CRYPTOSPORIDIUM PARVUM PROTEINS AND PEPTIDES INVOLVED IN STRAIN DIFFERENTIATION AND THEIR ANTIGENIC CHARACTERISTICS

by

ROXANA SANCHEZ INGUNZA

(Under the Direction of Ynés R. Ortega)

ABSTRACT

The present study evaluated the differences in protein expression between three Cryptosporidium parvum isolates, and identified C. parvum antigenic proteins and peptides. The C. parvum Iowa (IO), Arizona (AZ), and Moreum (MD) isolates’ protein profiles were compared by two-dimensional difference gel electrophoresis (2D-DIGE), and proteins which were differentially expressed and characterized each isolates were annotated. C. parvum antigenic proteins were determined by one-dimensional and two-dimensional separation of soluble C. parvum proteins coupled with western blot analysis. C. parvum antigenic proteins were recognized by IgG, IgM, IgA in human sera from Cryptosporidium infected individuals. Sera reactivity was compared with that of a group of serum samples from non-infected individuals. Antigenic peptides were predicted and peptides specific for these Cryptosporidium proteins were suggested. C. parvum AZ demonstrated significant difference in the expression of a 36 kDa protein with isoelectric point (pI) of 6.6 and a 43 kDa with pI of 6.36 (p < 0.05) while C. parvum IO showed significant abundance for a 46 kDa protein with pI of 6.91 with (p < 0.05) and C. parvum MD for 72 and 78 kDa proteins with pIs of 5.13 and 5.49 (p < 0.1), respectively. Six C. parvum antigenic proteins were significantly recognized by human sera from infected
individuals ($p < 0.05$). The molecular weights for these antigens ranged from 40 to 65 kDa while their isoelectric points ranged from 5.3 to 7.2. A *Cryptosporidium* serine/threonine phosphatase was identified by Tandem Mass Spectrometry (MS/MS) from those antigenic proteins. This is the first report confirming the existence of a *Cryptosporidium* serine/threonine phosphatase which is also involved in humoral immune response against cryptosporidiosis. Two *Cryptosporidium* antigenic peptides predicted from this protein might be able to distinguish *C. parvum* and *C. hominis* from other apicomplexans. These antigens should be further evaluated as potential diagnostic targets to control cryptosporidiosis.

INDEX WORDS:  *Cryptosporidium*, apicomplexans, 2D-DIGE, western blotting, mass spectrometry, antigenic proteins, antigenic peptides, serine/threonine phosphatase.
CRYPTOSPORIDIUM PARVUM PROTEINS AND PEPTIDES INVOLVED IN STRAIN DIFFERENTIATION AND THEIR ANTIGENIC CHARACTERISTICS

by

ROXANA SANCHEZ INGUNZA

B.S., Universidad Nacional Mayor de San Marcos, Peru, 1996
DVM, Universidad Nacional Mayor de San Marcos, Peru, 2002

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2009
CRYPTOSPORIDIUM PARVUM PROTEINS AND PEPTIDES INVOLVED IN STRAIN DIFFERENTIATION AND THEIR ANTIGENIC CHARACTERISTICS

by

ROXANA SANCHEZ INGUNZA

Major Professor: Ynes R. Ortega
Committee: Mark A. Harrison
Joseph F. Frank
Mark W. Jackwood
David S. Peterson

Electronic Version Approved:
Maureen Grasso
Dean of the Graduate School
The University of Georgia
December, 2009
DEDICATION

“En memoria de mi padre a quien recordamos siempre con mucho amor”

This work is entirely dedicated to my precious family, my husband Alan H. Icard, my children Jorge, Lana and Alexander and especially my mother, Maritza Ingunza, whose unconditional love and support always encourage me to fulfill my dreams.

Thanks my God for all your blessings
ACKNOWLEDGEMENTS

My sincere thanks go to Dr. Ynes Ortega and Dr. Carlos Chunga for their support and confidence in me.

I also would like to acknowledge my advisory committee Dr. Mark Jackwood, Dr. Mark Harrison, Dr. Joseph Frank, and Dr. David Peterson for their excellence in guidance this research.

I want to thank Dr. Tracy Andacht, Dr. Richard Winn, Dr. Hercules Moura, and Dr. Mark Compton for their technical advice during the conduction of the laboratory experiments in this study.

And, I sincerely thank my friends Patricia Torres, Deborah Hilt, Joan Berryman, and Ruth Ann Morrow for their constant encouragement during this intensive period of hard-work journeys and exciting life-transition.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1  INTRODUCTION AND LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>2  DIFFERENTIAL PROTEIN EXPRESSION OF <em>CRYPTOSPORIDIUM PARVUM</em> ISOLATES BY TWO-DIMENSIONAL DIFFERENCE GEL ELECTROPHORESIS (2D-DIGE)</td>
<td>52</td>
</tr>
<tr>
<td>3  <em>CRYPTOSPORIDIUM PARVUM</em> ANTIGENIC PROTEINS RECOGNIZED BY HUMAN SERA UNDER ACTIVE CRYPTOSPORIDIOSIS INFECTION</td>
<td>81</td>
</tr>
<tr>
<td>4  DETERMINATION OF <em>CRYPTOSPORIDIUM PARVUM</em> ANTIGENIC PEPTIDES FROM PROTEINS ASSOCIATED WITH HUMORAL IMMUNE RESPONSE IN HUMANS DURING ACUTE CRYPTOSPORIDIOSIS</td>
<td>118</td>
</tr>
<tr>
<td>5  CONCLUSIONS</td>
<td>153</td>
</tr>
</tbody>
</table>
LIST OF TABLES

| Table 2.1: | Differentially expressed *C. parvum* Iowa (IO) vs. *C. parvum* Arizona (AZ) proteins detected by Two-Dimensional Difference Gel Electrophoresis (2D-DIGE) .........................................................................................................................................................................................73 |
| Table 2.2: | Differentially expressed *C. parvum* Iowa (IO) vs. *C. parvum* Arizona (AZ) proteins detected by Two-Dimensional Difference Gel Electrophoresis (2D-DIGE) .........................................................................................................................................................................................74 |
| Table 2.3: | Differentially expressed *C. parvum* Moredum (MD) vs *C. parvum* Arizona (AZ) proteins detected by Two-Dimensional Difference Gel Electrophoresis (2D-DIGE) .........................................................................................................................................................................................75 |
| Table 2.4: | Differentially expressed *C. parvum* Moredum (MD) vs *C. parvum* Arizona (AZ) proteins detected by Two-Dimensional Difference Gel Electrophoresis (2D-DIGE) .........................................................................................................................................................................................76 |
| Table 2.5: | Differentially expressed *C. parvum* Moredum (MD) vs *C. parvum* Iowa (IO) proteins detected by Two-Dimensional Difference Gel Electrophoresis (2D-DIGE) .........................................................................................................................................................................................77 |
| Table 2.6: | Differentially expressed *C. parvum* Moredum (MD) vs. *C. parvum* Iowa (IO) proteins detected by Two-Dimensional Difference Gel Electrophoresis (2D-DIGE) .........................................................................................................................................................................................78 |
Table 3.1: Student’s T test results for the comparison between Group 1 (Cryptosporidium infected individuals) vs. Group 2 (non-infected individuals) of antigenic bands observed in one-dimensional western blot analysis.............................................111

Table 3.2: Student’s T test results for the comparison between Group 1 (Cryptosporidium infected individuals) vs. Group 2 (non-infected individuals) of protein spots of interest resolved by two-dimensional western blot..............................................112

Table 4.1: Identification of Cryptosporidium parvum proteins by Tandem Mass Spectrometry or MS / MS.................................................................................................144

Table 4.2: Antigenic peptides identified by the method of Kolaskar and Tongaonkar from Cryptosporidium parvum proteins identified in this study. .................................................145
### LIST OF FIGURES

| Figure 2.1: | Proteins of *Cryptosporidium parvum* Iowa (IO), Arizona (AZ), Moredum (MD) detected by Two-dimensional Difference Gel Electrophoresis (DIGE) | 79 |
| Figure 2.2: | Image of *Cryptosporidium parvum* internal standard in two-dimensional difference gel electrophoresis showing proteins differentially expressed between *C. parvum* isolates | 80 |
| Figure 3.1: | *Cryptosporidium parvum* Iowa antigens recognized by human sera collected after 3 consecutive *Cryptosporidium* infections | 113 |
| Figure 3.2: | *Cryptosporidium parvum* Iowa isolate antigenic bands detected by human sera in one-dimensional western blotting | 114 |
| Figure 3.3: | *Cryptosporidium parvum* Iowa isolate antigenic bands detected by human sera in one-dimensional western blotting and resolved in 10% polyacrylamide gels | 115 |
| Figure 3.4: | *Cryptosporidium parvum* antigenic proteins used in the statistical comparison between experimental groups | 116 |
| Figure 3.5: | *Cryptosporidium parvum* antigenic proteins recognized by human sera from non-infected individuals (Group 2) | 117 |
| Figure 4.1: | *Cryptosporidium parvum* proteins selected for identification by Mass Spectrometry | 148 |
| Figure 4.2: | Neighbor joining phylogenetic tree for the *Cryptosporidium* phosphoprotein phosphatase-related protein | 149 |
Figure 4.3: Neighbor joining phylogenetic tree for the Cryptosporidium actin protein ........150

Figure 4.4: Neighbor joining phylogenetic tree for the Cryptosporidium hypothetical protein Chro 70534 ..................................................................................................................151

Figure 4.5: Neighbor joining phylogenetic tree for the Cryptosporidium chaperone protein- related ........................................................................................................152
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

*Cryptosporidium parvum* is a protozoan parasite implicated in waterborne and foodborne diseases where water or foods are contaminated with fecal material from infected hosts. *Cryptosporidium* is responsible for gastrointestinal illness in humans and the disease can be anthroponotic or zoonotic in nature.

Currently, prevention methods do not completely eliminate the risk of human infection by *Cryptosporidium* through water use; accurate methods to measure oocysts viability and to assess *Cryptosporidium* infectivity are required. Few drugs for human cryptosporidiosis therapy exist and vaccines are not available. New *Cryptosporidium* molecular targets have to be evaluated in order to improve cryptosporidiosis diagnosis and therapeutics, as well as for finding alternatives to prevent the disease by vaccination.

In recent years, genomics and proteomics have opened alternative ways to assess the identification of *Cryptosporidium* molecular targets. The sequence of the *Cryptosporidium* genome facilitates proteomic studies and many *Cryptosporidium* proteins have been annotated. Among them, all of those proteins which are fundamental for parasite survival remain to be identified. Molecular differences between species or isolates also have been reflected in the peculiarities of cryptosporidiosis clinical manifestations. Those proteins which are involved in isolate differentiation and those which elicit immune response in the host, especially those with neutralizing activities, are viable candidates to be used as therapeutic targets, vaccines and/or
targets for diagnosis and identification of Cryptosporidium isolates in individuals with cryptosporidiosis, during outbreaks, and in foodstuffs or water.

