Quantitation of *Cryptosporidium hominis* Oocyst Shedding in a Rodent Model of Human Cryptosporidiosis by Real Time Polymerase Chain Reaction

By

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ABSTRACT
A real time or quantitative polymerase chain reaction (rtPCR or qPCR) was developed to quantitate Cryptosporidium hominis oocysts shed in the feces of experimentally infected gerbils serving as a model for human cryptosporidiosis. Primers specific for the Cryptosporidium oocyst wall protein (COWP), a gene with 99% conservation between C. hominis and C. parvum, were used to detect DNA extracted from both C. hominis and C. parvum. The concentration of oocysts in the experimental fecal samples was calculated by sequence detection software, comparing these samples to standard curves generated from DNA extracted from purified C. parvum or C. hominis oocysts. The data show a trend of similar quantities when comparing the number of oocysts per ml found using qPCR to the number counted microscopically by immunofluorescence assay. With further optimization, this protocol should be useful for quantitating oocysts in experimental fecal samples from other animal models of cryptosporidiosis resulting from C. hominis and C. parvum infection.
CONTRIBUTIONS

My own
In this project, I contributed to this group project as a project director. Though I did not perform the initial literature search, I performed subsequent literature searches and reviewed the papers previously found to determine the parameters to be used for quantitation of Cryptosporidium sp. oocysts through real-time polymerase chain reaction (rtPCR).

I prepared all samples, purified, fecal, and experimental, for DNA extraction before following an adaptation of a protocol already used in the laboratory. The original protocol for DNA extraction had already been developed for use in this laboratory. However, through literature searches it was determined that some changes needed to be made to this protocol. Deborah Schaefer and I collaborated in an attempt to find the best method of DNA extraction. Once the original protocol was changed, I adapted it to include crucial steps from the QiAamp® DNA Stool Mini Kit Handbook with one change (Protocol for Isolation of DNA from Stool for Pathogen Detection, 2001). I performed most of the DNA extractions myself, with a small amount of help from my fellow laboratory workers.

Once the DNA was extracted it was time to run the real-time or quantitative polymerase chain reactions (rtPCR or qPCR). I initiated contact with Ryan Sprissler at the Arizona Research Laboratories at the University of Arizona. He was able to explain how rtPCR worked as well as explain how to run the machine. After running two different plates in the rtPCR machine under the supervision of Arizona Research Laboratory employees, I was able to perform the remaining runs on my own. After each run was performed, I collected and analyzed the data. I discussed my results and ideas for the next step with Deborah Schaefer.

Deborah Schaefer
Deborah Schaefer performed the initial literature search, from which we have found several papers to be useful in protocol development. Aside from providing the initial protocols, which have since been adapted, Deborah has served as a collaborator and a backboard for bouncing ideas for development of the protocols and techniques utilized in this research. She also performed the initial microscopic counts for oocyst on the purified Cryptosporidium parvum and Cryptosporidium hominis oocysts used to create our purified and fecal DNA standard samples, as well as counting the oocysts present in the experimental samples to use as comparison for our rtPCR results.

M. Cerise
Once the protocol for DNA purification was established for the laboratory, Cerise assisted in extracting DNA from the rehydrated fecal samples.

Paige Chance
Once the protocol for DNA purification was established for the laboratory, Paige Chance assisted in extracting DNA from the rehydrated fecal samples.
INTRODUCTION

Statement of purpose
To develop a laboratory protocol that can be used to extract DNA from Cryptosporidium parvum and C. hominis oocysts shed in feces by Mongolian gerbils in experimental trials, as well as develop a protocol that is more efficient and effective in quantitating the number of C. parvum and C. hominis oocysts shed during these trials using real-time or quantitative polymerase chain reactions (rtPCR or qPCR).

Statement of relevance
According to the World Health Organization (WHO), diarrheal disease is the fifth leading cause of death worldwide after cardiovascular and respiratory diseases. Though this disease is the fifth cause of death in middle income countries and the second in low income countries, it is not found in the top ten causes of death in high income countries (World Health Organization, 2011).¹ Death is the most extreme result of diarrheal disease. Malnutrition, stunted growth and cognitive impairment have been observed in many cases involving chronic diarrheal disease in young children (Operario and Houpt, 2011). In many cases of diarrheal disease, an immunocompetent individual is able to clear the infection, designating the infection as self-limiting. Aside from the potential of dehydration, these individuals may not suffer any residual effects. Diarrheal disease can be caused by any number of pathogens including viruses, bacteria, helminths, and protozoa (Operario and Houpt, 2011). One pathogen found worldwide that acts in such a way, is the protozoan of the genus Cryptosporidium.

Cryptosporidium was first identified as a genus in 1907 by Ernest Edward Tyzzer. Though technology was limited at the time, he was able to understand that even though it appeared this parasite resided in the intestinal lumen, it was distinct from other coccidian parasites, like Eimeria falciformis, and not a developmental stage of E. falciformis (Tyzzer, 1910; Tzipori and Widmer, 2008). Tyzzer distinguished between two independent species, C. parvum and C. muris, though it is not known if these are the same as the species designated by those names today (Fayer, 2008; Tzipori and Widmer, 2008). It also was determined that this genus, Cryptosporidium, belongs to the phylum Apicomplexa.

