are denoted as follows: W-isolates collected from WLH, M-isolates collected from MDH, and A-all isolates.

Analysis	$Human^{W}$ & $Human^{M}$	$Human^{A}$ & $Cattle^{A}$
Nei's Genetic Distance (D)	0.012	0.030
F_{ST}	0.017	0.067

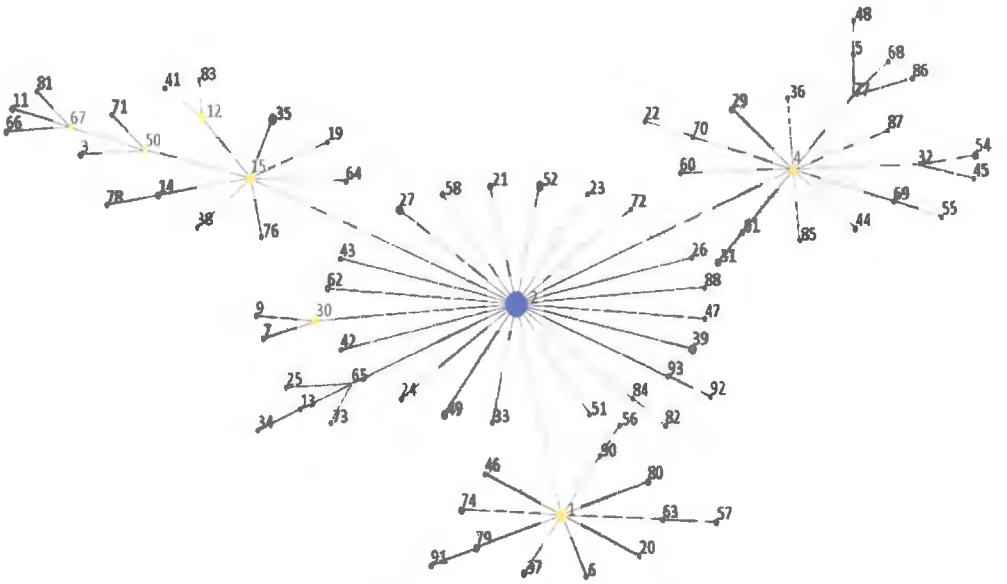

Figure 2. **eBURST diagram for isolates from human and cattle. The size of the dot represents the frequency of the MLT in the population. The blue dot is the founding genotype and the yellow dots are the founding genotypes for the subgroups.**

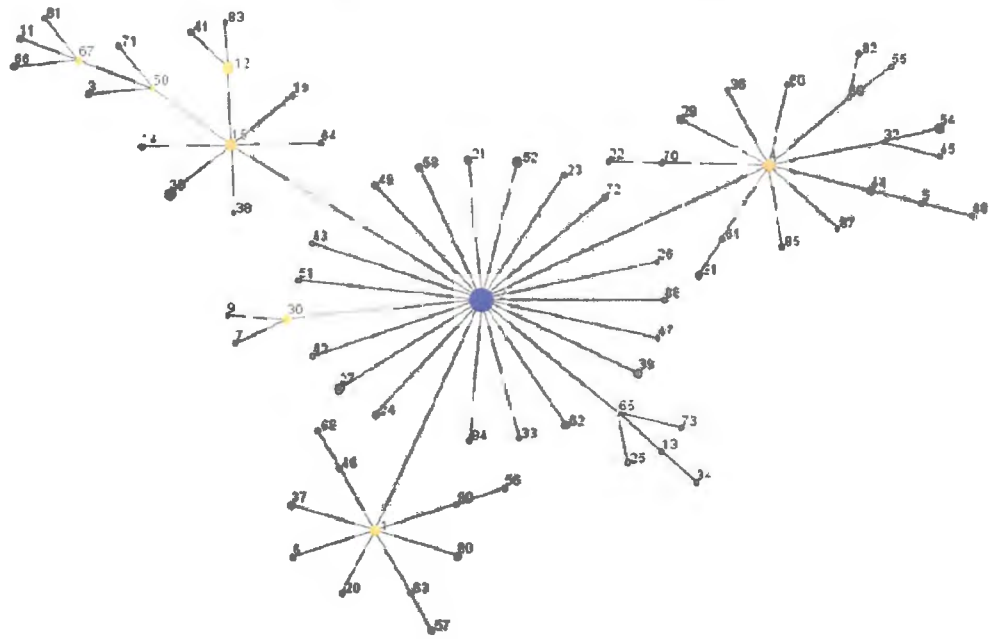

Figure 3. **eBURST diagram for isolates from humans. The size of the dot represents the frequency of the MLT in the population. The blue dot is the founding genotype and the yellow dots are the founding genotypes for the subgroups.**

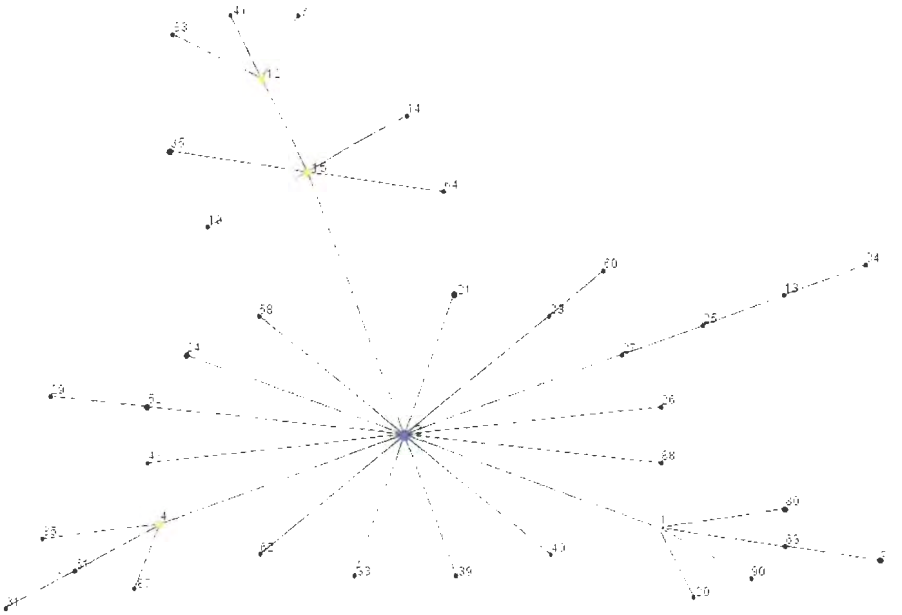

Figure 4. eBURST diagram for isolates from WLH. The size of the dot represents the frequency of the MLT in the population. The blue dot is the founding genotype and the yellow dots are the founding genotypes for the subgroups.

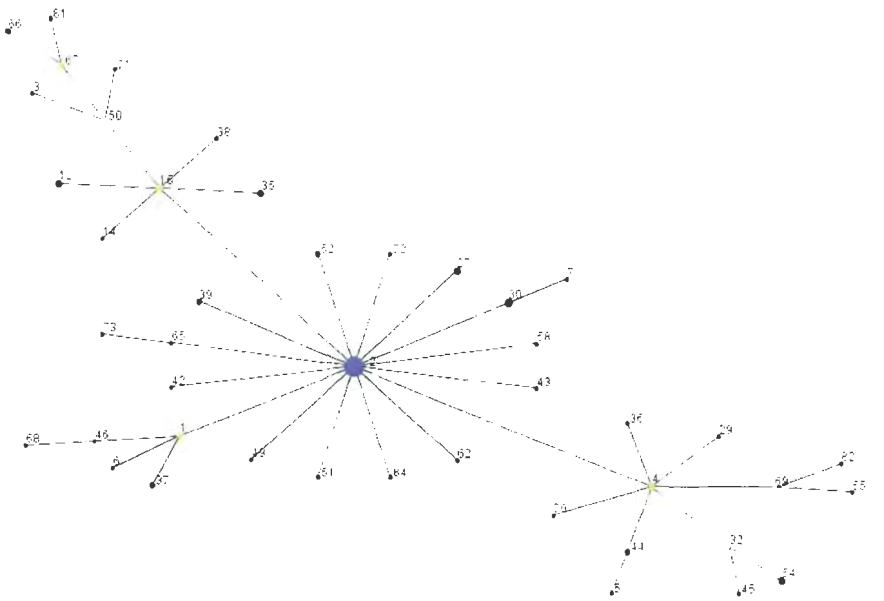

Figure 5. eBURST diagram for isolates from MDH. The size of the dot represents the frequency of the MLT in the population. The blue dot is the founding genotype and the yellow dots are the founding genotypes for the subgroups.