To reach the final goal of identifying new Cryptosporidium molecular targets for the diagnosis, epidemiological studies and control of cryptosporidiosis the present study focuses on determining Cryptosporidium parvum antigens recognized by the human immune system via two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) coupled with western blot analysis. In addition, C. parvum proteins were distinguished according to strain by differential fluorescent labeling. Finally, this study determined antigenic peptides from those C. parvum proteins recognized by the humoral immune system.

Description of the parasite and characterization of the disease as well as a review of the current diagnostic methodologies used for Cryptosporidium are as follows.

Microorganism description

The genus Cryptosporidium belongs to the phylum Apicomplexa, class Coccidia, order Eucoccidiorida and family Cryptosporidiidae. Cryptosporidium species have been isolated from fish, reptiles, birds, and mammals. C. hominis, C. parvum, C. andersoni, C. canis, C. felis, C.meleagridis, C. muris, C.suis and the Cryptosporidium cervine, horse, skunk and rabbit genotypes have been reported in humans; however, C. hominis and C. parvum are the most common species involved in human disease (Cama et al., 2008; Cama et al., 2003; Chalmers et al., 2009; Leoni et al., 2006; Muthusamy et al., 2006; Pedraza-Diaz et al., 2001b; Robinson et al., 2008).

The size of Cryptosporidium oocysts varies according to species, but accurate measurements cannot be delineated for each species. For instance, a size range of 5.5 – 5.0 x 5.0
– 3.7 μm (mean 5.2 x 4.3 μm) has been reported for C. parvum whereas significant variation was observed for C. hominis (Fall et al., 2003).

The genus name Cryptosporidium (meaning hidden sporocysts) was proposed by Tyzzer in 1910 for an organism he found in the stomach of a mouse (Marshall et al., 1997), and two years later he proposed the species name Cryptosporidium parvum for an organism observed in the small intestine of mice. It is possible that this microorganism was the one later recognized as C. muris. In 1976, Cryptosporidium was identified as a human pathogen.

Two genotypes of C. parvum were distinguished early: the human genotype and the bovine genotype (Xiao et al., 1999). The human genotype was designated a new species (C. hominis) after demonstrating different infection patterns between the bovine genotype and human genotype, and by considering differences in molecular and phylogenetic analysis of different loci (Morgan-Ryan et al., 2002). Subsequently, subtype families and subtypes have been identified for C. parvum and C. hominis isolates (Cama et al., 2008; Sulaiman et al., 2005).

Cryptosporidium is a major cause of disease in livestock and is classified as class B bioterror agent by the Centers for Disease Control and Prevention (CDC). The parasite is an enteropathogen of immunocompetent as well as immunocompromised individuals, and it is widespread distributed in nature (O'Donoghue, 1995).

Life cycle

Excystation of Cryptosporidium infectious oocysts occurs in the small intestine lumen after the ingestion of foods or water contaminated with feces from infected hosts or by direct contact with infected human or animal feces (Chalmers and Davies, 2009). The excystation is favored by the reducing conditions in the stomach and the exposure to pancreatic enzymes and
bile salts in the intestine; under experimental conditions the excystation has been observed in warm aqueous solutions (Chalmers and Davies, 2009).

Released sporozoites, four per infective oocyst, invade epithelial cells and transform into trophozoites, and all the intracellular stages after cell invasion are extracytoplasmic. During the formation of trophozoites a parasitophorus vacuole originates when thin extensions of the host cell membrane and an anterior vacuole formed in the parasite surround the organism. The sporozoite apical complex consists of rhoptries, dense granules, micronemes, and conoid, and participates in the parasitophorus vacuole formation. These organelles are localized in the anterior end of the parasite and in closed contact site with the host cell (Sam-Yellowe, 1996). In this region, vacuole structures form the feeder organelle.

Trophozoites multiply asexually (Merogony) to form types I and II meronts; with 8 and 4 merozoites, respectively. These stages are followed by sexual multiplication (Gamogony) which finally leads to the formation of oocysts by the fertilization of macrogamonts by microgametes. After fertilization, a trilaminar wall forms around the macrogamont and four sporozoites are formed by meiosis. Two types of oocysts have been observed, thin-walled oocysts, auto-infective life cycle form, or thick-walled oocysts form excreted in the feces. Sporulation occurs in the intestinal lumen where four sporozoites, not enclosed in sporocysts, are formed in each infective oocyst (Fayer, 2008; Marshall et al., 1997; O'Donoghue, 1995; Petersen, 1993).
Epidemiology

Clinical cryptosporidiosis

Susceptible populations:

Cryptosporidiosis has been reported in immunocompetent individuals as well as in the immunocompromised (Brockmann et al., 2008; Leoni et al., 2006). The disease has been observed in individuals who had close contact with calves (Preiser et al., 2003), individuals traveling overseas (Lazensky et al., 2008), children at day-care centers (CDC, 1984), the elderly, recreational water users or consumers of poor quality water (Chalmers and Davies, 2009), and people with HIV/AIDS (Cama et al., 2003). High-risk populations are those individuals with immune T-cell deficiency (cases of haematological malignacies, severe combined immunodeficiency syndrome, CD40 ligand deficiency), and HIV patients with low CD4+ counts (Chalmers and Davies, 2009; Hunter and Nichols, 2002).

In endemic regions, most cryptosporidiosis cases are found in individuals younger than 2 years who are thought to have been constantly exposed to Cryptosporidium at an early age. However, the infection is rarely observed in immunocompetent adults (Cama et al., 2008). In non-endemic areas the disease is observed in children as well as in adults.

Clinical description:

The disease incubation period ranged from 7 to 9 days in adult immunocompetent individuals (DuPont et al., 1995; Lazensky et al., 2008). During the Milwaukee outbreak in 1993, the estimated incubation time for cryptosporidiosis was 3 to 7 days (Eisenberg et al., 1998). The median incubation time was 5 days in individuals with pre-existing serum containing
anti-

Cryptosporidium antibodies, and some individuals remained asymptomatic while others shed oocysts in the stools (Chappell et al., 1999).

The clinical symptoms associated with cryptosporidiosis include watery or mucoid diarrhea, general malaise, abdominal pain, nausea, low-grade fever and vomiting (Cama et al., 2008; Lazensky et al., 2008; Sulaiman et al., 2005). Mild-to-moderate, sometimes prolonged, diarrhea with or without fever and without nausea and vomiting were associated with cryptosporidiosis in children at day-care centers in the United States. None required hospitalization, and some were asymptomatic (CDC, 1984). During the outbreak of cryptosporidiosis in Milwaukee, clinical illness in children was prolonged and associated with weight loss and abdominal cramps in laboratory-confirmed cases (Cicirello et al., 1997). Shedding time of Cryptosporidium hominis oocysts in stools was longer than the times reported for C. parvum, C. meleagridis, C. felis, and C. canis in children from Peru, and the oocysts shedding was longer in cases with diarrhea (17.4 days) compared to cases without diarrhea (10.3 days) (Xiao et al., 2001). In a different study of parasitic disease prevalence in rural areas in Peru, all of the children affected by cryptosporidiosis were symptomatic (Cordova Paz Soldan et al., 2006). Reinfections with different species were also observed in 11% of affected children and the median time between repeated infections was 10 months (range, 2.1 to 26 months) (Xiao et al., 2001). Secondary transmission of cryptosporidiosis has been observed in Brazil where 58% of households showed at least one case of secondary cryptosporidiosis while diarrhea was observed in only 4% of these cases (Newman et al., 1994). Evidence of clinical manifestation was also observed. Geographically diverse isolates of C. parvum varied in their ability to produce disease. Whereas the intensity of oocyst excretion and the incubation time were not significantly different between C. parvum Iowa isolate, C. parvum TAMU isolate and C. parvum
UCP isolate, a higher attack rate \((p = 0.04)\) was observed for the TAMU isolate (86%) compared to Iowa (52%) or UCP (59%). The ID\(_{50}\) also differed \((p < 0.05)\) between the TAMU (9 oocysts) and Iowa (87 oocysts) or UCP (1042 oocysts) isolates (Okhuysen et al., 1999).

In high-risk populations, chronic disease was observed, as well as atypical gastrointestinal disease and extra-intestinal disease (gall bladder, pancreatic tree or respiratory tract illnesses) (Chalmers and Davies, 2009; Hunter and Nichols, 2002). Diarrhea was associated with 47% of cryptosporidiosis cases in HIV/AIDS individuals (Cama et al., 2003). In this population, the diagnosis of cryptosporidiosis was correlated with clear survival disadvantage (Manabe et al., 1998). During the Milwaukee outbreak, symptomatic cryptosporidiosis was more common in HIV persons. This population, especially those patients with CD4\(^+\) lymphocyte counts lower than 200/\(\mu l\), suffered a more severe and prolonged disease than non-HIV individuals. Moreover, mortality rate in AIDS cases increased presumably due to the effect of *Cryptosporidium* as an underlying or contributing factor of death (Frisby et al., 1997; Hoxie et al., 1997).

### Seasonality

The occurrence of cryptosporidiosis cases in humans delineates disease seasonality. In Europe, a peak in *Cryptosporidium* infection was observed between September and November (autumn) and in Kuwait, more cases were detected during the cool season of November to April (Sulaiman et al., 2005; Wielinga et al., 2008). Many waterborne cryptosporidiosis outbreaks were reported during summer season in the United States, and human cryptosporidiosis cases increase in Latin America during the rainy season and summer (CDC, 1994a, b, 1996b; Javier Enriquez et al., 1997).
Cryptosporidium genotypes and subtypes associated with the illness

Children in Kuwait were predominantly infected by *C. parvum* (Sulaiman et al., 2005). In contrast, *C. hominis* was the species more frequently associated with cryptosporidiosis in children in Peru (Cama et al., 2008; Cordova Paz Soldan et al., 2006). The zoonotic family subtype *C. parvum* Iia was mostly detected in children in Kuwait while the anthroponotic family subtype *C. parvum* Iic was associated to *Cryptosporidium* infection in children in Peru.

In England, *C. parvum* and *C. hominis* were detected in the majority of the cryptosporidiosis human cases in one study (Leoni et al., 2006), as well as in immunocompetent individuals in Peru (Cama et al., 2003). *Cryptosporidium hominis* was detected in 82% of human cryptosporidiosis cases in Australia while the remaining 18% corresponded to *C. parvum* (O'Brien et al., 2008). Correlation with age was observed in The Netherlands where *C. parvum* was more often isolated from patients over 25 years old and *C. hominis* in patients from 0 to 9 of age; however, 80% of human cases were observed in children, and the detection of *C. parvum* in adults may be associated with exposure to cattle (Wielinga et al., 2008).