Cryptosporidium shares many characteristics with related organisms within the Apicomplexa, including a life cycle which has sexual and asexual phases, though the ultimate site of infection may vary, as well as a process for invading cells and unique apical organelles. Several species of Cryptosporidium are included amongst the few apicomplexan species known to be infective to humans through ingestion of cysts or oocysts (Yoshida et al., 2011). An exogenous stage, as well as several endogenous stages, including excystation, cell invasion, merogony, gamogony, and sporogony, can be observed during the complete life cycle of Cryptosporidium.

The exogenous stage of the Cryptosporidium life cycle begins when a fully sporulated oocyst is excreted in the host’s fecal matter. This form is environmentally resistant and infectious to its host species when ingested (Fayer, 2008). It is possible for the oocyst to remain viable in the environment for up to 176 days, though overall viability will decrease with time depending on the environmental conditions (Robertson et al., 1992).

Cryptosporidium begins the endogenous stage of its life cycle at the point of ingestion. Following this event, four sporozoites must escape from the oocyst before they are capable of infecting the host’s

¹ WHO classifies countries based on gross national income (GNI) per capita designations developed by World Bank. Low income countries have a GNI of less than $1,005, while middle income GNI ranges between $1,006-$12,275 (World Bank).
gastrointestinal tract. This process is known as excystation, by which a suture that begins at one pole of the oocyst opens to a point one-third to halfway around the oocyst, allowing the sporozoites to leave the thick oocyst shell. It is thought that this process may be enhanced by the stomach’s reducing conditions and the bile and enzymes found in the small intestine (Fayer, 2008).

Once the sporozoites have been released they begin to search for intestinal mucosal epithelial cells to invade. Cryptosporidium sporozoites secrete chemicals, including proteases, which allow them access to the epithelial cells forming the intestinal mucosa (Yoshida et al., 2011). When in contact with the host’s cell, the single rhoptry of the sporozoite will extend and allow parasite ligands to attach to the host-cell receptors (Fayer, General Biology, 2008). This begins the formation of a parasitophorous vacuole, and marks one of the differences between Cryptosporidium and other apicomplexan parasites. Most apicomplexan parasitophorous vacuoles are contained within the cytoplasm, while the parasitophorous vacuole for Cryptosporidium is found just beneath the cell membrane, but outside of the cytoplasm (Fayer, 2008). This has been given the term intracellular, but extracytoplasmic. The parasite develops a new organelle called a feeder organelle, which allows it to feed off of the nutrients gathered by the host-cell. It is at this point in the cell that the parasite changes from the crescent shape of the sporozoite to the more rounded form of the trophozoite (Fayer, 2008).

Cryptosporidium is able to undergo both asexual and sexual reproduction. Asexual reproduction, typically referred to as merogony, can result in either two or three types of meronts depending on the species of Cryptosporidium. However, the species that most commonly affect human health, C. parvum and C. hominis, only have two meront types (Fayer, 2008). Merogony is a result of the division of the trophozoite nucleus. In C. parvum infections, Type I meronts can develop between six and eight merozoites. Merozoites are also infectious to other host-cells, but are only viable in the endogenous stage (Fayer, 2008). These merozoites are capable of returning to Type I meronts after autoinfecting another host-cell or can continue their life cycle and become a Type II meront, typically resulting in four merozoites.

Type II meronts eventually develop into a microgamont or a macrogamont, the male and female gametocytes, respectively (Fayer, 2008). Sexual reproduction is referred to as gamogony. During this stage of the life cycle Cryptosporidium microgamonts will multinucleate and divide so that each nucleus develops into a “sperm-cell equivalent” (Fayer, 2008). The microgamont then fertilizes the uninucleated macrogamont or ovum equivalent. The nuclear contents of the microgamont are transferred to the nucleus of the macrogamont it fertilizes. This allows the nucleus of the macrogamont to undergo meiosis and develop into four sporozoites contained within an oocyst (Fayer, 2008).

The next stage, known as sporogony, allows the oocyst to sporulate and mature. The result is either a thin-walled or a thick-walled oocyst. Typically the thin-walled oocysts are involved in re-infecting the host through autoinfection, while the thick-walled oocysts are shed in the feces (Fayer, 2008). Once shed these oocysts remain in the environment until ingested by another suitable host, either animal or human.

Cryptosporidiosis is seen worldwide, in both developed and developing countries. The average number of Americans reportedly affected by this disease is only around a few thousand per year—between 3,000 and 4,000 in 2001 and 2002 (Beach, 2008). However the Centers for Disease Control (CDC) estimates that the actual number is much higher, possibly up to 300,000 cases a year. It is thought that cryptosporidiosis is underreported because of the fact that many people are asymptomatic or choose
not to seek medical care. However, often times those people who do see a physician are treated symptomatically without diagnostic tests to determine the pathogenic organism (Beach, 2008).