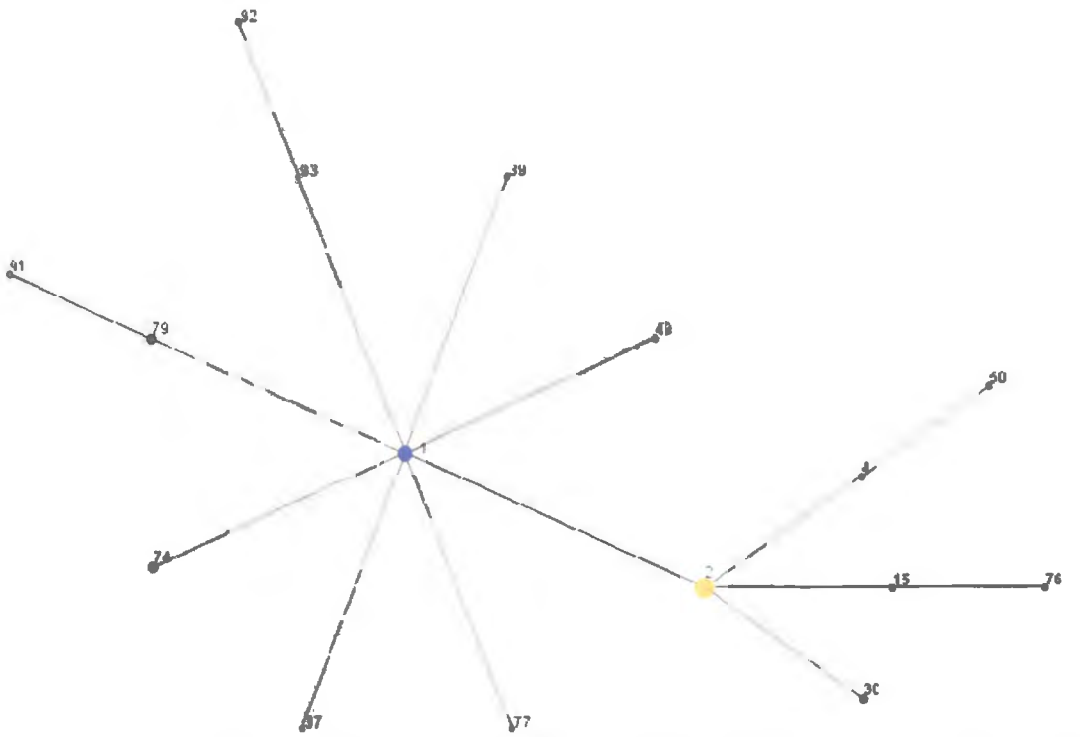

Figure 6. **eBURST diagram for isolates from cattle. The size of the dot represents the frequency of the MLT in the population. The blue dot is the founding genotype and the yellow dots are the founding genotypes for the subgroups.**

Discussion

Data from our comprehensive, multi-year study of *C. parvum* populations from humans and cattle in Minnesota, Wisconsin, and North Dakota support a panmictic population structure with high rates of sexual recombination. The number of MLTs identified in this study was relatively high; however, the number of alleles assigned to each locus was relatively low. Ninety three MLTs were identified in 213 completely typed isolates. Four loci (TP14, MSC 6-7, CP47, and MS5) all had only 3 alleles. This fits the expectation for a randomly mating population as described previously by Turner and others (Turner et al., 2007). Those authors reported that when recombination levels are

high and mutation levels are low, the overall number of MLTs would be high and the number of alleles assigned to a loci would be low (Turner et al., 2007).

eBURST diagrams in the present study were straggly and large. This type of eBURST pattern was reported previously to be typical of a high recombination to mutation ratio (Turner et al., 2007). Turner and others found that if the largest eBURST group contains 25% or more of the MLTs in the population or is a large straggly arrangement, that the eBURST ancestor-descendant links may not be accurate (Turner et al., 2007). While this indicates that the MLT ancestry in the present study may not be accurate, the eBURST does support a population with a high recombination rate.

Our finding that the *C. parvum* population in the upper Midwest is panmictic further adds to the complex picture of *C. parvum* population structures worldwide. In Scotland, the population was reported to be epidemic with slight variance at a local level (Morrison et al., 2008). In France, the population from humans was found to have a clonal structure, and the population in Haiti was found to be panmictic or epidemic depending on the host (Ngouanesavanh et al., 2006). Through LIAN, we found that the standard index of association was close to zero for every analysis. In complete panmixia the sIa value is zero and stochastic values around zero are also expected for panmixia (Haubold and Hudson, 2000). The P-values are large and the V_D is consistently less than the 95% critical value of V_D, which are evidence for panmixia.

The population structure for *C. parvum* in humans in the upper Midwest United States is not geographically or genetically sub-structured. Morrison et al. found no evidence for geographic sub-structuring in populations in Scotland (Morrison et al., 2008).

However, they did find genetic sub-structuring in 2 of their populations, Aberdeenshire and Dumfriesshire, when they included their outlier groups in the genetic analysis. Tanriverdi and others did not perform sub-structuring analysis. They did find that there was MLT clustering in many countries but it was not consistent throughout their study (Tanriverdi et al., 2008). We found no sub-structuring of the population of *C. parvum* from humans from Minnesota and Wisconsin. The Nei's genetic distance and F_{ST} were both very close to zero, which signify a panmictic population that has little to no sub-structuring. We found no sub-structuring by host in the present study. This is in contrast to the findings of Morrison and others who found that when *C. parvum* populations from cattle and humans were analyzed separately, there was a difference in population structure (Morrison et al., 2008). Those authors found that human populations in some regions were epidemic while cattle populations were panmictic. In the present study, the Nei's genetic distance and the F_{ST} support a finding that human and cattle populations in the region are closely related. This suggests that *C. parvum* populations in the region move freely between cattle and humans.

In summary, our data support a panmictic *C. parvum* population structure in Minnesota, Wisconsin, and North Dakota with no geographic, genetic, or host sub-structuring. This finding is important for our understanding of zoonotic transmission of *C. parvum* in the region.

CHAPTER 2. INFECTIVITY OF TWO *CRYPTOSPORIDIUM PARVUM* STRAINS IN A CELL CULTURE INFECTION MODEL

Abstract

Few *C. parvum* strains are propagated commercially and, therefore, most of what is known about the biology of this important pathogen is based on a handful of strains that may not be representative of the species. The most frequently used strains in research are the IOWA II and Moredun. The aim of this study was to determine the extent to which these strains vary in their infectivity of HCT-8 cells. HCT-8 cells were infected with IOWA II propagated in a calf (IOWA II-Calf), IOWA II propagated in a mouse (IOWA II-Mouse), and the Moredun propagated in a sheep. A quantitative RT-PCR approach was used to determine the expression of seven genes, two of which were markers for sexual stages. Fluorescence microscopy was used to enumerate life cycle stages. Sex stages appeared earliest in IOWA II-Mouse, followed by IOWA II-Calf, and Moredun. IOWA II-Calf had a larger number of trophozoites/meronts at 24 h p.i. than Moredun. These data show that *C. parvum* infectivity of HCT-8 cells is affected by the parasite strain and the host species used to propagate the parasite prior to infection.

Introduction

Cryptosporidiosis is a life-threatening diarrheal disease caused by *Cryptosporidium*. It does not have an effective treatment. Two species of *Cryptosporidium* contribute to disease in humans, *C. parvum* and *C. hominis* (Xiao et al., 2004). *C. hominis* infects humans exclusively, whereas *C. parvum* is found in humans and ruminants. *Cryptosporidium hominis* has proven difficult to propagate which has limited

research on this species. *Cryptosporidium parvum* can be propagated in neonatal calves and mice and is available commercially to researchers. However, relatively few strains of *C. parvum* are available for research with Iowa II and Moredun the primary strains available commercially in the US and Europe, respectively. Limiting most research to one species of *Cryptosporidium*, and to just a few strains within that species, creates a bias that may result in erroneous conclusions on the biology and pathogenesis of *Cryptosporidium*.

Over the past decade, experimental studies of *C. parvum* and *C. hominis* in humans and in cell culture have provided important information about *Cryptosporidium* infectivity. Hashim and others (2004) showed that *C. parvum* was less infective than *C. hominis* in an *in vitro* cell culture model. *C. hominis* has been shown to elicit an immediate IgG response in clinical studies, whereas *C. parvum* has not (Chappell et al., 2006; Okhuysen et al., 1998). The ID_{50} was also found to be low for *C. hominis* (10-83) and *C. parvum* (9-1042), but they have not been directly compared in a study. It has been shown previously that *C. parvum* strains do vary in their virulence for humans (Okhuysen et al., 1999).

This study addresses the infectivity and development of the IOWA and Moredun strains of *C. parvum* in cell culture. HCT-8 cells were infected with both strains and infection development was monitored using fluorescence microscopy to enumerate life stages and qPCR to quantify the expression of developmentally regulated genes. The data shows that the Iowa II and Moredun strain differ in their infectivity of HCT-8 cells.

Materials and Methods

Experimental design

- An *in vitro* cell culture model of infection was used to compare the infectivity of three commercially available isolates of *C. parvum*. These included the Iowa II strain propagated in a calf, the Iowa II strain propagated in a mouse, and the Moredun strain propagated in sheep. The hypothesis was that strains would vary in their infectivity.
- Infectivity was measured using quantitative RT-PCR to determine the expression of select genes or immunofluorescence microscopy to enumerate life cycle stages.