Zoonotic evidences

The *C. parvum* zoonotic subtype family Iia, associated with cryptosporidiosis in children (Sulaiman et al., 2005), was the most frequently isolated subtype from pre-weaned calves (Feng et al., 2007; Quilez et al., 2008). Cattle are a source of *C. parvum* and a risk factor for human infection. However, the transmission of *Cryptosporidium* from humans to animals was also suggested when the isolation of the same *Cryptosporidium* genotype was reported in a child and in a post-weaned calf in India (Feng et al., 2007).
Prevalence of *Cryptosporidium* in cattle decreases with age, the highest at younger age, and 41%, 26%, 12% and 5.5% prevalence have been reported for pre-weaned calves, post-weaned calves, heifers and milking cows, respectively. *Cryptosporidium parvum* is the most frequent isolate in pre-weaned calves while *C. andersoni*, *C. bovis*, *C. suis* and the *Cryptosporidium* deer-like genotype were detected in older animals (Amer et al., 2009; Fayer et al., 2007). A different study suggested no age correlation of *C. bovis* and *Cryptosporidium* deer-like genotype in cattle, the overwhelming *C. parvum* infection in young animals might be the cause of low occurrence of the other *Cryptosporidium* species in calves (Feng et al., 2007). The high prevalence of cryptosporidiosis in cattle farms was associated with the short time calves remain with their dams, therefore probably due to a shorter intake of maternal milk before weaning (Duranti et al., 2009). High prevalence of *C. parvum* in pre-weaned calves, 2 month or younger, constitutes a risk of *Cryptosporidium* transmission to humans (Fayer et al., 2007), especially to children attending agriculture fairs and petting zoos.

**Prevalence of the disease**

The prevalence of cryptosporidiosis in day-care centers during the outbreak in Milwaukee was 30% (Cordell et al., 1997), and the prevalence of *Cryptosporidium* in children from rural areas in Peru was 1% (Cordova Paz Soldan et al., 2006) when determined by the detection of oocysts in stools. However, reported seroprevalences range from 21% to 95% in humans (Newman et al., 1994; O'Donoghue, 1995).

*Cryptosporidium* infection was detected in 34.3% in immunocompromised populations in Poland (Bajer et al., 2008). The prevalence of *Cryptosporidium* in HIV-positive individuals in
Peru was 13.3%, and low counts of CD4$^+$ were associated with 87% of these cases (Cama et al., 2003).

The seroprevalence in children and adolescents was 31% in Brazil and children who attended day-care facilities were more likely to have antibodies against Cryptosporidium (Newman et al., 1994). In Oklahoma, the distribution of seroprevalences according to age was determined (Kuhls et al., 1994). Children under 5 years had 13% seroprevalence; children between 5 and 13 years have 38% seroprevalence, and 58% seroprevalence was observed in individuals from 14 to 21 years of age. There was not clear explanation for the increased seroprevalence in children older than 14 years; however, asymptomatic cryptosporidiosis seems to be more common in this population (Kuhls et al., 1994).

**Infective dose**

Median challenge infective doses of Cryptosporidium were lower for seronegative than for seropositive individuals, ranging from 75 to 3,000 and depending on the Cryptosporidium parvum isolate used in the experimental challenge in volunteers (DuPont et al., 1995; Okhuysen et al., 1999; White et al., 2000). The mean dose for individuals with pre-existing serum containing anti-Cryptosporidium antibodies ranged from 9,000 to 10,000 (Chappell et al., 1999; White et al., 2000). An increase in the ID$_{50}$ and decrease in oocysts shedding with little or no increments in IgG immune response after challenge was observed for volunteers with pre-existing IgG antibodies (Chappell et al., 1999).
Cryptosporidiosis outbreaks

Foodborne related outbreaks

The number of cases and incidence in the United States per 100,000 population for Cryptosporidium in 2008 were 1.036 and 2.25, respectively according to The Foodborne Diseases Active Surveillance Network (FoodNet) of CDC's Emerging Infections Program (CDC, 2009).

Different food products have been associated with Cryptosporidium outbreaks. In 1995 an outbreak of cryptosporidiosis linked to milk in school children was reported (Gelletlie et al., 1997). During this outbreak 48 of the 260 junior schoolchildren were infected. At first, plumbing work on the water system and contamination of the water supply was thought to be the source of infection but during a case control study it was determined that the only exposure significantly associated with illness was drinking school milk. Improper milk pasteurization was therefore more likely the cause of milk contamination.

An outbreak of cryptosporidiosis among students and staff attending a school agriculture fair in Central Maine in 1993 was associated with drinking fresh-pressed apple juice (Millard et al., 1994). Cryptosporidium oocysts were detected in the apple cider, apple cider press and one calf from the farm, which supplied the apples. In 1993, cryptosporidiosis also associated with the consumption of apple cider was reported in Ohio. In this report, the ozonation, process used for disinfection was presumed to be inadequate for killing Cryptosporidium (Brian G. Blackburn et al., 2006)

Consumption of chicken salad was implicated in a cryptosporidiosis outbreak in Minnesota in 1995 among attendees of a social event. The hostess, who prepared the salad,
operated a licensed day-care home, and may have contaminated the salad after being in contact with asymptomatic children infected with *Cryptosporidium* (CDC, 1996a).

In 1997, an outbreak of cryptosporidiosis was reported in Washington among banquet attendees in a restaurant. The epidemiological studies suggested that uncooked green onions were the source of infection. However, the possibilities of cross-contamination with other food items or contamination by food-handlers were also considered (CDC, 1997).

In 1998, 88 university students and 4 employees who ate at one cafeteria on the university campus in Washington DC showed gastrointestinal illness associated with *Cryptosporidium*. One employee at the cafeteria with the earliest onset of illness worked cutting up vegetables and fruit. This employee was in contact with children affected by diarrhea and vomiting before the outbreak and was implicated in the illness dissemination of the parasite. Food handling by infected people may therefore be considered in transmission of cryptosporidiosis (Quiroz et al., 2000).

**Waterborne related outbreaks**

The occurrence of waterborne outbreaks of cryptosporidiosis has influenced regulations concerning water treatment for human consumption. Currently, public water systems are regulated under the Safe Drinking Water Act (SDWA) of 1974 which was amended in 1986 and again in 1996. The microbial content of drinking water is regulated by EPA and the Surface Water Treatment Requirement Rule (SWTR) of 1989 and the Total Coliform Rule (TCR) of 1989. Additional protection against *Cryptosporidium* for systems that serve ≥ 10,000 persons is provided by the Interim Enhanced Surface Water Treatment Rule (IESWTR), promulgated by
EPA in 1998 after numerous cases of cryptosporidiosis were observed due to water contamination.

Drinking water has been associated with cryptosporidiosis (Lazensky et al., 2008). In addition, recreational waters have been linked to many cryptosporidiosis outbreaks and the contamination of waters for human use with Cryptosporidium oocysts has been assessed. For example, Cryptosporidium was found in 85% of raw water locations, with approximately 32% of viable oocysts, in river waters (2 to 112 oocysts/l), and in 97% of the surface water supplies (LeChevallier et al., 1991). In produce irrigation waters from Mexico, Cryptosporidium oocysts were detected in 48% of surface waters, and in 19% of wash-water tanks, the water was subsequently used for irrigation of crops (Chaidez et al., 2005).

In the United States, one hundred seventeen persons were infected after drinking contaminated water in Texas in 1984 (D'Antonio et al., 1985). In 1987, a chlorinated and filtered water supply was the source of an outbreak that occurred in Georgia where an estimated 13,000 people were affected (Hayes et al., 1989). Three outbreaks with 3,551 cases were observed between 1991 and 1992 in Oregon and Pennsylvania (CDC, 1993).

The first outbreak of cryptosporidiosis associated with recreational water in the United States was reported in 1994 among swimmers in a lake in New Jersey (Kramer et al., 1998). Between 1993 and 1994, 71.4% of the waterborne outbreaks related to recreational waters were caused by Cryptosporidium. Filtered and chlorinated lake water and untreated well water were associated with the five outbreaks affecting 403,271 people in Minnesota, Nevada, Washington, and Wisconsin (CDC, 1996c). In 1995, an outbreak in a water park in Georgia which affected 5,449 people was associated with contamination through fecal accidents, run-off water containing livestock feces or inadequate water chlorination (CDC, 1998). During 1997, nine
outbreaks were associated with recreational waters in pools or fountains (CDC, 2000), and between 2001 and 2002, three outbreaks, in Nebraska, Illinois and Massachusetts affecting 157, 358, and 767 people, respectively, were associated with fecal contamination of swimming pools (CDC, 2004). In 2003, an outbreak caused by *C. hominis* occurred in Kansas swimming pools and day-care centers, 617 people were affected, and this outbreak was the largest associated with recreational waters between 2003 and 2004 (CDC, 2006). Between 2005 and 2006, *Cryptosporidium* waterborne outbreaks associated with recreational waters affected 3,751 people (CDC, 2008).

**Treatment of Cryptosporidiosis**

**Chemical compounds**

No optimal treatment for cryptosporidiosis is currently available. Nitazoxanide, inhibitor of the pyruvate:ferredoxin oxidoreductase electron transferase activity (parasite anaerobic energy pathway), and the antibiotic paramomycin, an aminoglycoside, which binds to prokaryote ribosomes and decreases protein synthesis, are drugs prescribed for human cryptosporidiosis. Nitazoxanide has been approved by the United States Food and Drug Administration (FDA) for treatment of cryptosporidiosis in patients one year of age and older. Paramomycin has been partially effective against cryptosporidiosis in immunocompromised individuals, including children with leukemia, AIDS and HIV cases, and little benefits have been observed with nitazoxanide for patients with HIV (Abubakar et al., 2007; Bobak, 2006; Zardi et al., 2005). In HIV individuals, the use of antiretrovirals was associated with increased CD4⁺ counts and therefore had a positive effect against cryptosporidiosis (Hunter and Nichols, 2002).
Many molecules have been studied for their ability to inactivate *Cryptosporidium*. Some molecules, such as benzimidazoles, are not effective against *Cryptosporidium*. The protein residues in β tubulin related to benzimidazole susceptibility have not been found in *C. parvum* (Katiyar et al., 1994).

The effects of anti-cryptosporidial drugs are evaluated in cell cultures and in animal models. Fluorescence labeling of *Cryptosporidium* with monoclonal antibodies (mAbs) and the computerized imaging of infection foci in cell culture as well as the combination of cell culture and a qPCR targeting a fragment of *hsp70* gene of *C. parvum* have been suggested as alternative methodologies to evaluate the effect of anti-cryptosporidial drugs (Najdrowski et al., 2007; Shahiduzzaman et al., 2009a; Shahiduzzaman et al., 2009b).

Nitrogen containing biphosphonates (N-BP), which inhibit the biosynthesis of isoprenoids, have been tested against *C. parvum* in cell cultures and some molecules had better results, with low toxicity to the host cells, than those reported for paramomycin (Artz et al., 2008). Other molecules, such as miltefosine and curcumin, not previously-used against *Cryptosporidium*, reduced *Cryptosporidium* multiplication in cells (Shahiduzzaman et al., 2009a; Shahiduzzaman et al., 2009b). Drugs targeting the ATP-binding cassette (ABC) proteins and superoxide dismutases (SODs) have been suggested for treatment of cryptosporidiosis because these proteins are presumably relevant for parasite survival (Kang et al., 2008b; Zapata et al., 2002).