There are many different sources of cryptosporidial infections, which contribute to the high estimates of infection prevalence and cryptosporidial outbreaks. The method of transmission can vary depending on the species of Cryptosporidium. Humans are most commonly infected by either C. hominis or C. parvum, but C. hominis is responsible for the majority of cases. Cryptosporidium hominis infection generally occurs through person-to-person contact, contaminated food sources, and contaminated water sources, both potable and recreational (Nichols, 2008). The transmission routes of C. parvum include those above for C. hominis, as well as contact with animals and animal feces, as C. parvum is one of the potentially zoonotic species (Nichols, 2008). Though other species are not typically found in human cases of cryptosporidiosis, there have been occasional reports of C. meleagridis, C. felis, C. canis, C. muris, and C. suis infecting human hosts (Chako et al., 2010; Rajendran, et al., 2011). Aside from the primary means of zoonotic transmission being direct contact, zoonotic species can also be transmitted indirectly through waterway contamination (Nichols, 2008).

Waterborne transmission of Cryptosporidium typically occurs in one of two ways: direct ingestion of drinking water or exposure to recreational water. Transmission through drinking water is typically seen as an outbreak in areas where many people within a population use a single source of drinking water (Clancy and Hargy, 2008). The first known outbreak occurred in Braun Station, Texas in 1984. This outbreak only affected 200 people, while three years later 6000 people were diagnosed with cryptosporidiosis after drinking water which had been treated only with chlorine in the United Kingdom. However, one of the largest outbreaks of cryptosporidiosis occurred in Milwaukee, Wisconsin in 1993. It affected more than 403,000 individuals and lead to the deaths of over 100 people (MacKenzie et al., 1994; Clancy and Hargy, 2008). At the time of these outbreaks, this protozoan was not commonly known and professionals in the field believed the developed world was free from waterborne outbreaks. It was found that in many cases these outbreaks were due to either a disruption in the current disinfecting method of the water source or sewage contamination (Clancy and Hargy, 2008). Drinking water transmission can also correlate with recreational water transmission in certain countries where natural water sources are the only source of drinking water.

The second source of waterborne transmission is recreational water which includes marine waters, freshwater lakes, rivers and hot springs, as well as swimming pools, hot tubs, water parks, and fountains (Clancy and Hargy, 2008). Transmission can occur in these locales for a few reasons. The main reason can be attributed to the parasite’s fecal-oral route of transmission. People typically treat recreational waters like a public bath in the fact that they may immerse themselves fully. However, people seem to view these waters differently, wrongly assuming that the chlorination in “artificial, disinfected venues” (pools, hot tubs, etc.) essentially sterilizes the water (Beach, 2008). Another contributing factor is that oocysts are immediately infective once shed from the host and can be shed up to 50 days after a person’s symptoms of diarrhea stop. This means that people who have recently had cryptosporidiosis can be infecting others without knowing it. Fecal incontinence, especially in young children, can also contribute to oocyst counts in recreational waters. A few studies found that high percentages of children infected with Cryptosporidium will present asymptomatically (Beach, 2008). These outbreaks follow a seasonal pattern, with the number of infections dramatically increasing between July and September, with a peak towards the end of August and beginning of September (Beach, 2008). These infections are seen more frequently in children between the ages of one and four years of age, but are seen in all age groups. However, these are the reported data, and it is likely not complete because of the factors previously mentioned (Beach, 2008).
More recent studies have been finding that water used to irrigate agricultural fields is contaminating fresh food sources. It has been found that up to 36% of the water used for irrigation in the United States and Central America is contaminated with *C. parvum*. Spinach, a culprit for transmission of the pathogenic *Escherichia coli* O157:H7, is also being accused of harboring *C. parvum* oocysts (Wendel et al., 2009; Macarisin et al., 2010). Experimental laboratory tests showed that *C. parvum* oocysts strongly adhered to the root and leaves of the spinach plants and resisted being removed through vigorous washing. These tests even demonstrated the ability of oocysts to enter the stomata of the spinach leaves when they opened and remain trapped inside (Macarisin et al., 2010). In fall of 2008 a group of people fell ill after eating at the cafeteria of the Public Works Department in Helsinki, Finland. This outbreak was attributed to leafy greens contaminate with *C. parvum* (Ponka et al., 2009). While leafy greens will probably be suspect because of their easy potential for contamination, outbreaks of cryptosporidiosis have also been linked to raw meat. In the fall of 2006, *C. parvum* was suspected of causing gastroenteritis in a small group of people who had eaten Yukke, a “Korean-style beef tartar” (Yoshida et al., 2007).

Regardless of the potential source of transmission, *Cryptosporidium* oocysts must be ingested or inhaled for a person, or animal, to become infected. However, within as little as three days after ingestion of oocysts, people may become symptomatic. One study also showed oocysts present in the feces in as few as two days after ingestion, though with most cases shedding occurs five to ten days after infection (Warren and Guerrant, 2008). No matter the source of transmission, waterborne, foodborne, zoonotic, or anthroponotic, the symptoms of cryptosporidiosis are the same, though symptoms may vary from person to person. Some people are asymptomatic and can remain so throughout the course of their infection. Symptoms typically include: watery diarrhea, abdominal cramps, fever, anorexia, and vomiting (MacKenzie et al., 1994). Diarrhea is usually “watery, voluminous, and occasionally explosive and foul smelling” (Warren and Guerrant, 2008). In extreme cases, generally in the immunocompromised, death can also result due to dehydration.