Source and enumeration of oocysts and microscopy

Purified calf- and mouse-passaged IOWA II strain *C. parvum* oocysts (Henceforth referred to as IOWA II-Calf and IOWA II-Mouse) were obtained from Waterborne, Inc. (New Orleans, LA). Sheep-passaged Moredun strain oocysts (Henceforth referred to as Moredun) were obtained from the Moredun Research Institute, Edinburgh, Scotland. Oocyst stocks were enumerated by immunofluoresence microscopy. Purified oocyst stock was diluted in PBS, placed on the well of a microscope slide, and allowed to air dry. One drop of Crypt-a-Glo™ antibody reagent (Waterborne Inc, LA) was applied to air dried oocysts under low light. The slides were incubated in a humid chamber at 37°C for 30 minutes. Slides were rinsed two separate times in PBS in gyrating Coplin jars. After the slides dried, No-Fade mounting medium (Waterborne Inc, LA) was added to the well. A coverslip was applied and fixed in position using nail polish. The oocysts were examined

using a microscope with epifluorescence attachments with FITC specific excitation and emission filters at 400x magnification.

In vitro cell culture infection

Human ileocecal adenocarcinoma (HCT-8; ATCC CCL-244) cells were seeded into 6-well cell culture plates for the quantitative real-time PCR experiment or in Lab-Tek® II 4-chamber slides (Thermo Fisher Scientific, Waltham, MA) for immunofluorescence labeling. Cells were grown in filter sterilized RPMI 1640 with L-glutamine, 10% Opti-MEM, 2% fetal cattle serum, 1 mM sodium pyruvate, and 1X antibiotic/antimycotic solution. The pH of the growth medium was 7.4. Cells were grown at 37°C in a 5% CO_2 humidified incubator.

Oocysts were excysted prior to infection. For the qPCR experiment, 1.2×10^6 oocysts were used and 1.2×10^5 oocysts were used for the immunofluorescence labeling experiment. Oocysts were centrifuged at 20000 x g, the supernatant was removed, and the pellet was resuspended in 200 µL of ice cold 10% bleach. Following a 10 min incubation at 4°C, the oocysts were washed twice in PBS, resuspended in 100 µL 0.8% sodium taurocholate and incubated at 37°C for 30 min. Following incubation, the oocysts were washed once in PBS and resuspended in 500 µL of infection media (RPMI with 10% Opti-Minimum Essential Medium [InVitrogen, Carlsbad, CA], 10% Fetal Cattle Serum, 1X antibiotic/antimycotic solution [100X 10000 Units penicillin, 10 mg streptomycin, and 25 µL amphotericin B per mL], 1 mM sodium pyruvate, 50 mM glucose, 35 µg/mL ascorbic acid, 1 µg/mL folic acid, 4 µg/mL 4-aminobenzoic acid, 2 µg/mL calcium pantothenate). The excysted oocyst suspension was used to infect confluent HCT-8 cell monolayers in 2

mL total volume of infection media. Infected cells were incubated at 37°C in a 5% CO_2 humidified incubator.

Gene expression

The targeted genes can be categorized into two primary groups. They were either associated with sexual development or associated with host/parasite interactions. *Cryptosporidium* oocyst wall protein (COWP) 1 and COWP8 are known to be exclusively expressed during macrogamont stage of the life cycle (Spano et al., 1997a). Thrombospondin type 2 (TSP2) is thought to be associated with sexual development due to its expression at later stages of infection (Deng et al., 2002), but its function is not fully known. TSP8 is a micronemal protein that is translocated to the parasite surface upon interaction with host cells, therefore it is thought to have a role in attachment and invasion (Putignani et al., 2008). Thrombospondin related adhesive protein C1 (TRAPC1) contributes to gliding motility and adhesion to host cell surfaces (Deng et al., 2002; Spano et al., 1998; Sultan et al., 1997). GP900 is known to act as a ligand for a host cell receptor that is part of the attachment and invasion process (Barnes et al., 1998). Actin is a structural protein, which is important in the gliding motility of sporozoites and facilitates their invasion ability (Gordon and Sibley, 2005). It is constitutively expressed and has been used as a normalizing gene in previous studies (Abrahamsen and Schroeder, 1999).

A TaqMan® probe approach was used in qPCR (Applied Biosystems, Foster City, CA). The primer and probe sequences that were previously designed by the McEvoy lab are found in table 10.

Table 10. Primer sets and TaqMan® probes for targeted loci

Primer Sets	Sequence
18SqPCR-F	5'-CAG CTT TAG ACG GTA GGG TAT TGG-3'
18SqPCR-R	5'-CGA ACC CTA ATT CCC CGT TAC- 3'
TRAPC1qPCR-F	5'-GGA GGA GGA TGA GAC AAA TTA TCA AT-3'
TRAPC1qPCR-R	5'-TTT CTT GAA CAT ATT CTG AGT CTT GATCT-3'
COWP8F-69	5'-AAG AAT GTT GAG ATC GAC CCA GTT-3'
COWP8R-69	5'-GCA CAT TTG ACC GTC AGT TAA AGT-3'
COWP1F-75	5'-GAA ACT GCG AAC AAC TCA TTC G-3'
COWP1R-75	5'-CAC CAG CCT GAG GTC TAT AAT ATC C-3'
ACTINF56	5'-GGC AGG TGT TGC AGG TGA T-3'
ACTINR56	5'-GAC CAA CGA TCG ATG GGA AA-3'
TSP8F-66	5'-CTC CTT GCT CAG CTT CTT GTG A-3'
TSP8R-66	5'-GGA GCA GAG TGG GTC AAT TCT C-3'
GP900qPCR-F	5'-CAT CCA CCG GGT TTA GAG TTG-3'
GP900qPCR-R	5'-GGG TTG AAT GGA CAA TTA GGG TAT-3'
TSP2qPCR-F	5'-AGC ATG TGA CCA TTC GGT TTC-3'
TSP2qPCR-R	5'-TTT GCG CCG CTA CTA TTG TTA TAC-3'
TaqMan® Probe	Sequence
18SqPCR PROBE	VIC CCG TGG CAA TCA CMGBNFQ
TRAPC1 PROBE	6FAM CTT TGA TCA ATC TTC TGC TACTMGBNFQ
COWP8 PROBE	6FAM CTG TAT GCC CAT CAG GATMGBNFQ
COWP1 PROBE	6FAM TCA CCA CCA ACG ATG GMGBNFQ
ACTIN PROBE	6FAM ACG CTC CAA GAT GTGMGBNFQ
TSP8 PROBE	6FAM AGG GCT TAC AAT CAG GACMGBNFQ
GP900 PROBE	6FAM TCT ATC ACT GGT CTC CCA ACMGBNFQ
TSP2 PROBE	6FAM TCA GGA CGA CTG TTA TAC TAMGBNFQ

RNA extraction

Infections were stopped by replacing infection media with the same volume of RNAlater RNA stabilization reagent (QIAGEN, Valencia, CA). RNA was extracted using an adapted protocol from the RNeasy® Plus Mini Kit (QIAGEN). Following the removal of

RNAlater, 600 µL RLT Plus lysing buffer with 1% beta-mercaptoethanol, which immediately inactivates RNases to ensure isolation of intact RNA, was added to each well. Wells were scraped and the homogenate was transferred to a QIAshredder column (QIAGEN). The QIAshredder column quickly homogenizes the lysate further, resulting in a higher RNA yield. Columns were centrifuged at maximum speed and the flow-through was transferred to a gDNA eliminator column to remove genomic DNA. The gDNA eliminator columns were centrifuged for 30 seconds at 9300 x g. To provide optimal binding conditions for RNA, a 500 µL aliquot of RNase free 70% ethanol was added to the flow-through and 700 µL of the sample was added to an RNeasy spin column and centrifuged at 9300 x g. Any remaining sample was added to the RNeasy column and centrifuged. The RNeasy column binds all of the RNA to its membrane. Any flowthrough during the washing steps was discarded because it contained the removed contaminants. To remove any DNA contamination, the RNeasy column was with DNase. A 350 µL aliquot of Buffer RW1 was added to the RNeasy spin column to improve RNA binding, and columns were centrifuged at 9300 x g for 15 seconds. An 80 µL aliquot of DNase treatment (QIAGEN) to each column and they were incubated at room temperature for 15 minutes. DNase treatment was composed of 10 µL DNase stock and 70 µL Buffer RDD per tube. The column was washed three times to remove contaminants. A 350 µL aliquot of Buffer RW1 was added to the column and centrifuged at 9300 x g for 15 seconds. Buffer RPE was added twice in 500 µL aliquots and centrifuged at 9300 x g, first for 15 seconds and then for 2 minutes. The long centrifugation dries any residual ethanol on the membrane. If any flow-through contacted the RNeasy column, it was placed in a new 2 mL collection tube

and centrifuged at max speed for 1 minute to prevent interference in downstream reactions. To elute the RNA, the RNeasy column was placed in a 1.5 mL microcentrifuge tube and 50 µL of RNase-free water was added to the columns. The columns were centrifuged at 18300 x g for 1 minute. The RNA flow-through was placed at -80°C until required. To determine the purity, RNA was analyzed with a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA).

Complementary DNA synthesis

RNA (2µg), and random hexamers (2µg) (Promega, Madison, WI) were combined in a sterile RNase free microcentrifuge tube in 15 µL of RNase free water. To denature the RNA, the tubes were heated to 70°C for five minutes before being cooled to 4°C. A master mix was prepared containing 200 units of Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega), 1X M-MLV buffer (Promega), 0.5 mM dNTP (Promega), and RNase free water to increase the volume to 10 µL. A master mix without reverse transcriptase was used as a control for genomic DNA. A 10 µL aliquot of the appropriate master mix was added to the correct tube containing RNA template. The reverse transcription was carried out at 37°C for 1 hour in a thermocycler followed by a 10 minute incubation at 70°C to inactivate the reverse transcriptase. Samples were maintained at 4°C until needed.