**Immunological molecules**

The use of immunological agents against *Cryptosporidium* also has been evaluated for their ability to inactivate the parasite *in vitro* and ameliorate the disease in animal models. For
instance, anti-fibronectin serum was shown to be effective in reducing infectivity and host-cell attachment, in protozoan parasites, such as *Trypanosoma* and *Leishmania*; and cycloheximide, a protein synthesis inhibitor, reduced *C. parvum* infectivity in cell cultures (Rosales et al., 1995).

Oral passive immunotherapy with a monoclonal antibody (mAb) against *C. parvum* sporozoites and merozoites antigens of 75 and 215 kDa, and *C. parvum* sporozoite antigens of 150 and 175 kDa significantly reduced *C. parvum* intestinal infection in congenitally athymic nude mice (Bjorneby et al., 1991). In another report, the *C. parvum* sporozoite and merozoite surface antigen-1 (SAG-1) recognized by neutralizing mAb was used to obtain SAG-1 immune bovine serum and was tested for its ability to reduce *C. parvum* intestinal infectivity in mice. This immune serum failed to prevent *C. parvum* infection or terminate persistent infection in SCID mice and BALB/c mice whereas a hyper-immune bovine serum showed effectiveness in suckling BALB/c mice (Tatalick and Perryman, 1995).

In severe combined immunodeficient (SCID) mice, hyper-immune colostrum antibody administered during cryptosporidiosis significantly reduced shedding of oocysts as well as gastrointestinal and biliary illness caused by *Cryptosporidium* (Riggs et al., 1994). In addition, hyperimmune bovine colostrum containing anti-*Cryptosporidium* immunoglobulins inhibited *C. parvum* infectivity *in vitro* and demonstrated protection for calves (Doyle et al., 1993).

**Disinfection**

The observation of infection foci in cell culture was used to assess the effect of different disinfectants on *Cryptosporidium* oocysts (Weir et al., 2002). The 6% hydrogen peroxide solution completely reduced the infectivity of *Cryptosporidium* after 13 to 33 minutes of exposure, and a 1000-fold reduction was observed after 4 minutes. Infectivity was also
significantly reduced after 13 minutes of exposure to 37% methanol. However, the 6% sodium hypochlorite, 70% ethanol, and 70% isopropanol did not reduce infectivity of *C. parvum* after 33 minutes of exposure.

**Water treatment**

During water treatment, coagulation, flocculation, and sedimentation were effective to remove > 90 % *Cryptosporidium* oocysts before filtration and disinfection (Keegan et al., 2008). The effect of free chlorine, chlorine dioxide and ozone has been tested for water treatment (Pereira et al., 2008). The disinfectant effect was assessed by measuring the inactivation of *C. parvum* oocysts in excystation assays. The highest rate of inactivation for free chlorine was 49.04% when using 2 ppm concentration for 120 min (CT = 240); a 90.56% inactivation rate was reached using 5 ppm concentration of chlorine dioxide for 120 min (CT = 450), and 0.24 mg/l concentration of ozone was enough to increase the inactivation rate up to 100%.

Eighteen percent reduction of *C. parvum* infectivity was observed in water samples with a level of turbidity of 5 NTU after 8 h of exposure time to a gamma radiation intensity of 600 W/m² while a 70% reduction was observed at the same turbidity level but with 12 h of exposure time to a gamma radiation intensity of 900 W/m² (Gomez-Couso et al., 2009). Gamma irradiation was also evaluated for its effect on *C. parvum* sporozoites, and a dose of 10 kGy gamma radiation was suggested for disinfection purposes (Lee et al., 2009).

**Parasite Inactivation**

Temperatures below freezing reduce *Cryptosporidium* viability due to physical damage. To inactivate 99.99% of *Cryptosporidium* oocysts 853 days at -4°C or 64 days at -22°C is
required (Peng et al., 2008). *Cryptosporidium* lost infectivity when it was exposed to temperatures between 45 °C to 55°C for five minutes (Anderson, 1985).

**Inactivation in foods**

High temperature short time treatment (HTST) applied to milk and water seeded with *C. parvum* oocysts completely inactivated *Cryptosporidium*. The temperature reached was 71.1°C, and milk and water were treated for 5, 10 and 15 seconds at this temperature. *C. parvum* oocysts recovered from HTST pasteurized water and milk were not infective to mice (Harp et al., 1996).

The use of UV light was evaluated during depuration of oysters. The average viability measured by *Cryptosporidium* oocysts staining with 4’,6’-diamidino-2-phenyl indol (DAPI) fluorogenic dye and inclusion/exclusion propidine iodide (PI) vital dye was significantly reduced in oyster tissues after 48 h depuration in clean artificial sea water coupled with UV treatment compared to only depuration in clean artificial sea water. Viability was reduced from 53.7±2.5% to 17.6±3.7% during this experiment. Incomplete inactivation of *Cryptosporidium* in oysters’ tissues was may be related to immobilization of them within shellfish gills and hemocytes (Sunnotel et al., 2007).

**Diagnosis**

**Clinical Diagnosis**

Microscopic examination of stools for *Cryptosporidium* oocysts has been used for diagnosis of cryptosporidiosis. For microscopy, multiple staining procedures have been employed (O'Donoghue, 1995); however, acid-fast staining, Ziehl-Neelsen or Kinyoun, where
oocysts are distinguished by their bright red color, is the most common staining technique used in diagnosis (Black et al., 1996).

Even though staining-based microscopy is broadly used, technician expertise is always required to identify the parasite. Immunoassays have resulted in better sensitivity and specificity than the chemical staining methods (Arrowood and Sterling, 1989). However, microscopic identification of chemically as well as immunologically stained Cryptosporidium oocysts does not differentiate species, or viable oocysts.

Among immunoassays, direct fluorescent antibody test (DFA) demonstrated better sensitivity than the immunochromatographic lateral flow immunoassay and enzyme immunoassays (EIA) rapid assays and the acid-fast staining for the detection of Cryptosporidium in stool samples (Johnston et al., 2003). However, the use of these methods for screening for low cryptosporidiosis prevalence populations is not recommended.

Specificity and cross-reactivity of the antibodies used for immunolabeling and immunoassays determines the sensitivity of these methods. Lack of sensitivity has been suggested for direct immunofluorescent assays when oocysts in experimentally infected individuals who showed symptoms of cryptosporidiosis were not detected (Okhuysen et al., 1999).

In a flow cytometry experiment, the detection limit was $2 \times 10^5$ Cryptosporidium parvum oocysts/ml in fecal samples (Barbosa et al., 2008). Quantification and discrimination from debris are possible in flow cytometry analysis and flow cytometry with or without cell sorting were shown to be useful for the identification of Cryptosporidium oocysts in samples stained by immunofluorescent antibody (Hsu et al., 2005).
Immune response

Humoral and cellular immune response have been observed during cryptosporidiosis. Intestinal epithelial cells are stimulated to produce inflammatory molecules, such as chemokines, during *Cryptosporidium* infection (Chen et al., 2005), and these molecules provoke migration of inflammatory cells to the infection areas (Zadrozny et al., 2006). T-cells were also essential for elimination of the parasite according to studies conducted in mice (Heine et al., 1984). IFN-γ and CD4⁺ T-cell lymphocytes are involved in the immune response against *Cryptosporidium* (Hunter and Nichols, 2002). Regarding the humoral immune response, IgA, IgG and IgM in sera from humans and animals have recognized several *Cryptosporidium* antigens, especially proteins of 15, 17, 23, and 27 kDa (Moss et al., 1998; Reperant et al., 1994).

Immune response assessment:

An indirect ELISA for the detection of anti-*Cryptosporidium* antibodies against the *C. parvum* protein 23 (Cp23) was designed and optimized (Wang et al., 2009). This ELISA method was more sensitive than microscopic examination for determining recent exposure to *C. parvum* in cows. ELISA was suggested to be the most practical method for monitoring *Cryptosporidium* infection in HIV individuals (Kaushik et al., 2008). ELISA sensitivity and specificity were comparable to PCR for this population. The Cp23 has also been used in dot blot analysis to screen calves and cows for anti-Cp23 antibodies (Shayan et al., 2008). Cp23 used in these analyses was a recombinant product. In a different study, a recombinant 17 kDa *Cryptosporidium* protein recognized by human sera from cryptosporidiosis cases, and monoclonal antibodies were also used to design an ELISA test (Priest et al., 2000).
Oocyst purification

*Cryptosporidium* oocysts can be purified from suspensions with other particles by density gradient centrifugation, concentration steps or immunomagnetic separation (Fayer et al., 2000; Xiao et al., 2001). Magnetic beans, flow cytometry and immunomagnetic separation have been used to recover and quantify *Cryptosporidium* oocysts (Sunnotel et al., 2007). However, detection of oocysts by methods which include immunomagnetic separation can be affected by pH, turbidity, divalent cations, algae, iron in water/food samples due to interference with antibody binding (Smith and Nichols, 2009).

Molecular diagnosis

Different PCR-based methods have been described for the identification of *Cryptosporidium* in human and environmental samples. Nested PCR coupled with RFLP or sequencing has been useful to differentiate *Cryptosporidium* genotypes and subtypes (Bajer et al., 2008; Coupe et al., 2005; Elwin et al., 2001; Leone et al., 2009; Leoni et al., 2006; Pedraza-Diaz et al., 2001a; Peng et al., 1997; Wielinga et al., 2008). Multiplex real time-PCR designed for the detection in stools of *Entamoeba histolytica, Giardia lamblia* and *C. parvum / C. hominis* has also been used for patients with traveler’s diarrhea (Ten Hove et al., 2009). This analysis identified more positive cases when compared to microscopy and antigen detection tests.

Even though PCR analysis simplifies the identification of *Cryptosporidium*, detection can be affected by contaminants, inhibitory molecules; such as humic acid, fulvic acid and polyphenolics; encountered in water or other samples, and it does not differentiate viable or infective *Cryptosporidium* oocysts (Smith and Nichols, 2009).
Cryptosporidium identification in foods

Orange juice, apple cider, whole milk, strawberries, parsley and lettuce experimentally contaminated with Cryptosporidium oocysts were evaluated by nested PCR targeting the 18S rRNA to compare two different DNA extraction methods (Frazar and Orlandi, 2007). When 5 to 50 oocysts were inoculated, the method based on FTA filters yielded more false negatives than the method based on the application of a DNA extraction kit. Detection of 15% and 59 % positive samples were reported from the FTA filter method and the DNA extraction kit methods, respectively. PCR results were affected by pH, inhibitors and matrix interference with the immunomagnetic separation. The CpR1 gene was also a target for a PCR to detect Cryptosporidium in milk. In this method the PCR product was hybridized with a digoxigenin-labeled oligonucleotide and detected with a chemiluminescent substrate. This test detected one to ten oocysts in milk samples (Laberge et al., 1996).

Cryptosporidium oocysts have been detected by immunofluorescence in shellfish from estuarine and coastal waters in the North American Atlantic coast (Fayer et al., 2003). The parasite was detected in 3.7 % of the shellfish samples, and C. parvum, C. hominis, and C. meleagris were identified.