In the immune competent host, most infections are self limiting. This is due in part to innate immunity (McDonald, 2008). Much of what is known about host immunity to *Cryptosporidium* has been demonstrated using murine studies, especially with severe combined immunodeficient (SCID) mice. The immune responses typically begin in the epithelial cells where the infection begins. These cells produce inflammatory molecules known as chemokines which stimulate inflammatory responses. Enterocytes are stimulated by this inflammation and begin their work generating mechanisms of microbial inactivation (McDonald, 2008). Though inflammatory responses can stimulate cell apoptosis, *Cryptosporidium* parasites have found a way to manipulate cells into avoiding apoptosis until their life cycle within that cell is complete. Of course this infection is self-limiting in immune competent people, meaning there are more specific immune responses involved.

One murine study demonstrated the importance of T-cells, specifically CD4⁺ T-cells, in ridding the body of cryptosporidiosis (McDonald, 2008). This finding is significant for the effect that cryptosporidiosis plays in patients with human immunodeficiency virus (HIV) infection and acquired immune deficiency syndrome (AIDS). This retrovirus survives by replicating within immune cells, like CD4⁺ T-cells (Dalgleish et al., 1984). When the virus has finished its replication, the cell then lyses, freeing countless more viral particles to infect more cells. This depletes the host’s own immune responses and makes them more susceptible to and unable to clear other infections, like cryptosporidiosis (McDonald, 2008). However, since the development of antiretroviral drug therapies, cryptosporidiosis presents less of a life-threatening hazard to individuals infected with HIV.
Cryptosporidium can be found in a variety of environments that humans come into contact with on a daily basis. Because there are so many opportunities for humans to ingest oocysts and become infected, an effective treatment could be highly useful. Currently there are many different drugs used in the treatment of cryptosporidiosis in both humans and animals. However, none of them are parasite specific or capable of completely treating this infection when in vivo (Shahiduzzaman and Daugschies, 2012). The search is still on to find a treatment that will show efficacy in cell cultures, as well as in vivo during clinical trials (Shahiduzzaman and Daugschies, 2012). In the development of anticryptosporidial monoclonal antibody-biocide fusion proteins in our laboratory, an effective and efficient means of quantitating oocysts shed by animal models in experimental trials is necessary.

Some of the many species of Cryptosporidium have undergone multiple name changes and classifications since their identification, including C. parvum and C. hominis (Fayer, 2008). Because of the similarities in location of endogenous stages and morphology of both endogenous and exogenous stages, many scientists relied on the standard for naming parasites of the Apicomplexa phylum—host specificity. This meant that each time Cryptosporidium was discovered in a new host species it was given its own species name (Fayer, 2008; Fayer, 2010). However, at least in mammalian hosts, there are many species which can each infect multiple different hosts (Tzipori and Widmer, 2008). The advent of genomic sequencing has made it easier to determine the relatedness between the different genotypes of each species and subspecies. In fact the different genotypes of C. parvum are now recognized as the individual species: C. hominis, C. bovis, and C. suis (Fayer, 2008). These species share genes that have been highly conserved across the species. Currently there are nineteen valid species of Cryptosporidium based on molecular, biologic, and morphologic characteristics, though C. parvum and C. hominis pose the greatest threat to human health.

**METHODOLOGY**

**Creating Standards**

Standards of Cryptosporidium hominis and Cryptosporidium parvum DNA were created from two sources: purified C. hominis and C. parvum oocysts and oocyst-negative fecal samples seeded with a known number of these oocysts. DNA was extracted from the purified oocysts two different ways.

The first extraction was performed after washing $1 \times 10^8$ C. hominis oocysts per mL (Tzipori, used three months past the shed date) and $1 \times 10^8$ C. parvum oocysts per mL (used two months past the shed date) with sodium hypochlorite as per the protocol established in the laboratory. The C. hominis and C. parvum oocysts were then excysted by adding 0.75% taurocholic acid/Hank’s Buffered Saline Solution (HBSS) and 0.5% taurocholic acid/HBSS respectively and placed in a shaking 37°C water bath for one hour. Once excysted the oocysts were washed twice with phosphate buffered saline (PBS) and centrifuged at 16,000x $g$ for 10 minutes before being resuspended in 0.5% SDS/PBS. Five freeze/thaws were then performed using liquid nitrogen (LN$_2$) and a 37°C water bath. The DNA was then extracted using the “Blood and Body Fluid Spin Protocol” from the QIAamp® DNA Mini Kit and QIAamp DNA Blood Mini Kit Handbook with the following changes:

- In Step 2 there is the addition of 4μl of RNaseA (DNase free) before proceeding to Step 3. The optional Step 9a is included.
- In Step 10 Buffer AE is used rather than distilled water and 200μl are run through the spin column twice rather than once. Also an additional 100μl of Buffer AE are added to the
between the two species. Since most of the samples to be analyzed are from a single source, it would not be possible to distinguish between the species. The DNA was extracted from the samples and used in a serial dilution to test the purity of the COWP gene primers.