Quantitative RT-PCR

The TaqMan® probes (Applied Biosystems, Foster City, CA), previously designed by the McEvoy lab, were diluted to a 2.5 µM working stock. The cDNA was diluted according to the primer efficiency testing results. The IOWA II-Calf and IOWA II-Mouse strains had

optimal results with a 1:256 cDNA dilution for the 18S loci and a 1:4 cDNA dilution for all other loci. The sheep-passaged Moredun strain had better results with a 1:64 cDNA dilution for 18S and undiluted cDNA for the remaining loci. The qPCR master mix was composed of 1X Gene Expression master mix (Applied Biosystems), 180 nM 5' and 3' primers (Table 10), 250 nM TaqMan® probe (Table 10), and RNase free water up to a volume of 18 µL per sample. The quantitative PCR conditions consisted of an initial uracil-DNA glycosylase incubation for 2 min at 50°C, ultra-pure AmpliTaq Gold DNA Polymerase activation for 10 min at 95°C, followed by 40 cycles of denaturing for 15 seconds at 95°C and annealing and extending for 1 min at 60°C. Reverse transcriptase positive controls from uninfected wells, reverse transcriptase negative samples for each time point, and water controls were included to determine if the samples were free of contamination.

The Applied Biosystems (Foster City, CA) 7500 real-time PCR software was used to analyze the raw qPCR data. A comparative cycle threshold (Ct) method was used to analyze the data. Δ Ct is achieved by subtracting the ct of the target gene from the ct of the normalizing gene. Δ Δ Ct was calculated to measure the fold change in expression by arbitrarily selecting a calibrator. The calibrator serves as the physiological reference and enables the gene expressions changes in different over time to be compared to one another. We graphed our data using GraphPad Prism 5 (La Jolla, CA).

Immunofluorescence microscopy

A t 2, 6, 12, 24, 48, and 72 h post infection, the media is removed from the slides previously mentioned in section 3.3, and the monolayers were fixed with 1 mL of 4% formaldehyde for 30 min. Following fixation monolayers were washed twice with 1 mL

PBS. The monolayers were permeablized with 1 mL of 1% Triton X-100 in PBS at room temperature for 10 min. The Triton was removed and the monolayers were blocked with 1 mL of 0.5% cattle serum albumin (BSA) in PBS at room temperature for 10 min. BSA was removed and the monolayers were incubated in 1 mL of 1 µg/mL of conjugated Lectin VVL-biotin at room temperature for 30 minutes. The Lectin-VVL biotin was removed and the monolayers were washed three times with 1 mL of PBS. The monolayers were incubated with 1 mL of 1 µg/mL of conjugated Streptavidin Alexa Fluor 555 at room temperature for 30 min in the dark. Wells are washed twice with 1 mL PBS the chamber was removed and slides were mounted with 8 µL of No-Fade mounting medium (Waterborne). Slides were examined using at 600x magnification using an Olympus BX61 microscope with epifluorescence attachments and excitation and emission filters for TRITC. To enumerate the trophozoites/meronts, 20 fields were randomly chosen for each well. The total number of infected cells was counted and measured using image analysis software. Round parasite stages that were 2-4 µM in diameter were classified as trophozoite/meronts.

Data analyses

There were duplicate infections and two wells for each infection at every h p.i. in both experiments. The duplicate wells were averaged for each infection and the average of both infections was used to calculate the Δ Δ Ct value. The variance for the Δ Δ Ct values between the duplicate infections for each h p.i. was graphically represented by error bars. Each infected well was enumerated twice to verify the numbers of total parasites and trophozoites/meronts.

Results

Intracellular gene expression

Real-time PCR was used to quantify expression of 7 genes using at 2, 6, 12, 24, 48, and 72 h p.i.. For all genes targeted, expression was higher for IOWA II strain than Moredun strain. As a result of the lower gene expression for the Moredun strain, higher concentrations of total RNA were required to obtain a detectable signal in qPCR.

Cryptosporidium oocyst wall protein (COWP) 1 and COWP8 were targeted as markers for sexual stage development. For IOWA II-Mouse, IOWA II-Calf, and Moredun, COWP8 was not expressed at significant levels until 24 hours and the peak expression was observed at 72 hours p.i. (Figure 7). Between 24 and 48 h p.i., there was a 30 and 425 fold increase in COWP8 expression for Iowa II-Calf and IOWA II-Mouse, respectively. COWP8 expression in Moredun was observed to decrease during the same time period.

COWP1 expression was determined for IOWA II-Calf and Moredun and showed similar patterns of expression to COWP8 (Figure 8). COWP1 expression increased 30 fold between 24 and 48 h and a further 2.4 fold between 48 and 72 h in IOWA II-Calf. Moredun showed 4.7 and 24 fold increases in COWP1 expression for the time periods 24-48 h and 48-72 h, respectively.

The function of *C. parvum* Thrombospondin 2 (TSP2) is not known; however, it appears to be associated with sexual stages of development (Deng et al., 2002). Similar to COWP1 and COWP8, peak TSP2 expression was observed at 72 h p.i. for all isolates tested (Figure 9). TSP2 expression increased 7.4 fold between 24 and 48 h and a further 1.6 fold between 48 and 72 h in IOWA II-Calf. IOWA II-Mouse showed a 28 fold increase between

24 and 48 h and a 3.9 fold increase from 48 to 72 h. Moredun showed a 1.1 fold decrease from 24-48 h and a 24.8 fold increase for 48-72 h.

Thrombospondin-related adhesive protein (TRAP) has been associated with mediating host-parasite interactions and gliding motility and is expected to be predominantly expressed during asexual stages (Baum et al., 2006; Spano et al., 1998; Sultan et al., 1997). TRAP expression was observed to vary among IOWA II-Mouse, IOWA II-Calf and Moredun (Figure 10). IOWA II-Mouse was the only isolate with detectable TRAP expression at 2 and 6 h with peak expression observed at 24 h for this isolate. Peak TRAP expression in IOWA II-Calf and Moredun was observed at 48 h.

Cryptosporidium parvum TSP8 is a micronemal protein with a putative role in attachment and invasion of host cells (Putignani et al., 2008). TSP8 expression peaked at 2 h for IOWA II-Mouse, IOWA II-Calf and Moredun and patterns of expression did not vary substantially among these isolates (Figure 11).

GP900 is a glycoprotein that has been shown to be involved in the attachment and invasion process (Barnes et al., 1998). GP900 expression patterns varied among the three isolates (Figure 12). Expression peaked at 12 h for the IOWA II-Calf and Moredun isolates, while it peaked at 24 h for the IOWA II-Mouse isolate. A second peak in GP900 expression was observed at 48 h for Moredun.

Actin is a major structural protein in *Cryptosporidium* (Gordon and Sibley, 2005). Actin expression was determined for IOWA II-Calf and Moredun isolates (Figure 13). The isolates showed similar actin expression patterns.

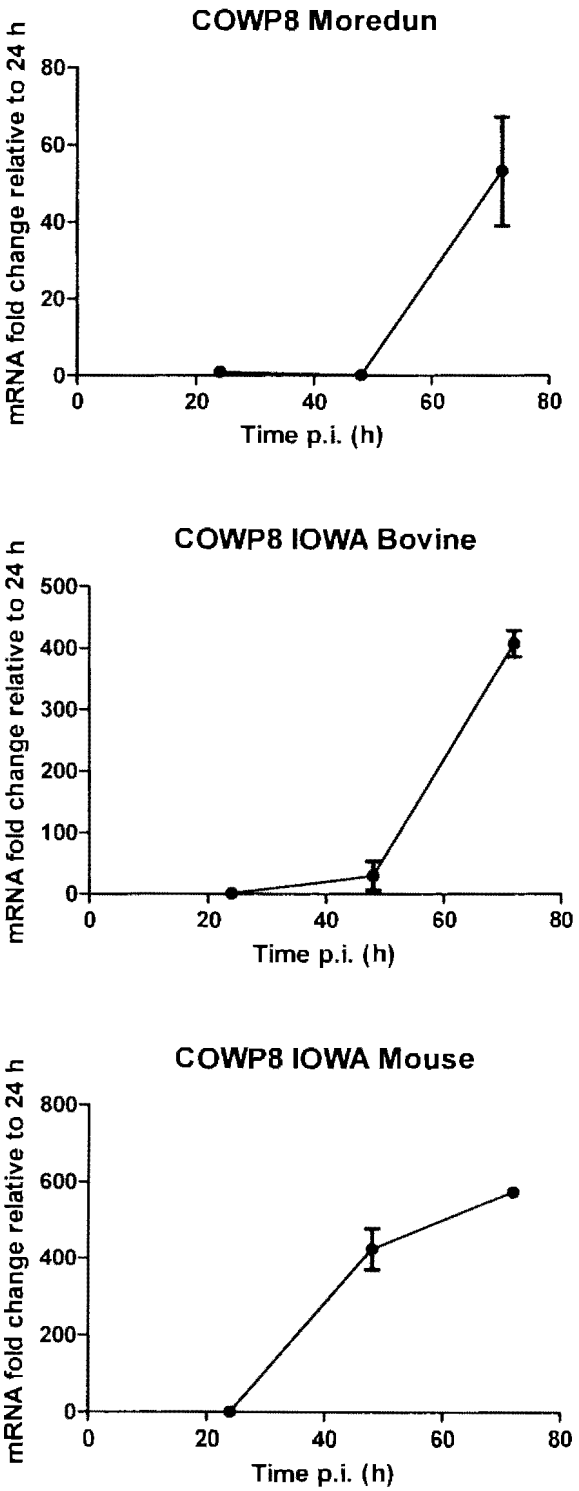

Figure 7. Gene expression levels for COWP8 relative to the 24 hour time point for the infection of HCT-8 cells using oocysts from *C. parvum* Moredun, IOWA II-Calf, and IOWA II-Mouse strains.