Cryptosporidium identification in water

The methods 1622 and 1623 of the US EPA were designed to identify Cryptosporidium in water samples by filtration, elution, immunomagnetic separation (IMS) and immunofluorescence (IF) assay microscopy. Confirmation is done by the 4’,6-diamidino-2-phenylindole (DAPI) staining and differential interference contrast (DIC) microscopy. The DAPI staining method is also used to indicate parasite viability, however it can overestimate...
viable counts (Black et al., 1996). One advantage of this method is the possibility to detect intact as well as empty *Cryptosporidium* oocysts, since empty oocysts also can be considered as an indicator of contamination of water with *Cryptosporidium* oocysts (Smith and Nichols, 2009).

The method cannot determine the *Cryptosporidium* species or oocyst infectivity, and many interference substances can affect the concentration and detection steps of this method, such as the presence of autofluorescent structures, dinoflagellates and algae (USEPA, 2001a, b). Recovery rates of 72% and 42% were reported for the EPA methods 1623 and 1622, respectively (LeChevallier et al., 2003; Simmons et al., 2001).

The 18S rRNA gene based nested PCR RFLP method with immunomagnetic separation has been evaluated for the identification of *Cryptosporidium* oocysts in water samples. Using this method, it was determined that *Cryptosporidium* oocysts from wildlife were the major contaminants of storm waters, while *Cryptosporidium* oocysts from humans and domestic animals were found in raw surface waters and waste water (Xiao et al., 2004).

The binding of *C. parvum* oocysts to an antibody-covered gold-coated crystal surface monitored with a quartz crystal microbalance with dissipation monitoring (QCM-D) biosensor has been also used to detect the parasite in water samples. The detection limit was ~1 oocyst/ml; however, non-biological and biological interference substances, such as humic and fulvic acid or bacteria, respectively, were shown to affect the level of detection by this method. Therefore, its effectiveness at detecting *Cryptosporidium* in water samples needs to be improved (Poitras et al., 2009).

The immobilization of biotin, streptavidin, and biotinylated secondary polyclonal anti-mouse antibody over a gold surface was conducted for the immunodetection of *C. parvum* oocysts in water samples. The analyte was the residual anti-*C. parvum* IgM, and changes in the
refractive index on the monolayer after analyte binding was real-time detected and inversely corresponded to the oocyst concentration in the media (Kang et al., 2008a). This method was less effective at detecting oocysts in tap water and reservoir water samples compared to those in a buffer solution. The unfavorable conditions for the immune reactions in field water samples were suggested as the explanation for the observed reduction in sensitivity (Kang et al., 2008a).

**Determination of parasite viability**

The determination of parasite viability is important in foods and water since it indicates potential sources of *Cryptosporidium* infection. Some methods have been evaluated. For instance, the oocyst excystation followed by a DNase treatment and sporozoite DNA extraction was used to distinguish live from dead oocysts using PCR to amplify the *Cryptosporidium* outer-wall protein (COWP) gene (Wagner-Wiening and Kimmig, 1995). DNA from the excysted sporozoites was detected when an initial count of 100 oocysts was processed. PCR sensitivity was improved to detect 10 oocysts when the number of PCR amplification cycles was increased.

Fluorescent *in-situ* hybridization (FISH) targeting the 18S rRNA of *Cryptosporidium* was conducted to detect the parasite and assess its viability. However, degradation of the 18S rRNA might not be fast enough, and as such this RNA could be still detected even in non-viable parasites (Jex et al., 2008b). Oocyst viability was also determined by nucleic acid sequence based amplification (NASBA) (Baeumner et al., 2001). With this method, it was possible to detect 5 viable oocysts in buffer solutions, and 50 oocysts from an original volume of 10 L water samples.

The *Cryptosporidium* heat shock protein 70 (*hsp70*) mRNA has been used to determine parasite viability in water samples (Smith and Nichols, 2009; Stinear et al., 1996). *Hsp70*
mRNA induced by heat stress in *Cryptosporidium* was hybridized to oligo (dT)$_{25}$ which were linked to magnetic beads before RT-PCR detection. Some interference was observed for reservoir and river waters; however, the detection of a single viable oocyst was achieved by this method.

The Cp2 mRNA, which corresponds to a *Cryptosporidium* membrane-associated protein, was evaluated by quantitative real-time PCR, and its efficiency was compared with the use of *hsp* 70, COWP, β tubulin and 18S rRNA genes to determine *Cryptosporidium* viability (Lee et al., 2008). With heat-killed oocysts, CP2 and *hsp*70 showed heat lability, and the COWP and β tubulin genes were not expressed while the 18S rRNA gene persisted. The number of viable oocysts was overestimated with the 18S rRNA gene and cross-reactivity with *Toxoplasma* was observed for CP2, β tubulin, and *hsp*70.

**Molecular characterization of *Cryptosporidium***

The *C. parvum* genome is comprised of 9.1 Mb of DNA sequences assembled in 8 chromosomes, with an estimated number of 3,807 genes (Trasarti et al., 2007). Only a few of them have been studied for the diagnosis and identification of *Cryptosporidium* genotypes and subtypes. Housekeeping genes are preferred for genotyping, and genes with rich hypervariable regions are more useful for *Cryptosporidium* subtyping. The internal transcriber spacer regions (ITS) have been proven to be suitable for subtyping to some extent. Genotyping and subtyping of *Cryptosporidium* isolates are important for epidemiological studies during outbreak investigations.
Genotyping

PCR methods targeting the COWP, 18S rRNA, *hsp70*, gp60, ML1, ML2, β tubulin and TRAP genes coupled with restriction fragment length polymorphism (RFLP) and sequencing analysis have been used to genotype *Cryptosporidium* from stools and environmental samples (Bajer et al., 2008; Coupe et al., 2005; Elwin et al., 2001; Leone et al., 2009; Leoni et al., 2006; Pedraza-Diaz et al., 2001a; Peng et al., 1997; Wielinga et al., 2008).

In human samples, *Cryptosporidium* species have been identified by nested PCR targeting a segment of the 18S rRNA gene and restriction analysis with endonucleases *SspI* and *VspI* (Cordova Paz Soldan et al., 2006). *Cryptosporidium andersoni*, *C. parvum* and *Cryptosporidium* deer-like genotype were differentiated by a nested PCR targeting the 18S rRNAs and restriction analysis with the endonuclease *MboII* (Feng et al., 2007), and *C. hominis*, *C. parvum*, *C. meleagris*, and *C. muris* were identified by nested PCR targeting the 18S rRNA gene and RFLP with the *AsnI* enzyme (Gatei et al., 2003). Denaturing gradient gel electrophoresis (DGGE) has also been used to identify polymorphism in *Cryptosporidium* spp (Satoh and Nakai, 2007). This PCR based technology was designed to evaluate a partial sequence of the 18S rRNA gene. A nested-PCR targeting this gene followed by the digestion of the amplicon with the *SspI* and *ApoI* restriction enzymes was then used for comparison. PCR/RFLP did not distinguish *C. andersoni* and *C. muris* while the DGGE method showed different band patterns for these species. In addition, many *Cryptosporidium* species could be genotyped by single strand conformation polymorphism (SSCP) following PCR targeting the 18S rRNA gene (Jex et al., 2007a).

*Cryptosporidium felis*, *C. canis*, *C. andersoni*, *C. suis*, *C. muris*, and *C. cervine* genotype were not identified by a PCR/ RFLP analysis designed to amplify a segment of the COWP gene
(Leoni et al., 2006; Spano et al., 1997), and a nested PCR targeting the gp60 gene followed by sequencing did not amplify DNA from *C. bovis*, *C. andersoni*, or *Cryptosporidium* deer-like genotype (Feng et al., 2007).

Sequencing analysis of the *hsp*70 gene identified *C. parvum*, *C. canis*, *C. felis*, and *C. meleagridis* (Xiao et al., 2001). Single nucleotide polymorphisms of the *hsp* 70 gene were used to differentiated *C. parvum* and *C. hominis* by a microarray approach (Straub et al., 2002).

A PCR-based high-resolution melting curve analysis of the *Cryptosporidium* ITS2 sequence was evaluated as a rapid, high-throughput diagnostic and genetic analysis tool (Pangasa et al., 2009). *Cryptosporidium hominis*, *C. parvum*, and *C. meleagridis* were identified by this method. This qualitative approach has the potential to detect “mixed” infection.

**Subtyping**

Subtyping of *C. parvum* and *C. hominis* is done by the sequence analysis of the gp60 gene. This gene has tandem repeats of the serine-coding trinucleotide TCA, TCG or TCT at the 5’ end, and this characteristic allows classification of isolates according to their serine-coding trinucleotide repeats. Subtypes families of *C. parvum* (IIa, IIb, etc) or *C. hominis* (Ia, Ib, etc), and the number of tandem repeats are indicated in the subtype name. This subtyping tool does not amplify DNA from other related *Cryptosporidium* species, such as *C. canis* and *C. felis*. The *C. parvum* subtype Ila is common in animals (zoonotic genotype), the *C. parvum* subtype IIC is mostly isolated from humans (anthroponotic genotype) (Xiao, 2009). The *C. hominis* family subtype Ib was the only one associated with diarrhea and nausea, vomiting and general malaise, suggesting this subtype family as more pathogenic than subtype families Ia, Id and Ie (Cama et al., 2008).
A multilocus microsatellite subtyping analysis was proposed to evaluate epidemiological data from cryptosporidiosis outbreaks. Almost all of the isolates from one strain identified during a waterborne outbreak in England were clustered around the main focus for this outbreak. However, the variation due to presence of different lineages or evolution of strains could not be elucidated by this method (Hunter et al., 2008).

For large-scale population, scanning SSCP following a nested-PCR targeting the gp60 gene, and sequencing of selected amplicons was evaluated (Jex et al., 2007b). The method was compared to PCR methods targeting the Cryptosporidium ITS-2 sequence and the 18S rRNA gene followed by sequencing. The variation obtained was greater in gp60 gene than the ones observed for the ITS-2 and 18S rRNA gene PCR methods, and sequencing of the amplicons resulted in different Cryptosporidium subtypes. In another study, SSCP and sequencing following PCR targeting the 18S rRNA and hsp70 genes yielded only 2% and 1.2% variability between C. parvum and C. hominis isolates, respectively (Jex et al., 2008a). Using this methodology, the most dominant subtypes were the Ib for C. hominis and IIA for C. parvum in the UK (Jex and Gasser, 2008).

**Cryptosporidium antigenic proteins**

Cryptosporidium antigens of 15-17 and 23 kDa have been suggested as markers of early cryptosporidiosis infection for different species, and the immune response against these antigens has been evaluated in one-dimensional western blots (Reperant et al., 1994).

Other Cryptosporidium proteins were identified by their reactivity with hyperimmune colostrum anti-Cryptosporidium antibodies. Hyperimmune colostrum antibodies recognized proteins from 25 kDa to 200 kDa in C. parvum oocysts, sporozoites and merozoites (Riggs et al.,
1994), and in another report, two major antigens were recognized by hyperimmune colostrum, a >900 kDa and a 250 kDa protein (Doyle et al., 1993). Some antigenic proteins were able to elicit immune protection against cryptosporidiosis. Monoclonal antibodies (mAbs) against *C. parvum* antigens of 75, 150, 175, and 215 kDa were protective for mice (Bjorneby et al., 1991).