Realizing that with the previously mentioned DNA extraction, the original oocyst counts were provided by the suppliers, we needed to make new standards with accurate pre-extraction oocyst counts in order to have standards that fit our needs. Thus we extracted DNA from new pure samples of both C. hominis (3.9x10⁷ oocysts per mL) and C. parvum (1.3x10⁸ oocysts per mL). The protocol was changed slightly after literature review (Alonso et al., 2011). In this paper, rather than excysting the Cryptosporidium oocysts directly, fifteen freeze thaws were performed using LN₂ and a warm water bath (37°C). The oocysts were washed once and then resuspended in 200μl 0.5% SDS/PBS before the freeze thaws were performed. The DNA extraction followed the same process as listed above.

With the second source of Cryptosporidium DNA, a frozen sample of calf feces, free of Cryptosporidium spp. and other pathogens was thawed and liquefied by adding either autoclaved PBS or autoclaved high performance liquid chromatography water (HPLC). These liquefied feces were then aliquoted 1mL into each 1.5mL microfuge tubes. They were seeded with sequential known numbers of C. parvum or C. hominis oocysts, and one tube for each of the diluents was left free of oocysts as a negative control. This occurred at two different times because of the differing availability in C. parvum and C. hominis oocysts. For the C. parvum standards, the DNA was extracted from 200μl of the seeded feces following the “Protocol for Isolation of DNA from Stool for Pathogen Detection” from the QIAamp® DNA Stool Mini Kit Handbook with one change (Protocol for Isolation of DNA from Stool for Pathogen Detection, 2001). This change occurred in Step 3 where instead of heating the suspension for five minutes at 70°C, the sample underwent fifteen freezes/thaws in LN₂ and a 37°C water bath. After receiving the C. hominis oocysts, the oocyst free feces were seeded with a known number of oocysts and the extraction was performed in the same manner as for the C. parvum samples with one crucial difference: all the centrifugation times were increased by a factor of three to account for the limited g force (x g) of the tabletop microcentrifuge being used. Also to make the extraction process a little more similar to the original process performed for the purified oocysts, 4μl RNase A (DNase free) was added after combining the supernatant and proteinase K.

**Selection and Preparation of Primers**

It was determined that there were two different genes, the DNA J-like protein (GenBank No. AF188857) and the Cryptosporidium oocyst wall protein (COWP) (GenBank No. AF248743), that have been successfully utilized as qPCR primers for both C. parvum and C. hominis (Altschul et al.; Fontaine and Guillot, 2002; Guy et al., 2003). Using the National Center for Biotechnology Information’s (NCBI) basic local alignment search tool (BLAST), it was determined that the genes for both the DNA J-like protein (GenBank no. XM 661034.1) and COWP were highly conserved between C. hominis and C. parvum, as both genes had a minimum 98% similarity between the species (Altschul et al.). It was decided that the COWP gene would be best suited to the purposes of this research because this segment had been used for PCR detection of Cryptosporidium in many other papers and the DNA sequences needed were easily found. Because of the conservation of both these genes between the two species they would be equally useful for detecting either species in a sample. However, it would not be possible to distinguish between the species. Since most of the samples to be analyzed are from animals experimentally infected with one species or the other in our research studies (generally not both), it as unnecessary to distinguish between the two.
The first primers received were desalt purified, indicating that though the primers are pure, steps could be taken to increase purity. However, these primers would still indicate whether or not the gene sequence chosen would be appropriate for the research at hand. To obtain the proper concentration of primers a dilution was first made by adding HPLC water at a rate of 10μl for every nanomole of the primers. A small amount of this primer dilution was diluted once more at a similar rate of 10μl HPLC water for every microliter of the first dilution, yielding a 10 micromolar concentration, the concentration recommended for real time PCR.

Because the purity of the primers could have been increased, the next primers ordered were high performance liquid chromatography water (HPLC) purified. These primers were also diluted through the same process as the desalt purified primers yielding a 10 micromolar concentration.

**DNA Purification/Extraction from Experimental Samples**

The feces collected for experimental analysis came from Mongolian gerbils infected with *C. hominis*. Because the fecal samples were dehydrated, as is normal gerbil feces, rehydration of the samples was required before DNA could be extracted. Approximately one gram of dry gerbil feces was placed in a 15mL centrifuge tube along with 5.0ml of autoclaved PBS. These samples were briefly vortexed to allow the feces maximal contact with the PBS. They were then soak for at least twenty-four hours and vortexed and stirred with a spatula to ensure complete mixture of the sample.

The process for DNA extraction followed the steps as outlined in DNA Extraction “Protocol for Isolation of DNA from Stool for Pathogen Detection” from the QIAamp® DNA Stool Mini Kit Handbook (Protocol for Isolation of DNA from Stool for Pathogen Detection, 2001). Because the fecal samples were rehydrated and not liquefied approximately 200 milligrams of the sample was measured and placed into a 2mL microfuge tube. The remainder of the steps followed the same protocol as used for extracting DNA from the calf feces (described below) for standard samples.