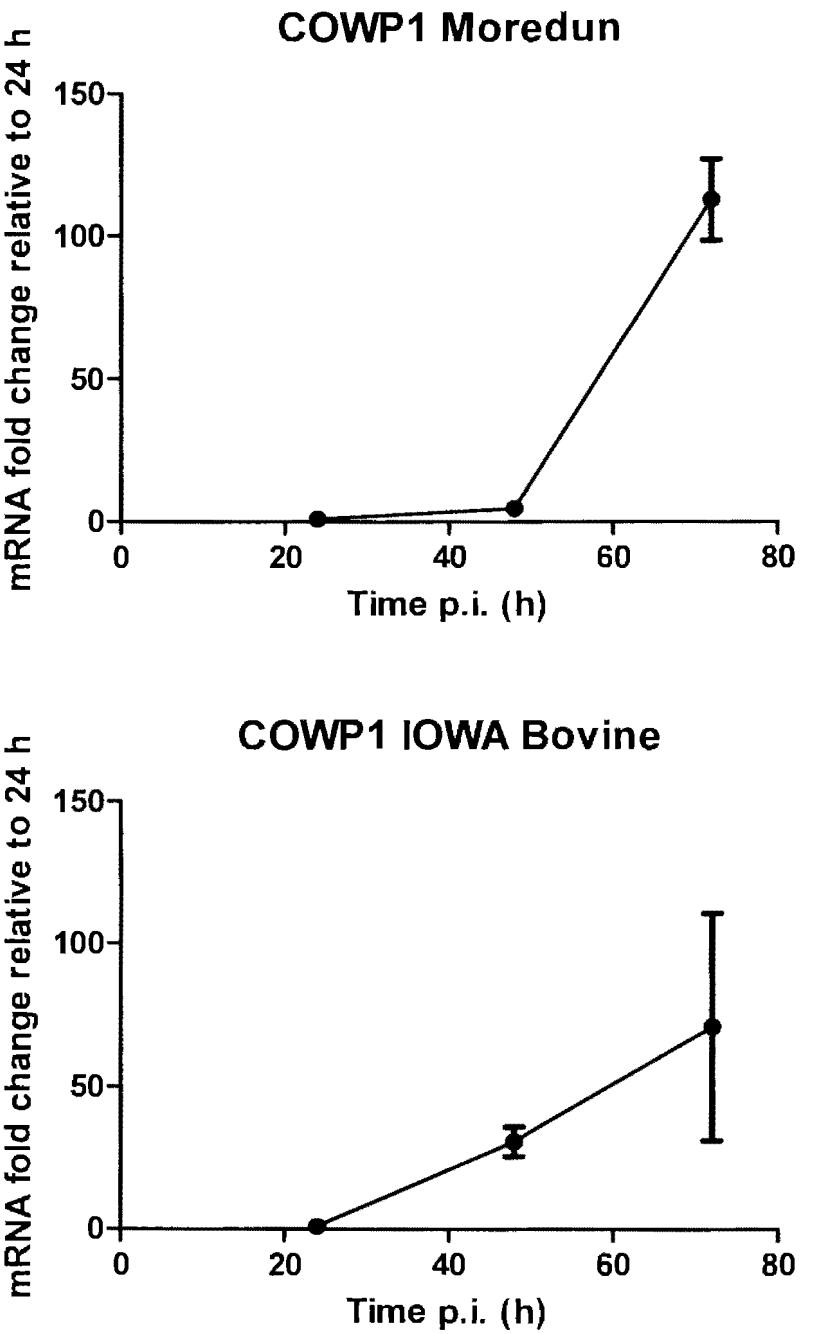

Figure 8. Gene expression levels for COWP1 relative to the 24 hour time point for the infection of HCT-8 cells using oocysts from *C. parvum* Moredun and IOWA II-Calf strains.

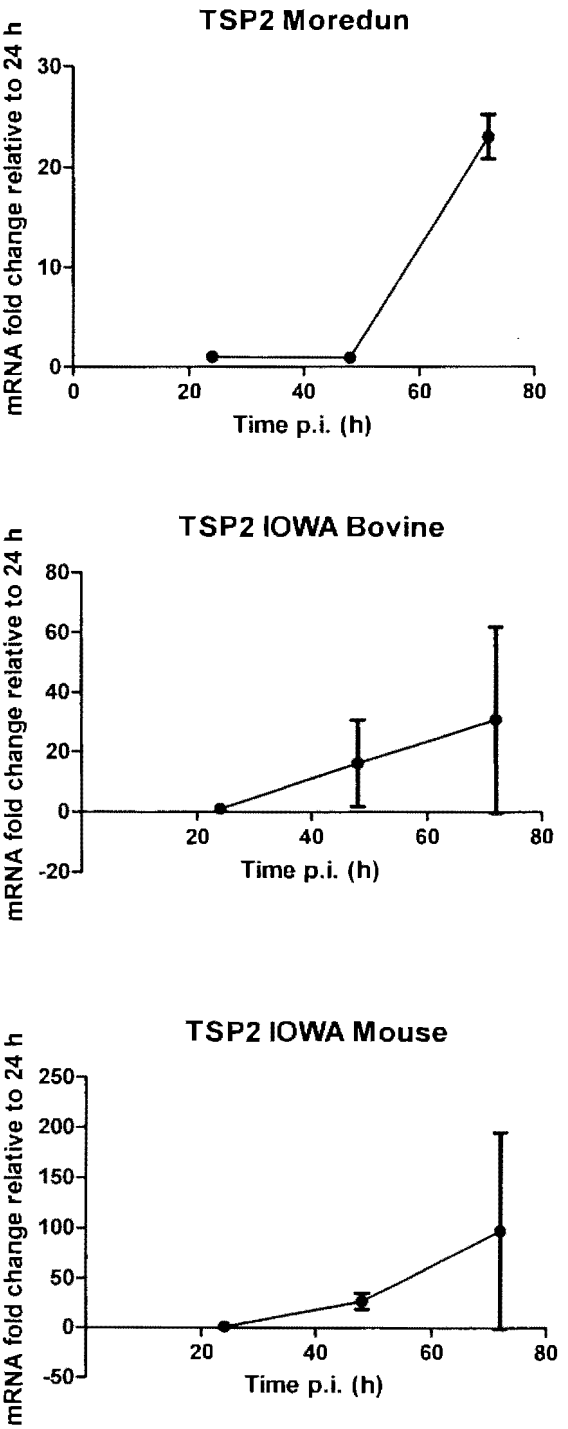

Figure 9. Gene expression levels for TSP2 relative to the 24 hour time point for the infection of HCT-8 cells using oocysts from *C. parvum* Moredun, IOWA II-Calf, and IOWA II-Mouse strains.

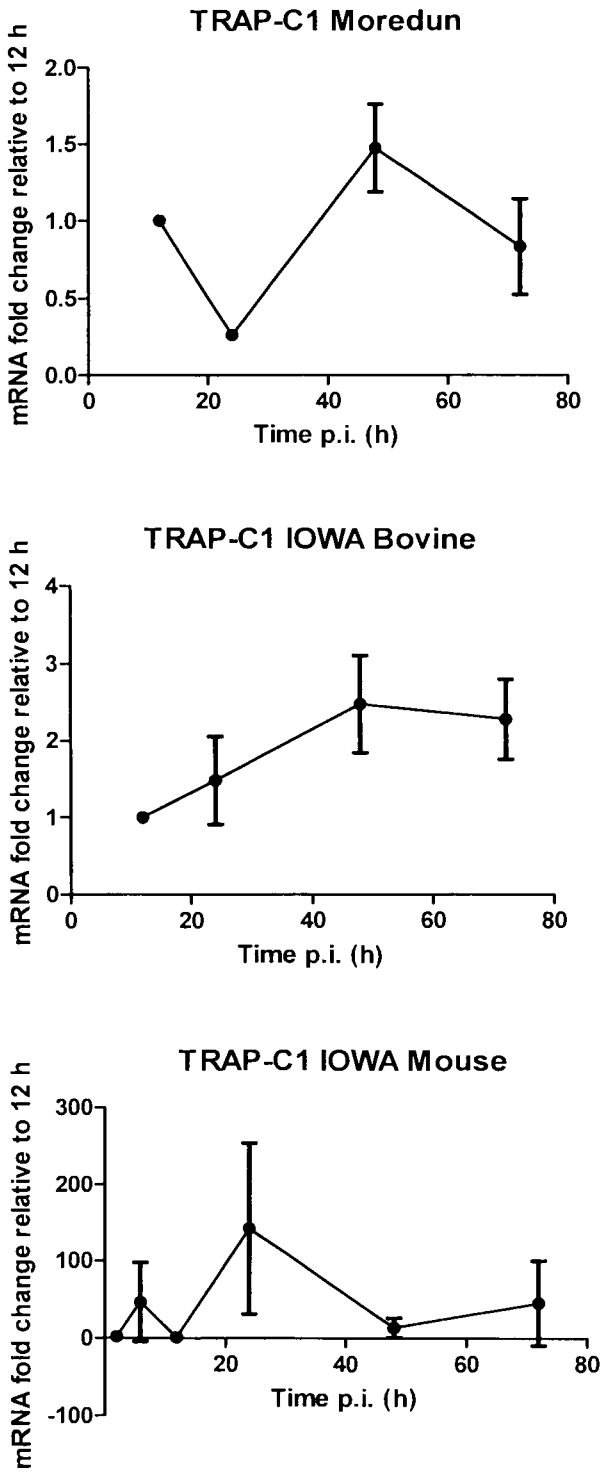

Figure 10. Gene expression levels for TRAP-C1 relative to the 12 hour time point for the infection of HCT-8 cells using oocysts from *C. parvum* Moredun, IOWA II-Calf, and IOWA II-Mouse strains.