*Cryptosporidium* sporozoite antigens in the apical complex and surface pellicle were identified by screening of DNA expression libraries (Petersen et al., 1992). These proteins had molecular weights (MWs) of >500, 68/95, 45, 23, 15/35 kDa.

MAbs against sporozoite surface epitopes have been evaluated for their therapeutic effect on cryptosporidiosis. The anti-CSL mAb 3E2 was useful to reduce but not eliminate persistent cryptosporidiosis in mice models, and the mAb18.44 and mAb17.41 neutralized *Cryptosporidium* infectivity in a mouse model (Riggs et al., 2002; Uhl et al., 1992).

Regarding genotype differentiation, mAbs produced against *C. parvum* Iowa isolate p23 and GP900 showed different affinities for the corresponding *C. hominis* proteins. Nucleotide differences in the p23 and GP900 genes resulted in amino acid differences of the respective proteins between *C. parvum* and *C. hominis*. The authors suggested these differences are associated with host specificity (Sturbaum et al., 2008).

**Proteomic studies**

More than 300 *Cryptosporidium* sporozoite proteins from 5 to 150 kDa and pI from 4 to 8.5 were identified by two-dimensional gel electrophoresis (Mead et al., 1990). A protein of 106 kDa and a protein family of 40 kDa were useful for the differentiation of four *C. parvum* isolates.

In 2007, twenty six proteins expressed during excystation of *Cryptosporidium* oocysts compared to non-excysted *Cryptosporidium* oocysts were identified by MS and iTRAQ™
reagent (Applied Biosystems, Foster City, CA) isotype labeling and the C. parvum genomic database (Snelling et al., 2007). Cryptosporidium sporozoites proteins were also identified by two-dimensional gel electrophoresis/ MALDI-TOF or MS/MS, 1D gel-LC-MS/MS and MudPit, resulting in 282, 642 and 1,154 proteins, respectively. They represented 32% of the predicted Cryptosporidium genome (Sanderson et al., 2008).

Objectives and brief description of the study

Proteomics development in the last several years is providing abundant information related to Cryptosporidium protein expression. Consistent identification of Cryptosporidium proteins and antigenic proteins by different approaches facilitates the selection of specific molecules to be further evaluated for their usefulness in the diagnosis and therapeutics of cryptosporidiosis.

Working on this trend, this study was designed to first compare protein profiles of C. parvum Iowa, Arizona and Moredum isolates by two-dimensional difference gel electrophoresis (DIGE) in order to determine isolate-specific proteins of C. parvum which may aid in the understanding of biological characteristics associated with parasite behavior and/or host-parasite interaction and specifically associated to these Cryptosporidium isolates. In addition, this study evaluated and compared the C. parvum antigenic proteins recognized by human sera from people suffering from Cryptosporidium infection with those recognized by human sera from people with no history of cryptosporidiosis infection and without any clinical symptom associated with the disease. Antigenic proteins were determined by one-dimensional and two-dimensional western blotting. Selection of proteins which could be useful for diagnosis of acute cases of cryptosporidiosis was based on significant differences in antigenic reactivity between
experimental groups. Antigenic proteins were identified by Tandem Mass Spectrometry (MS/MS) by screening Cryptosporidium genomic databases, and theoretical antigenic peptides were selected from high-scoring Cryptosporidium peptide sequences when they were aligned with sequences available at the non-redundant NCBI database.
References


Cama, V.A., Bern, C., Sulaiman, I.M., Gilman, R.H., Ticona, E., Vivar, A., Kawai, V.,
Vargas, D., Zhou, L., Xiao, L., 2003, Cryptosporidium species and genotypes in HIV-

CDC, 1984, Cryptosporidiosis among children attending day-care centers--Georgia,
Pennsylvania, Michigan, California, New Mexico. MMWR Morb Mortal Wkly Rep
33, 599-601.

MMWR CDC Surveill Summ 42, 1-22.

CDC, 1994a, Cryptosporidium infections associated with swimming pools--Dane County,

CDC, 1994b, From the Centers for Disease Control and Prevention. Cryptosporidium
infections associated with swimming pools--Dane County, Wisconsin, 1993. JAMA
272, 914-915.

CDC, 1996a, Foodborne outbreak of diarrheal illness associated with Cryptosporidium

CDC, 1996b, From the Centers for Disease Control and Prevention. Outbreak of
cryptosporidiosis at a day camp--Florida, July-August 1995. JAMA 275, 1790.

MMWR CDC Surveill Summ 45, 1-33.

MMWR CDC Surveill Summ 47, 565-567.

MMWR CDC Surveill Summ 47, 1-34.


Hunter, P.R., Wilkinson, D.C., Lake, I.R., Harrison, F.C., Syed, Q., Hadfield, S.J.,
Chalmers, R.M., 2008, Microsatellite typing of Cryptosporidium parvum in isolates

Javier Enriquez, F., Avila, C.R., Ignacio Santos, J., Tanaka-Kido, J., Vallejo, O., Sterling,
C.R., 1997, Cryptosporidium infections in Mexican children: clinical, nutritional,

Jex, A.R., Gasser, R.B., 2008, Analysis of the genetic diversity within Cryptosporidium
hominis and Cryptosporidium parvum from imported and autochtonous cases of
human cryptosporidiosis by mutation scanning. Electrophoresis 29, 4119-4129.

Jex, A.R., Pangasa, A., Campbell, B.E., Whipp, M., Hogg, G., Sinclair, M.I., Stevens, M.,
Gasser, R.B., 2008a, Classification of Cryptosporidium species from patients with
sporadic cryptosporidiosis by use of sequence-based multilocus analysis following

Jex, A.R., Ryan, U.M., Ng, J., Campbell, B.E., Xiao, L., Stevens, M., Gasser, R.B., 2007a,
Specific and genotypic identification of Cryptosporidium from a broad range of host
species by nonisotopic SSCP analysis of nuclear ribosomal DNA. Electrophoresis 28,
2818-2825.

Jex, A.R., Smith, H.V., Monis, P.T., Campbell, B.E., Gasser, R.B., 2008b, Cryptosporidium—
biotechnological advances in the detection, diagnosis and analysis of genetic

Jex, A.R., Whipp, M., Campbell, B.E., Caccio, S.M., Stevens, M., Hogg, G., Gasser, R.B.,
2007b, A practical and cost-effective mutation scanning-based approach for


Cryptosporidium antigens following experimental infection in humans. J Infect Dis 178, 827-833.


Gasser, R.B., 2009, High resolution melting-curve (HRM) analysis for the diagnosis

Pedraza-Diaz, S., Amar, C., Nichols, G.L., McLauchlin, J., 2001a, Nested polymerase chain
reaction for amplification of the Cryptosporidium oocyst wall protein gene. Emerg
Infect Dis 7, 49-56.

Pedraza-Diaz, S., Amar, C.F., McLauchlin, J., Nichols, G.L., Cotton, K.M., Godwin, P.,
Iversen, A.M., Milne, L., Mulla, J.R., Nye, K., Panigrahl, H., Venn, S.R., Wiggins,
R., Williams, M., Youngs, E.R., 2001b, Cryptosporidium meleagridis from humans:

Peng, M.M., Xiao, L., Freeman, A.R., Arrowood, M.J., Escalante, A.A., Weltman, A.C.,
among Cryptosporidium parvum isolates: evidence of two distinct human

Peng, X., Murphy, T., Holden, N.M., 2008, Evaluation of the effect of temperature on the
die-off rate for Cryptosporidium parvum oocysts in water, soils, and feces. Appl
Environ Microbiol 74, 7101-7107.

Pereira, J.T., Costa, A.O., de Oliveira Silva, M.B., Schuchard, W., Osaki, S.C., de Castro,
E.A., Paulino, R.C., Soccol, V.T., 2008, Comparing the efficacy of chlorine, chlorine
dioxide, and ozone in the inactivation of Cryptosporidium parvum in water from


Riggs, M.W., Cama, V.A., Leary, H.L., Jr., Sterling, C.R., 1994, Bovine antibody against Cryptosporidium parvum elicits a circumsporozoite precipitate-like reaction and has


Sam-Yellowe, T.Y., 1996, Rhoptry organelles of the apicomplexa: Their role in host cell invasion and intracellular survival. Parasitol Today 12, 308-316.


Trasarti, E., Pizzi, E., Pozio, E., Tosini, F., 2007, The immunological selection of recombinant peptides from Cryptosporidium parvum reveals 14 proteins expressed at the sporozoite stage, 7 of which are conserved in other apicomplexa. Mol Biochem Parasitol 152, 159-169.


Wielinga, P.R., de Vries, A., van der Goot, T.H., Mank, T., Mars, M.H., Kortbeek, L.M.,
van der Giessen, J.W., 2008, Molecular epidemiology of Cryptosporidium in humans


Xiao, L., Bern, C., Limor, J., Sulaiman, I., Roberts, J., Checkley, W., Cabrera, L., Gilman,
R.H., Lal, A.A., 2001, Identification of 5 types of Cryptosporidium parasites in

Xiao, L., Escalante, L., Yang, C., Sulaiman, I., Escalante, A.A., Montali, R.J., Fayer, R.,
Lal, A.A., 1999, Phylogenetic analysis of Cryptosporidium parasites based on the

in water by PCR-RFLP. Methods Mol Biol 268, 163-176.

Neutrophils do not mediate the pathophysiological sequelae of Cryptosporidium
parvum infection in neonatal piglets. Infect Immun 74, 5497-5505.

Cryptosporidium parvum ABC protein family. Mol Biochem Parasitol 120, 157-161.

Zardi, E.M., Picardi, A., Afeltra, A., 2005, Treatment of cryptosporidiosis in
CHAPTER 2

DIFFERENTIAL PROTEIN EXPRESSION OF CRYPTOSPORIDIUM PARVUM ISOLATES

BY TWO-DIMENSIONAL DIFFERENCE GEL ELECTROPHORESIS (2D-DIGE)¹

¹Sanchez-Ingunza, Roxana and Ynés R. Ortega. To be submitted to Infection and Immunity.
Abstract

Cryptosporidium parvum Iowa (IO), C. parvum Arizona (AZ) and C. parvum Moredum (MD) protein profiles were compared by two-dimensional difference gel electrophoresis (2D-DIGE). C. parvum proteins were extracted from $10^6$ oocysts, and the soluble fractions were differentially labeled with cyanine fluorescent dyes CyDye DIGE Fluor Cy5 and Cy3. C. parvum AZ demonstrated significant difference in the expression of a 36 kDa protein with isoelectric point (pI) of 6.6 and a 43 kDa with pI of 6.36 ($p < 0.05$) while C. parvum IO showed significant abundance for a 46 kDa protein with pI of 6.91 ($p < 0.05$) and C. parvum MD for 72 and 78 kDa proteins with pIs of 5.13 and 5.49 ($p < 0.1$), respectively, thereby indicating that these proteins are useful for distinguishing these isolates. The association of the expression of these proteins with the differences in the parasite behavior between isolates, especially at the parasite infectivity level, may be elucidated to support the suitability of using these polypeptides for differential diagnosis.
**Introduction**

*Cryptosporidium* is a protozoan parasite associated with food and waterborne illness (CDC, 1996, 2008). The disease is associated with self-limited symptoms in immunocompetent individuals. However, it can be life-threatening for the immunocompromised (Chalmers and Davies, 2009; Hunter and Nichols, 2002; Leoni et al., 2006). Limited treatment options are currently available (Bobak, 2006; Zardi et al., 2005), and there is a need of new *Cryptosporidium* molecular targets for treatment of cryptosporidiosis as well as for diagnosis and strain differentiation.