**Performing Real Time Polymerase Chain Reactions**

All reactions were run in triplicate using the Applied Biosystems 7300 Real-Time PCR System. Each reaction well contained DNA to be tested, SYBR green mastermix, HLPC water, forward and reverse primers for the COWP gene, and purified bovine serum albumin (BSA). The qPCR control wells were also run in triplicate. One control contained only HPLC water to minimize background signals that might arise from contaminated HPLC water or the optical plate itself. The other control, a non template control, contained all the same ingredients as the experimental reaction wells except the DNA (HPLC water was used to keep the volume equal to the rest of the reactions). This was used to control for primer dimerization within the reactions. Many of the reactions were performed with a total reaction volume of 25 μl, while one plate was run with reaction volumes of 50 μl. All the wells were contained within a 96-Well Optical Reaction Plate (with barcode) from Applied Biosystems. The plate was then covered and sealed with a MicroAmp™ Optical Adhesive Film Kit from Applied Biosystems.

The parameters used in running the real-time PCR were determined by examining the annealing temperature of the primers, past research, trial and error. Many of the initial reactions of 25 μl were run with the following settings: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute (Guy et al., 2003). When first testing the primers, these settings worked well. However, after realizing the purpose of much of the research in our laboratory is to minimize or eliminate cryptosporidiosis, there would need to be more distinction between the smaller quantities of oocysts
shed in the samples. This was accomplished by changing the parameters to add another step to the 40 cycles, after 1 minute at 60°C the reactions then underwent 1 minute at 72°C (Spano et al., 1997).

**Analysis of Raw Data**

Analysis of the data was performed using two programs: Applied Biosystems 7300 Sequence Detection Software (SDS) and Microsoft Excel. The SDS not only collected data from the real-time PCR, but it also had tools that allowed the data to be analyzed without being exported to another file format. However, graphic representation of results is always useful and SDS was not appropriate for making multiple graphs. Because of this the data, once initially analyzed were exported from SDS to Microsoft Excel, where graphic representations of data were easily created.

To obtain linear graphic representations of the data collected, which allows for correlations to be observed, the log (base ten) of the quantity of oocysts needed to be calculated and graphed against the threshold cycle (Fig. 1). The data were simple to place in a graph when all quantities of oocysts were known. However, when testing experimental fecal samples, the linear curves were generated using SDS. This generated a quantity of oocysts per ml of reconstituted feces. In order to find the number of oocysts per ml of the rehydrated feces the SDS generated number needed to be multiplied by a dilution factor. This dilution was calculated by dividing the sum of the weight of feces in grams and the volume of PBS added in ml by the weight of feces in grams.

\[
\text{dilution factor} = \left( \frac{\text{amount of feces to be rehydrated (g) + volume of PBS used (ml)}}{\text{amount of feces to be rehydrated (g)}} \right)^2
\]

Once the oocysts per ml of pre-hydration feces is calculated the results can be compared to the experimental results calculated through immunofluorescence assay (IFA). This is done by taking the \( \log_{10} \) of the quantity of oocysts per ml. These numbers can then be compared directly. By dividing the \( \log_{10} \) of the IFA results, by the \( \log_{10} \) of the qPCR results, a single factor (log factor) can be determined. The average of the log factors for qPCR 96-well plate can be calculated \( \bar{x} \log \text{factor} \), and used in converting the qPCR quantities to a quantity of oocysts shed in a single day.

\[
\log_{10}(\text{oocysts in 1.0 ml of pre-hydration feces qPCR}) = q
\]

\[
\log_{10}(\text{oocysts in 1.0 ml of pre-hydration feces IFA}) = f
\]

\[
\frac{f}{q} = \log \text{factor}
\]

\[
\text{oocysts per day} = \left[ 10^{(q-\bar{x}\log \text{factor})} \right] \times \text{total weight of feces for the day (g)}
\]

Once the total oocysts per day are calculated this number can be compared to the number of oocysts per day calculated using IFA quantities. This number can also be used to calculate the entire amount of oocysts shed over the course of the trial by adding the quantities calculated for each day of a single experimental group.

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2 When rehydrating feces 1.0 grams of dry feces is equal to 1.0 ml.
RESULTS
The first run of the qPCR was to test our desalt purified primers’ specificity for C. hominis and C. parvum, as well as the degree of primer dimerization, a condition in the reaction where the primers bind with one another preferentially rather than the DNA sequences they were created to bind with. This was performed using DNA extracted from purified C. hominis and C. parvum oocysts, though the exact quantities of oocysts were unknown when this DNA was extracted. The curve was generated by performing serial dilutions of the extracted DNA (Fig. 1). This allowed relative DNA concentrations to be known, even if the initial quantity was not. The following equations of a line were found for C. hominis: $y = -0.2887x + 4.1254$ and C. parvum: $y = -0.2776x + 3.8291$. The correlation between each of the data points were as follows, for C. hominis: $R^2 = 0.9951$ and C. parvum: $R^2 = 0.9945$. The dissociation curve (Fig. 2) serves as an indicator of primer dimerization. The single large peak that occurs around 78°C is a result of the amplified cryptosporidial DNA dissociating. The smaller individual peaks that occur around 72°C may have resulted from the no template control wells. Because they lack the DNA extracted from either C. parvum or C. hominis, the peak is indicative of a small amount of primer dimerization.

Figure 1. qPCR results for C. hominis and C. parvum DNA at a 1:2 dilution.