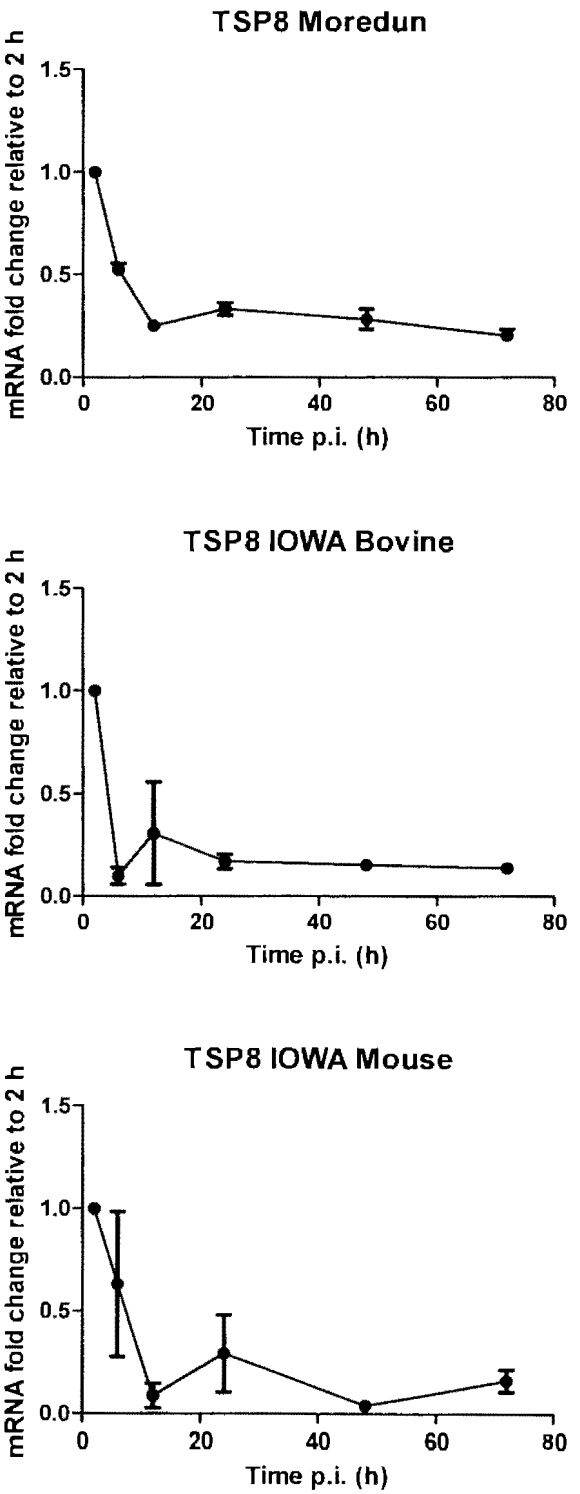

Figure 11. Gene expression levels for TSP8 relative to the 2 hour time point for the infection of HCT-8 cells using oocysts from *C. parvum* Moredun, IOWA II-Calf, and IOWA II-Mouse strains.

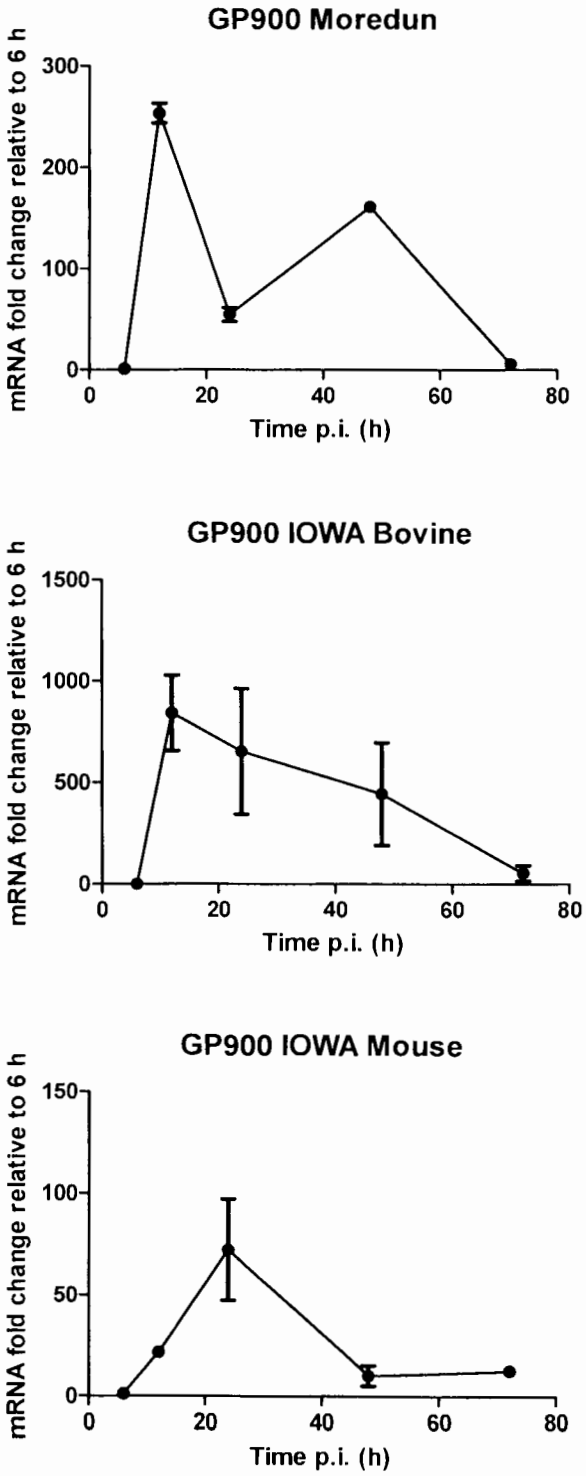

Figure 12. **Gene expression levels for GP900 relative to the 6 hour time point for the infection of HCT-8 cells using oocysts from *C. parvum* Moredun, IOWA II-Calf, and IOWA II-Mouse strains.**