The study of *Cryptosporidium* protein expression aids in the identification of potential molecular targets. Some proteins relevant for cryptosporidiosis control interventions have been suggested based on their participation in host-parasite interactions. For example, the *Cryptosporidium parvum* gp40/15, a mucin-like glycoprotein formed by the glycoproteins gp40 and gp15 present on the *Cryptosporidium* sporozoite surface as well as GP900 has been proven to participate in host-cell invasion (Cevallos et al., 2000; O'Connor et al., 2007). In addition, a protein-carbohydrate interaction associates GP900 with a *C. parvum* protein of 30 kDa (p30), and this p30 protein, which is localized to the apical region of sporozoites, binds to intestinal epithelial cells and is also involved in infection of epithelial cells by *Cryptosporidium* (Bhat et al., 2007). Many of those proteins involved in parasite infectivity have been identified as *Cryptosporidium* antigenic proteins recognized by hyperimmune colostrum antibody or sera antibody (Jenkins et al., 1993; Riggs et al., 1994).

The identification of surface proteins presumably involved in host immune system recognition and proteins useful for strain or species differentiation has been assessed by many
methodologies. In previous investigations, polypeptides of 16, 25 to 27, 37, 72 to 73, 86, and 240 kDa that may be unique to *C. parvum* were detected on *Cryptosporidium* surface $^{125}$I radiolabeled proteins in one-dimensional (1-DE) analysis. In this study, proteins which were common among *C. parvum*, *C. baileyi*, and *C. muris* were polypeptides of 32, 57, 120, 145-148, and 285-290 kDa (Tilley et al., 1990). In a different study, the identification of surface proteins labeled in intact oocysts with $^{125}$I resulted in bands of 15.5, 32, 42, 47.5, 79, and 96 kDa. In addition, cell wall preparations after excystation were analyzed in 1-DE gels and stained with Coomassie Blue stain, and bands of 47.5, 55, 66, and 130 kDa were observed. However, few of these proteins were recognized by convalescent human sera, in which antigenic proteins were of 23 and 32 kDa (Lumb et al., 1988).

Identification of differential proteins was hampered by the low detection capabilities of early methodologies. For instance, *Cryptosporidium* isolates from 4 geographically unrelated human cases of cryptosporidiosis with clear diarrhea did not show major difference among protein patterns in 1-DE analysis (Lumb et al., 1988).

*Cryptosporidium parvum* Iowa, Alabama, Peru, Louisiana and Mexico isolates have been compared by analyzing their protein profiles by two-dimensional gel electrophoresis (2-DE) coupled with silver staining. Sporozoite proteins (15 µg) from $2 \times 10^9$ oocysts were used in this study. Proteins from the whole oocysts were not evaluated because sonication of oocysts during the protein extraction step caused smearing in the gels and reduced protein spot resolution. From 300 to 400 polypeptides were detected, molecular weights (MWs) and isoelectric points (pIs) ranged from 5 to 150 kDa and from 4 to 8.5, respectively. A 106 kDa protein distinguished the Peru, Louisiana-Alabama and Iowa isolates by the differences in pIs, and a family of 5 proteins of 40 kDa distinguished the Mexico isolate. Protein glycosylation, phosphorylation, acetylation
or amino acid differences were suggested as causes for the 106 kDa pI shifting (Mead et al., 1990). Differences between parasite isolates have also been reported at the genetic and clinical level. For instance, the *C. parvum* Moredum (MD) isolate ID$_{50}$ value was approximately 300 oocysts in a study conducted with 16 seronegative volunteers (Okhuysen et al., 2002) while the ID$_{50}$ for *C. parvum* IO isolate was reported as 132 oocysts in a study conducted with 29 seronegative volunteers (DuPont et al., 1995), and 87 oocysts (confidence interval: CI = 48.67 – 126) in a different study with 29 volunteers (Okhuysen et al., 1999). An earlier median onset of illness (3 days vs. 7 days, $p < 0.05$) and longer duration of diarrhea (169 vs. 93 hours, $p < 0.05$) were observed for *C. parvum* MD isolate compared to the *C. parvum* IO isolate in one comparative study (Okhuysen et al., 2002).

Gene sequences have been compared to elucidate genetic variation among *C. parvum* IO isolates used in experimental settings. *C. parvum* isolates were obtained from experimentally infected calves from Arizona, Idaho, CDC, Maryland and Louisiana, and sections of the genes encoding for the *C. parvum* gp60, a predictive telomeric-zinc protease (ZPT), a putative hydroxyproline-rich glycoprotein (DZ-HRGP), and a hypothetical predictive protein were amplified by PCR and sequenced. The multilocus neighbor-joining tree showed two distinct clades. One was formed by the Arizona isolates from 2004 and 2005, a waterborne isolate from Louisiana, and the sequence of *C. parvum* chromosome 6 reported by Bankier *et al* in 2003 (Bankier et al., 2003). The other clade was formed by Arizona isolates from 1989 to 1996, Idaho, CDC, Maryland, and the sequences in the whole *C. parvum* IO isolate genome published by Abrahamsen *et al* in 2004 (Abrahamsen et al., 2004; Cama et al., 2006). Displacement by exogenous parasites during passages was suggested for those strains which were not closely related to *C. parvum* IO isolate but were identified as such for experimental purposes.
Regarding protein studies, sequencing of the *C. parvum* chromosome 6 was useful to annotate 474 protein-coding genes whereas no function could be assigned for 55% of the predictive proteins. However, several genes which may encode mitochondrial peptides were annotated as well as 45 potentially apicoplast targeting proteins (Bankier et al., 2003).

The complete *C. parvum* IO genome sequence was elucidated by shotgun proteomics and aided in the estimation of 3,807 protein-encoding genes. The authors reported the loss of plastid genome and plastid-associated metabolic pathways including the type II fatty acid synthases and isoprenoid synthetic enzymes, as well as enzymes involved in mitochondrion metabolism, such as Krebs cycle enzymes. However, the finding of plant-like and bacterial-like enzymes, and the lack of de novo biosynthetic capacity for purines, pyrimidines and amino acids were suggested as useful information to evaluate therapeutic molecules (Abrahamsen et al., 2004). In a different genomic study, the *Cryptosporidium* animal-type O-linked glycosylation pathway and the more than 30 predicted surface proteins having mucin-like segments unique to the apicomplexan lineage as well as those proteins with clear phylogenetic affinity to bacterial homologs could be also relevant for therapeutic interventions or diagnosis (Templeton et al., 2004).

More recently, in order to provide in vivo evidence for the expression of protein predicted from genomic databases, the protein expression in non-excysted and excysted *C. parvum* oocysts was determined by LC-MS/MS and N-terminal labeling using iTRAQ™ reagents (Applied Biosystems, Foster City, CA). In this study, insoluble fractions were analyzed by shotgun proteomics. By these methods, 303 *C. parvum* proteins were identified, and 56 were annotated as hypothetical proteins. Of the total, the expression of 26 proteins was significantly increased during excystation, among them were ribosomal proteins and heat shock proteins. Five of those were specific for the genera *Cryptosporidium* and all were identified as sporozoite secreted
proteins. These proteins were: hypothetical protein Cgd3_3370, hypothetical garp protein, putative secreted protein with signal peptide EAK90189, hypothetical protein with signal peptide EAK88888, and hypothetical protein Cgd5_1370 (Snelling et al., 2007). In another study, the *C. parvum* sporozoite protein repertoire was evaluated by MALDI-TOF, 2-DE LC-MS/MS, 1-DE LC-MS/MS, and multi-dimensional protein identification technology (MudPIT) analysis. A total of 1,237 non-redundant proteins were identified. Clusters of proteins with the same identification analysis were observed in 2-DE. These proteins could be post-translational modified proteins or isoenzymes. In 2-DE analysis, the identified proteins corresponded to the expression of 115 individuals *Cryptosporidium* genes. The MudPIT analysis showed the greater number of expressed proteins. A total of 1,154 non-redundant proteins were identified by this methodology. Each method identified unique proteins, 47% unique proteins for MudPIT, 6% for 1-DE LC-MS/MS, and 0.7% for 2-DE analysis, highlighting the relevance of using multiple methods to identify proteins. Up to 40% of proteins were not associated with a specific biological function. This study provided evidence for a functional mitochondrion in *Cryptosporidium* because enzymes involved in metabolism and carrier proteins associated with a mitochondrion were detected. In addition, microneme proteins, such as TRAP-CI, GP900, and Cpa135, as well as surface proteins which may play a role in parasite-host cell interactions were also detected. Only 20% of the proteins detected had corresponding mRNA transcript, and evidence of proteins for half the genes with transcriptional data available could not be detected (Sanderson et al., 2008).

In addition, 1,971 protein families restricted to the *C. parvum* and *C. hominis* were determined, and apicomplexan protein domains were analyzed from proteomics databases to generate information about those domains not previously reported or restricted in the phylum.
Sixteen and forty-four species-specific domain combinations were identified for *C. hominis* and *C. parvum*, respectively. For instance, the C1-like domain, rich in cysteine and histidine residues, determined in proteins associated with chromatin were indicated as being restricted to *C. parvum* (Wasmuth et al., 2009).

Protein profile comparisons between *Cryptosporidium* strains using more methodologies could facilitate information about biological characteristics associated with the parasite. For this reason, this study was designed to evaluate *C. parvum* IO, *C. parvum* AZ and *C. parvum* MD protein profiles by two-dimensional difference gel electrophoresis (2D-DIGE) in order to assess differential protein expression and to identify strain-specific proteins.

**Objective**

To compare the *C. parvum* Iowa, *C. parvum* Arizona and *C. parvum* Moredum protein expression profiles for the identification of polypeptides which may be useful in strain differentiation.

**Material and Methods**

Oocyst protein repertoires of *C. parvum* Iowa (IO), *C. parvum* Arizona (AZ) and *C. parvum* Moredum (MD) isolates were compared by two-dimensional difference gel electrophoresis (2D-DIGE). Subsequently, protein abundance was calculated from image analysis and statistically analyzed between strains.
Strains

Six *C. parvum* isolates were used in this study, three *C. parvum* IO, two *C. parvum* AZ, and one *C. parvum* MD isolates. The oocysts of *C. parvum* AZ isolates were shed from calves experimentally infected in 2005. The oocysts of two *C. parvum* IO isolates were obtained from passages in calves in 2005 and 2009. All of these isolates were obtained from The Sterling Parasitology Laboratory, Department of Veterinary Science and Microbiology in Tucson, Arizona. Oocysts were provided in antibiotic solution containing 0.01% Tween 20, 100 U of penicillin, and 100 μg of gentamicin per ml, and were kept at 4°C until analyzed.