Figure 2. Dissociation curve from qPCR indicating minimal primer dimerization as seen by the single large peak around 78°C, and minimal peaks seen around 72°C. The three peaks at 72°C are from the no template control reactions. The remaining lines are from reactions run with different amounts of DNA from purified C. parvum and C. hominis oocysts.

Figure 3. Amplification plot of DNA from purified C. parvum oocysts compared to DNA from C. parvum seeded in clean calf feces.
The next qPCR run was performed comparing DNA from purified *C. parvum* oocysts, of a known quantity, and DNA from *C. parvum* oocysts seeded in clean calf feces. The results of this reaction were inconclusive (Fig. 3). As can be observed in Figure 3, there is a significant amount of background noise from the beginning of the reaction, through cycle 29. There is also no significant correlation ($R^2=0.3327$) when generating a standard curve from these reactions. This reaction was duplicated in an attempt to generate results with a stronger correlation.

This second run (Fig. 4) yielded slightly more conclusive results with a correlation of $R^2=0.7268$ for the data from the DNA from purified *C. parvum* oocysts. However, the correlation for the DNA from calf feces seeded with *C. parvum* was still low with $R^2=0.5647$. There was also a difference in the slopes of the lines for the different sources of *C. parvum* oocysts. The slope was -0.1948 for the DNA from the purified oocysts, while the slope was -0.6064 for the DNA extracted from the oocysts seeded within the calf feces. The dissociation curve for this run indicated a greater degree of primer dimerization than the initial run, with over half of the wells having a small peak between 70°C and 75°C. However, there were many larger peaks around 78°C, indicating the desired gene was being amplified during the reaction. This dissociation curve is similar to the one seen in Figure 5.

Following the test of *C. hominis* DNA standard, new primers, HPLC purified, were obtained and tested to determine whether a purer primer 3

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3 These calf feces had been frozen for 3 years and were free of *Cryptosporidium* and other diarrheal etiologic agents.

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would yield more conclusive results, and reduce the degree of primer dimerization in the reactions. There was no decrease in the primer dimerization. In fact, it increased from the initial primer test (Fig. 2). The dissociation curve can be seen in Figure 5. The standard curve from these primers resulted in a relatively high correlation ($R^2=0.9135$) for the DNA extracted from *C. parvum* oocysts seeded in negative calf feces, while there was a much smaller correlation ($R^2=0.6313$) in the qPCR data for the DNA extracted from purified *C. parvum* oocysts. The slope of the lines still did not match with the slope for the DNA extracted from oocysts seeded within negative calf feces being -0.3537. The slope for the data points from the DNA extracted from was -0.1932.

The HPLC primers were also tested to be sure that they interacted with the DNA extracted from *C. hominis* oocysts seeded in negative calf feces (Fig. 6). DNA from *C. parvum* oocysts, was also run as a control as the primers had already been determined to react with this DNA. The lines generated from both sets of DNA samples generated similar equations of the lines: *C. hominis* $y=-0.3629x + 14.731$; *C. parvum* $y=-0.3254x + 14.117$. The $R^2$ values were 0.8782 for *C. hominis* and 0.7935 for *C. parvum*.

Looking at the past data and the purpose of this project, it was noticed that this technique could be more useful if there was a larger distinction between the threshold cycles of small quantities of oocysts. In an attempt to achieve this distinction an elongation step was added to the qPCR reaction times. The results of this reaction, run using DNA extracted from *C. parvum* oocysts seeded in negative calf feces, were compared to the previous data points from the same DNA when the reaction was run to compare this DNA to the DNA extracted from purified *C. parvum* oocysts. Viewing the graph in Figure 7, it can be seen that the elongation step reduces the threshold cycle. This is further...
exemplified by looking at the equations of the lines for these data points. Without the elongation step the y-intercept is 16.426 ($R^2=0.8509$). However, the addition of the elongation step lowers the y-intercept to 15.486 ($R^2=0.8895$).

When analyzing the experimental data, it was determined to use standard curves generated by DNA extracted from both purified *C. hominis* oocysts and *C. hominis* oocysts seeded in negative calf feces. As seen in Table 1, there is a significant difference between the quantities of oocysts calculated between the two DNA extraction methods. The next step was to determine which standard yielded results that more closely resembled those calculated through IFA.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Cycle Threshold</th>
<th>Quantity per ml of diluted feces</th>
<th>Average quantity of oocysts per ml of diluted feces</th>
<th>Cycle Threshold</th>
<th>Quantity per ml of diluted feces</th>
<th>Average quantity of oocysts per ml of diluted feces</th>
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<tbody>
<tr>
<td>T2 G1 5/10/10</td>
<td>34.1</td>
<td>118.72</td>
<td>196.64</td>
<td>34.1</td>
<td>774.72</td>
<td>1529.86</td>
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<td>34.14</td>
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<td>1529.86</td>
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<td>196.64</td>
<td>32.5</td>
<td>3071.04</td>
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<tr>
<td>T2 G1 5/11/10</td>
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<td>249.46</td>
<td>280.62</td>
<td>33.01</td>
<td>1901.67</td>
<td>2180.36</td>
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<tr>
<td>T2 G1 5/11/10</td>
<td>32.77</td>
<td>294.15</td>
<td>280.62</td>
<td>32.77</td>
<td>2252.29</td>
<td>2180.36</td>
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<td>T2 G1 5/11/10</td>
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<td>130.97</td>
<td>33.55</td>
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<tr>
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<td>124.2</td>
<td>130.97</td>
<td>34.03</td>
<td>900.86</td>
<td>986.45</td>
</tr>
</tbody>
</table>