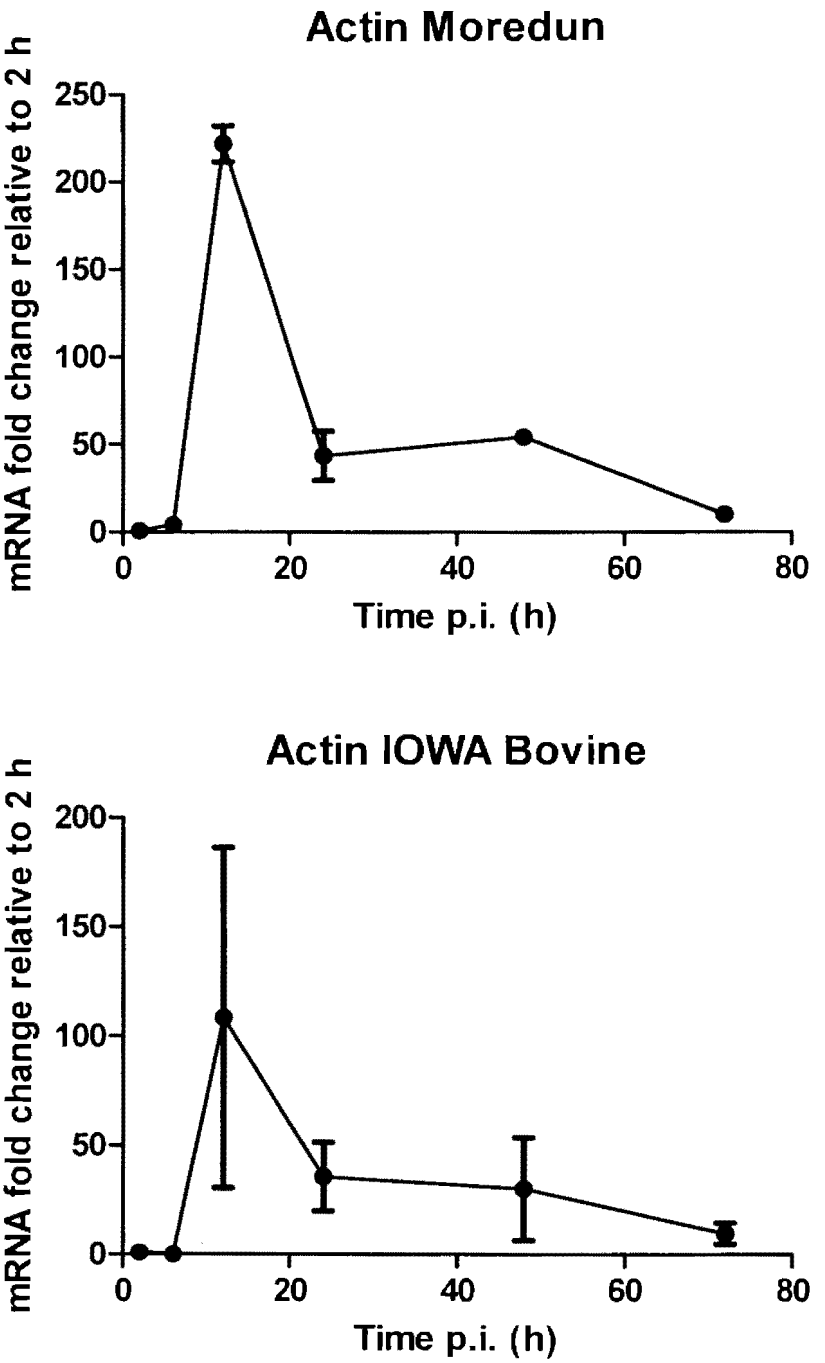

Figure 13. Gene expression levels for Actin relative to the 2 hour time point for the infection of HCT-8 cells using oocysts from *C. parvum* Moredun and IOWA II-Calf strains.

Immunofluorescence microscopy

The lectin-VVL assay detects glycoproteins on zoites (sporozoites and merozoites) and in intracellular parasite stages. The assay was used to quantify IOWA II-Calf and Moredun strains in HCT-8 cells at 24 h p.i. The size and shape of the parasites was determined using image analysis software. Round parasite stages in the range 2-4 µM were identified as trophozoite/meront stages. Trophozoites measure 2.7 x 2.7 µM in diameter and meronts measure 3.1 x 2.8 µM in diameter, so we were looking for infected foci that had a size between 2-4 µM (Thompson et al., 2005). Parasites less than 2 µM were determined to have not yet entered the trophozoite/meront stage.

The total number of parasites in 20 fields was greater for IOWA II-Calf than Moredun (Table 11). Furthermore, a higher prevalence of trophozoite/meront stages was observed in IOWA II-Calf (44%) than Moredun (9%). Representative images for each strain at 24 h p.i. are shown in Figures 14 and 15.

Table 11. A comparison of the trophozoites/meronts count between two *C. parvum* strains using fluorescent microscopy at 24 hours.

C. parvum strain	Total # of parasites	# of trophozoites/meronts	% trophozoites/meronts
IOWA II-Calf	191	84	44%
Moredun	157	14	9%

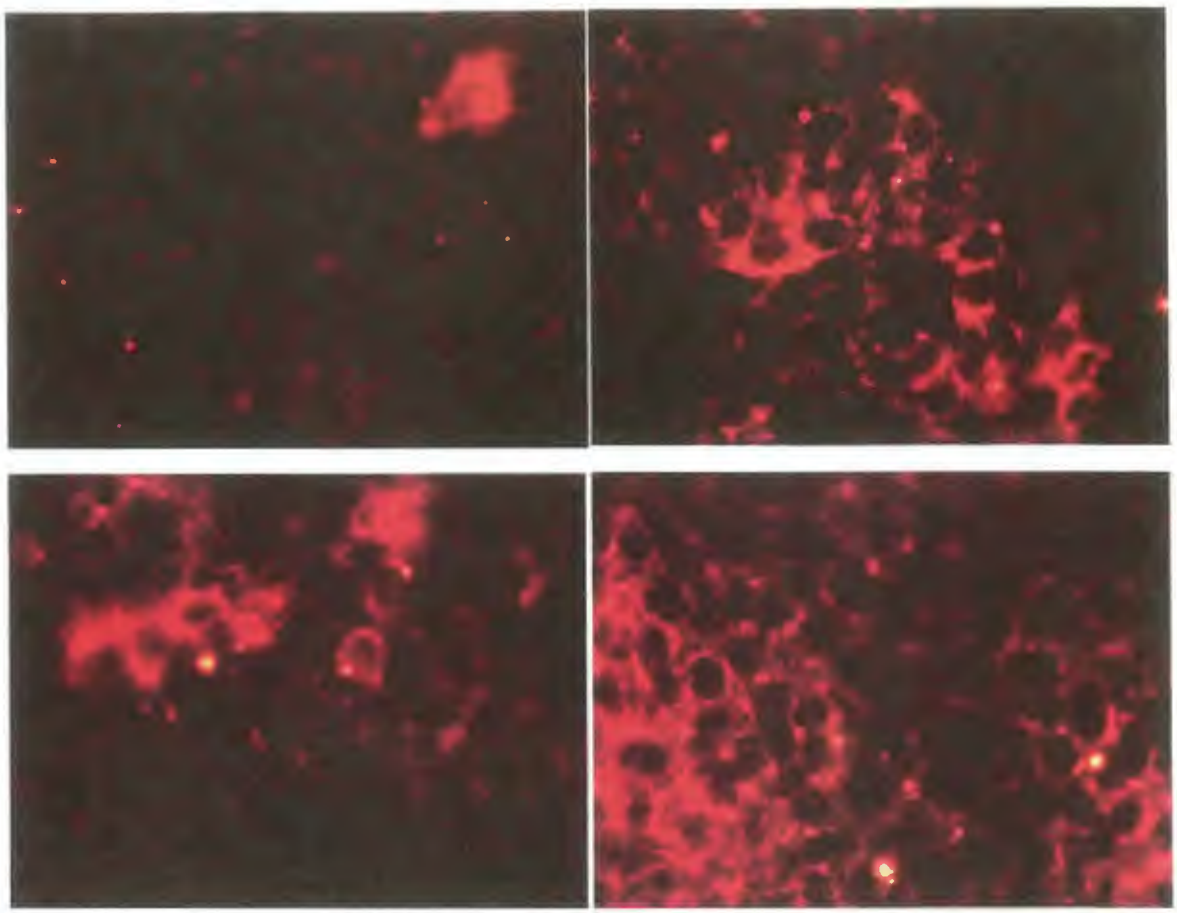

Figure 14. **Lectin-VVL staining of primary HCT-8 cell monolayers infected with Moredun *C. parvum*. Images were taken using a TRITC filter and fluorescent microscopy with a 60X lens. Total magnification was 600X.**

Discussion

This study has shown that the *in vitro* infectivity of *C. parvum* varies intraspecifically and this variation may be the result of *C. parvum* strain differences and/or differences in the host used for propagation. This finding has implications for our understanding of *C. parvum* biology and suggests that care should be taken when interpreting data from *C. parvum* infectivity studies.