Table 1. Comparison of Experimental Data Using Two Different Standard Curves. Data from the experimental fecal samples of the second gerbil trial. (T2=Trial 2; G1=Group 1)

In comparing the results of oocyst quantitation using the qPCR standard curves of *C. hominis* DNA extracted from both purified oocysts and oocysts seeded in negative calf feces to the quantities calculated microscopically using IFA, a trend was noticed which will be elaborated on later in this report.
### QUANTITY OF OOCYSTS

<table>
<thead>
<tr>
<th>METHOD OF QUANTITATION</th>
<th>SAMPLE</th>
<th>QUANTITY</th>
<th>AVERAGE # OOCYSTS</th>
<th>OOCYSTS PER ML FOR SAMPLE</th>
<th>TOTAL OOCYSTS PER DAY</th>
<th>TOTAL OOCYSTS PER GROUP</th>
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</thead>
<tbody>
<tr>
<td>IFA</td>
<td>T2 G1 5/10/10</td>
<td>39, 36, 34</td>
<td>36.33</td>
<td>7.79E+04</td>
<td>2.28E+05</td>
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<td>77, 62, 59</td>
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<td>1.42E+05</td>
<td>9.41E+05</td>
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<td></td>
<td>T2 G1 5/12/10</td>
<td>59, 69, 60</td>
<td>62.67</td>
<td>1.33E+05</td>
<td>7.20E+05</td>
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<tr>
<td>DNA from purified C. hominis oocysts</td>
<td>T2 G1 5/10/10</td>
<td>118.72, 115.48, 355.72</td>
<td>196.64</td>
<td>1.22E+03</td>
<td>3.62E+05</td>
<td>2.09E+06</td>
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<td></td>
<td>T2 G1 5/11/10</td>
<td>249.46, 294.15, 298.24</td>
<td>280.62</td>
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<td>130.97</td>
<td>7.86E+02</td>
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<td>DNA from C. hominis in calf feces</td>
<td>T2 G1 5/10/10</td>
<td>774.72, 743.81, 3071.04</td>
<td>1529.86</td>
<td>9.45E+03</td>
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<td>T2 G1 5/12/10</td>
<td>718.51, 1339.98, 900.86</td>
<td>986.45</td>
<td>5.92E+03</td>
<td>3.64E+05</td>
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</tbody>
</table>

**Table 2. Comparison of Quantitation Methods.** This table compares the oocyst counts of experimental samples containing *C. hominis* oocysts by the different methods of quantitation.

**DISCUSSION**

**Primers:** In the process of this work the primers for qPCR were purified two different ways: desalt and using HPLC. The first run of qPCR used to test the desalt primers revealed a minimal amount of primer dimerization, while also working well to amplify the COWP gene within the *C. parvum* and *C. hominis* DNA. Because of the slight primer dimerization, an attempt to obtain more “pure” primers, HPLC purified primers were purchased. Contrary to the initial thought, these HPLC purified primers had a greater amount of primer dimerization than the desalt purified primers. However, both primers have greater activity increasing the desired gene than they do interacting with one another. It would be best to use the desalt purified primers, but either would effectively amplify the COWP gene and quantitate the number of oocysts within a given sample.

**Standards:** The standard curve generated from the DNA of purified oocysts correlation slightly more ($R^2 = 0.9965$) than the standard curve generated from the DNA of oocysts seeded within a clean sample of neonatal calf feces ($R^2 = 0.9433$). This further indicates the usefulness of both standards in quantitating *C. hominis* oocysts in experimental fecal samples, though it indicates the equation of the line used to calculate the quantities of oocysts will be more accurate when using the DNA from purified *C. hominis* oocysts. The original reason behind using DNA from seeded fecal samples to generate a standard curve was to mimic the inhibitors within the experimental samples. However, by using calf feces another variable was entered into the equation. First was the age of the animal the feces were taken from. The calf sample was collected from a neonate, while the experimental gerbil samples are collected after the animals reach an adult age. Also the feces being tested are from a different species with different gastrointestinal tracts. However, the process for extracting DNA has removed many of the PCR
inhibitors. This is evident in the similarity of the resulting quantities of oocysts when using standard curves generated by either source of *C. hominis* DNA.

Both sources of *C. hominis* DNA, from purified oocysts and oocysts seeded in negative calf feces, yielded oocysts quantities that followed a trend similar to the experimental *C. hominis* oocysts counts determined through IFA. Thus far only three experimental samples have been quantitated using qPCR, and even with the trend these data show, more experimental samples are needed to determine if the trend is significant. More data points might also help to determine which standard, DNA from purified *C. hominis* oocysts or DNA from *C. hominis* oocysts seeded in negative calf feces, would be more appropriate for these samples.
REFERENCES