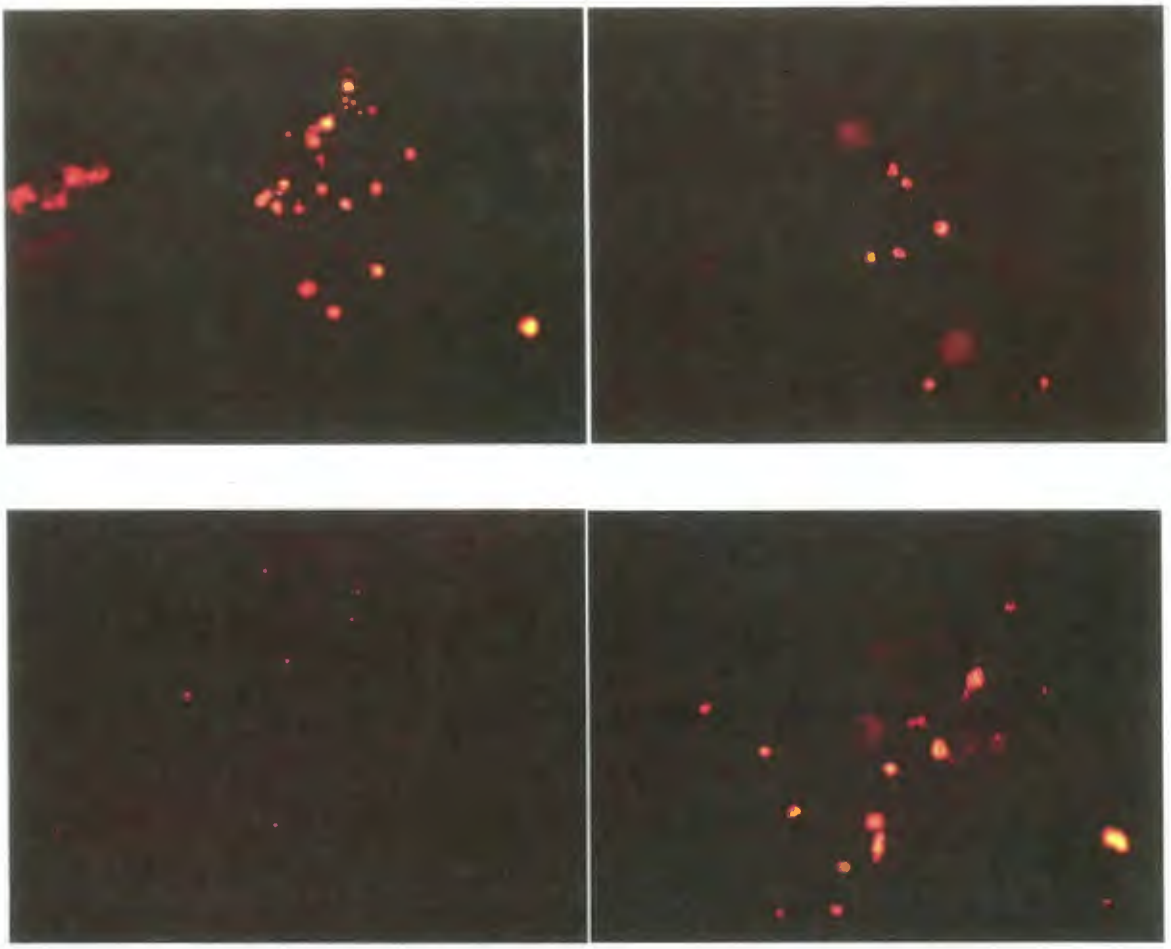

Figure 15. Lectin-VVL staining of primary HCT-8 cell monolayers infected with IOWA II strain *C. parvum*.

The IOWA II strain of *C. parvum* resulted in a greater number of trophozoite/meront stages 24 h p.i. and reached sexual stages earlier than the Moredun strain. Contrary to our findings, previous studies by Okhuysen and others determined the infectivity of both the IOWA II and Moredun strains in human subjects (DuPont et al., 1995; Okhuysen et al., 1999; Okhuysen et al., 2002) and they found that the Moredun strain had a shorter incubation period and more severe clinical symptoms than the IOWA II strain. Their infections demonstrated that the ID_{50} was lower for the IOWA II strain (87 oocysts) compared to the Moredun strain (300 oocysts) (Okhuysen et al., 1999; Okhuysen

et al., 2002). These apparently contradictory findings suggest that *in vitro* infectivity of HCT-8 cells may not be a good predictor of infectivity *in vivo*. Alternatively, given the time elapsed between the studies by Okhuysen and the present study, the IOWA II strain and the Moredun strain may have undergone changes in their infectivity. Interestingly, the Moredun strain was until recently only propagated in sheep; however, for the past three years it has been propagated in both sheep and calves. Dr. Elisabeth Innes at the Moredun Research Institute has reported observing a reduction in infectivity of the Moredun strain during that period (Innes, 2009).

COWP1 and COWP8 are *Cryptosporidium* oocyst wall proteins that have been shown previously to be expressed only during sexual stages (Spano et al., 1997a). As such, their expression levels represent a useful marker for sex stage development. Sex stages appeared at 48 h for the IOWA II strain but did not appear until 72 h for the Moredun strain. Our COWP1 expression data for the IOWA II strain are similar to those previously reported by Abrahamsen and Schroeder (1999). Those authors observed COWP1 expression increasing at 48 h, similar to the present study, before decreasing at 72 h. The expression peaked for both strains at 72 h in the present study.

Actin expression levels were similar for the IOWA II and Moredun strains. Furthermore, the expression patterns were similar to the pattern previously reported by Abrahamsen and Schroeder (1999). The expression levels for both strains are similar to this study and have their peak expression at 12 hours.

The host used to propagate the *C. parvum* IOWA II strain affected infectivity in cell culture. The difference was less pronounced than the difference observed between the

IOWA II and Moredun strains. Different suppliers were used for the IOWA II-Calf and IOWA II-Mouse propagated isolates. A recent study showed that IOWA II isolates propagated by different labs and commercial suppliers had evolved to become genetically distinct, making them essentially different strains (Cama et al., 2006). However, in a follow up interview of the suppliers used for the present study it was determined that Bunchgrass Farms supplied Waterborne Inc. with the oocysts that were used to infect mice (Prichard, 2009). The IOWA II-Mouse isolate supplied by Waterborne Inc. was therefore passaged in a calf by Bunch Grass Farm immediately prior to the mouse passage. This greatly reduces the probability that genetic differences contributed to the observed phenotypic differences in cell culture. Propagation in different hosts may, however, cause changes in the epigenetic landscape of the isolate, and these changes may affect the behavior of the isolate in subsequent infections. While, little is currently known about *Cryptosporidium* epigenetic regulation, such a finding would have significant implications for zoonotic transmission of *C. parvum*.

In summary, our data found intraspecific variation in *C. parvum in vitro* infections. Other studies should keep these findings in mind when choosing their experimental design and interpreting their results.

GENERAL DISCUSSION

Chapter 1 has addressed intraspecific heterogeneity in the important human and animal pathogen, *C. parvum*. We have found that the *C. parvum* population causing human and animal disease in the Midwestern states of North Dakota, Minnesota, and Wisconsin is panmictic with no evidence for substructuring by host or geography. Panmixia requires frequent sexual recombination which in turn requires co-infection of a host with two different *C. parvum* strains. Therefore, transmission dynamics of *C. parvum* in the region must result in frequent co-infections with different strains. It has previously been reported that a high incidence of co-infections occurs in areas with high rates of transmission (Tanriverdi et al., 2006). The lack of host substructuring in the present study suggests that transmission is likely to be zoonotic. Zoonotic transmission is further supported by the relatively high incidence of *C. parvum* among humans in Wisconsin (Feltus et al., 2006) and Minnesota (McEvoy et al., 2007). Anthroponotic transmission is also possible; however, most cases of anthroponotic transmission in the literature have been associated with poor hygiene and/or urban areas where there are dense populations (Hunter et al., 2004; Newman et al., 1994).

A potential limitation of the present study is its limitation to clinical cases. To date, reports of *C. parvum* infectivity have been limited to clinical cases and little information exists regarding subclinical infections in humans and cattle. Tanriverdi and others found genetically distinct populations in cattle that were established over a short period of time. The authors proposed that that these distinct populations varied in their virulence, though no further evidence was provided to support that claim (Tanriverdi et

al., 2006). Subpopulations in humans may also occur based on variations in virulence traits; however, further research would be required to test this hypothesis.

Chapter 2 addressed the effect of propagation host and parasite strain on *C. parvum* infectivity in an *in vitro* model of infection. The IOWA II strain, which is commercially available in the US, developed more rapidly in HCT-8 cells than the Moredun strain, which is commercially available in the UK. The IOWA II strain reached sexual stages approximately 24 h before the Moredun strain. To a lesser extent, differences were also observed between IOWA II strains propagated in different hosts. IOWA II propagated in a mouse reached sexual stages earlier than IOWA II propagated in a calf. The observed differences have significant implications for how we interpret research on this important human pathogen. Most of what we know about the infectivity and basic biology of *Cryptosporidium* has been based on research using a few commercially available strains of *C. parvum*, with the assumption that conclusions reached using one strain can be extended to all strains of the species.

Observed differences in infectivity between strains could be due to genetic differences. However, Bankier and others determined that of the 13 predicted genes on chromosome 6 with published sequences, 10 were identical for the Moredun and IOWA II strains, and the other 3 only had one or two amino acid differences (Bankier et al., 2003). This suggests that that IOWA II and Moredun are genetically similar and that genetic differences are unlikely to explain to observed differences in infectivity.

In the absence of a genetic explanation for the observed phenotypes, epigenetic regulation must be considered. All three isolates used in this study were propagated in

different hosts (mouse, calf and sheep), with each host creating a different environment for the parasite. These different environments could have influenced the epigenetic landscape of *C. parvum*, affecting gene regulation and the phenotype of subsequent developmental stages. Epigenetic regulation has been found to influence gene expression in other apicomplexans and could be the primary cause of the variability among different *C. parvum* strains. Epigenetic regulation is a reversible change in the chromatin and nuclear location of genes (Lopez-Rubio et al., 2007). This change can create an up or down regulation in gene expression. Studies have shown that *Plasmodium falciparum* and *Toxoplasma gondii* are possibly epigenetically regulated. Using acetylation-specific antibodies and immunoblotting, Miao and others found that some histones for *P. falciparum* increased in acetylation at the trophozoite stage, whereas others were throughout the erythrocytic cycle (Miao et al., 2006). Gissot and others found histones with extensive methylation and acetylation in *T. gondii*, which were directly linked to active promoters that were 5′ orientation specific (Gissot et al., 2007). Similar upstream 5′ regions were found by Mullapudi and others in *C. parvum* (Mullapudi et al., 2007). Most of the details for this type of regulation are unknown; specifically its physiological influence; however, epigenetic regulation cannot be discounted as a cause of the observed phenotypic differences in our study.

Together, the findings from the two studies support the hypothesis that *C. parvum* is a heterogeneous species. Although the *C. parvum* population in the upper Midwest is freely mating, populations in other parts of the world have been described as clonal or epidemically clonal. Therefore, local factors affecting transmission dynamics are likely to

be important. At the individual isolate level, genetic similarity does not necessarily translate to similar infection phenotypes and this needs to be considered when interpreting research findings.

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