One *C. parvum* IO and the *C. parvum* MD ATCC isolates were purchased from the Biodefense and Emerging Infections Research Resources Repository (Manassas, VA) in 2008. Oocysts from these two isolates were provided in 2.5% potassium dichromate, and were kept at 4°C until analyzed.

Reagents

Thiourea, urea and 87% glycerol were acquired from GE Healthcare Biosciences (Pittsburgh, PA); CHAPS, ASB-14, DTT, and DL-Lysine were purchased from Sigma Aldrich (St. Louis, MO); tris base was obtained from Fisher Scientific (Pittsburgh, PA); iodoacetamide, SDS, and Bio-lyte® 3-10 ampholytes were purchased from Bio-Rad (Hercules, CA).

Protein extraction

*Cryptosporidium parvum* oocysts were enumerated with a Neubauer hemocytometer (VWR Scientific, Suwanee, GA), and a fraction of 10^6 oocysts were used for protein extraction. Oocysts were washed five times in 1 ml distilled sterile water by suspension and centrifugation
at 6,000 ×g for 3 min each time. Oocysts were sonicated twenty times in 300 μl of sterile water for 3 ± 0.5 seconds at level 5 using a Sonic Dismembrator Model 100 (Fisher Scientific, Pittsburgh, PA). The sample volume was reduced to 20 μl in a Speed-Vac (Savant Instruments, Holbrook, NY) at 30°C, and the protein concentration was measured by the Bradford method (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Extracted proteins were diluted out in 100 μl of protein solubilization buffer (2M thiourea, 7M urea, 2% w/v CHAPS, 2% w/v ASB14, 15 mM tris) and were kept in this buffer at room temperature overnight.

Proteins were purified from salts, lipids, phenolics, nucleic acids, and charged detergents by using the 2D clean-up kit (Amersham Biosciences, Pittsburgh, PA) and resuspended in 31.25 μl of solubilization buffer. Samples were centrifuged at 12,000 ×g for 5 min and the supernatant was subsequently used in the following steps.

Soluble protein fractions were labeled with cyanine fluorescent dyes CyDye DIGE Fluor Cy5 and Cy3 (Amersham Biosciences, Pittsburgh, PA). Briefly, samples in solubilization buffer were labeled with 200 pmol of CyDye in the dark on ice and for 30 min, and the reaction was stopped by adding 10 mM Lysine for a 10 min incubation time in the dark. Cy5 labeled samples were combined with Cy3 labeled samples, and 62.5 μl of IPG buffer (2M thiourea, 7M urea, 2% w/v CHAPS, 2% w/v ASB14, 0.018M DTT, and 0.2% v/v Bio-lyte® 3-10 ampholytes) were added to each combination before isoelectric focusing (IEF).

**Internal standard**

An internal standard, used to facilitate comparative analysis of protein spots resolved in DIGE analyses, was prepared by combining equal amounts of each Cy3-labeled *C. parvum* strain
protein sample. Each Cy5-labeled sample was combined with 32.5 μl of the internal standard before IEF.

**Isoelectric focusing (IEF) and second dimension SDS-PAGE analysis**

Seven cm long strips of 3-10 immobilized pH gradient (IPG) (Amersham Biosciences, Pittsburgh, PA) were loaded with 125 μl of sample and actively rehydrated at 50 V for 12 h, followed by isoelectric focusing using a Protean IEF Cell (Bio-Rad, Hercules, CA) for a total of 16 kVh (ramped to 500 V for 5 min, held at 500 V for 55 min, ramped to 1000 V for 5 min, held at 1000 V for 55 min, ramped to 2000 V for 5 min, held to 2000 V for 55 min, ramped to 4000 V for 1 h, held at 4000 V for 2 h 30 min).

The cysteine sulfhydryls were reduced and carbamidomethylated while the proteins were equilibrated by 10 min incubation of the focused strips in equilibration buffer (1.5M tris, 6M urea, 87% v/v glycerol, 10% w/v SDS) supplemented with 1% w/v DTT followed by incubation in equilibration buffer supplemented with 2.5% w/v iodoacetamide for 10 min. Second dimension SDS-PAGE was run on 12% polyacrylamide gels at 200 V for 40 min by using a Mini-Protean 3 cell electrophoresis unit (Bio-Rad, Hercules, CA).

**Experimental design and data analysis**

Two sets of images were analyzed all together in a single DeCyder project. CyDye labeled proteins from the *C. parvum* isolates were combined to obtain images of two protein profiles in one single polyacrylamide gel. For the first set of images, three replicates of the combination of *C. parvum* IO and *C. parvum* AZ differentially-labeled proteins were processed. The isolates from 2005 were used in these combinations. In two replicates, *C. parvum* IO
proteins were labeled with Cy3 while *C. parvum* AZ proteins were labeled with Cy5. In the third replicate, *C. parvum* IO proteins were labeled with Cy5 while *C. parvum* AZ proteins were labeled with Cy3.

For the second set of images, two replicates of protein samples from the three *C. parvum* isolates: IO, AZ, and the MD labeled with Cy5 were combined with the internal standard before IEF. *C. parvum* IO isolates from 2008 and 2009, *C. parvum* AZ isolate from 2005 and *C. parvum* MD isolate form 2008 were used to make these combinations.

The labeled components of each gel were individually imaged using mutually exclusive excitation/emission wavelengths in a Typhoon 9410 scanner and the Image Quant LT ® software (Amersham Biosciences, Pittsburg, PA). The quantification of protein spots, estimation of molecular weights (MWs) and isoelectric points (pIs) for the protein of interest, and statistical analysis were performed by using the DeCyder-DIA Differential in Gel Analysis and the DeCyder-BVA Biological Variation Analysis softwares (Amersham Biosciences, Pittsburgh, PA) as follows: Gel images were loaded in to the DeCyder-DIA Differential in Gel Analysis software. The program was run to identify up to 10,000 protein spots at a threshold of 2-model standard deviation (SD). Real protein spots were differentiated from dust particles visually by the inspection of the maximum volumes, maximum pick heights, areas, and the maximum slope values for the spots. Images were exported to the DeCyder-BVA Biological Variation Analysis software where protein spots were matched among gels using the standard images and distinctive protein spots observed in common among gel images.

MWs and pIs for nine proteins were estimated by comparison with a low molecular weight protein standard (Bio-Rad, Hercules, CA) and by using the Immobiline DryStrip pH 3-10
gradient graph (Amersham Biosciences, Pittsburgh, PA). The software automatically calculated MWs and pIs for all the protein spots detected.

Proteins of interest were selected from the statistical comparison between experimental groups. For the analysis, *C. parvum* AZ images were assigned to Group 1, *C. parvum* IO images were assigned to Group 2, and *C. parvum* MD images were assigned to Group 3. The three groups were compared by the Student’s T-test and significant differences in protein spot abundance between groups were indicated for *p*-values lower than 0.10.

**Results**

**Protein extraction**

The $10^6$ *C. parvum* oocysts used for the two-dimensional difference (2D-DIGE) analysis corresponded to 1.4 µg of protein. Only the soluble part was labeled and processed by IEF and SDS-PAGE. Pellets were not analyzed due to the difficulty encountered at running the first dimension separation which caused bad gel resolution in the second dimension (data not shown).

**Protein detection**

The DIGE gels with differentially labeled proteins are shown in Figure 2.1. The proteins of interest are depicted in Figure 2.2 and Tables 2.1, 2.2, 2.3, 2.4, 2.5, and 2.6. The average number of *C. parvum* IO and AZ protein spots was 250 while only 70 protein spots were observed for *C. parvum* MD strain. Molecular weights (MWs) and isoelectric points (pIs) ranged from 19 to 150 kDa and 4.0 to 8.0, respectively.
Between *C. parvum* AZ and *C. parvum* IO, 51 protein spots were found significantly different while 34 of them were increased for *C. parvum* AZ. Among these proteins, a 36 kDa protein with pI of 6.6 (Spot Nº 159) and a 43 kDa protein with pI of 6.36 (Spot Nº 161) were the most abundant for *C. parvum* AZ compared to the other two strains. In addition, the gel images of *C. parvum* AZ isolate demonstrated an increment in the fluorescent labeling for three protein spots. Two of these proteins had MWs of 52 kDa and pIs of 5.72 and 6.0 (Spots Nº 122 and 119, respectively), and one protein showed a MW of 59 kDa with a pI of 5.7 (Spot Nº 93). Also observed was a significant difference in abundance for these polypeptides between *C. parvum* AZ and *C. parvum* IO or *C. parvum* MD (*p* < 0.05). Regarding the *C. parvum* IO protein profile compared to the protein profiles of the AZ and MD isolates, the major protein expressed by this isolate was a protein of 46 kDa with a pI of 6.91 (Spot Nº 151).

*C. parvum* AZ and *C. parvum* MD had 30 different protein spots whereas 21 differences were detected between *C. parvum* IO and *C. parvum* MD. Of them, proteins of 78 and a 72 kDa with pIs of 5.49 and 5.13 (Spots Nº 29 and 55, respectively) were found to be more expressed (*p* < 0.1) in the *C. parvum* MD protein profile compared to the profiles of the other two isolates.

**Discussion**

Protein profiles of *Cryptosporidium parvum* Iowa (IO), *C. parvum* Arizona (AZ) and *C. parvum* Moredum (MD) were evaluated by two-dimensional difference gel electrophoresis (2D-DIGE) in order to determine differences in protein expression which might explain variations in genomic or clinical characteristics previously reported for these isolates (Cama et al., 2006; DuPont et al., 1995; Okhuysen et al., 1999; Okhuysen et al., 2002).
Proteins extracted from *C. parvum* oocysts were chosen for evaluation even though more research interest has been previously focused on the study of proteins extracted from the invasive stage of the parasite: the sporozoite (Lumb et al., 1988; Mead et al., 1990; Sanderson et al., 2008; Snelling et al., 2007). Investigators have used a large number of oocysts (From $10^8$ to $10^9$ *C. parvum* oocysts) for protein extraction to improve their test detection levels. However, *C. parvum* specimens are difficult to procure, especially when some isolates are not normally propagated for experimental use, such as the case of the *C. parvum* AZ isolate.

One relevant reason for using sporozoite proteins was the fact that some protein antigens were detected in sporozoites when they were excysted from the oocysts and not from sonicated oocysts as demonstrated in an early study conducted in 1994 (Riggs et al., 1994). However, using very high-throughput assays, such as liquid chromatography coupled with mass spectrometry, differences in protein expression between non-excysted and excysted *C. parvum* oocysts were observed for not more than 30 proteins. Presence or absence of these proteins in the aforementioned parasite stages was not evaluated (Snelling et al., 2007). In addition, oocyst excystation is conducted under laboratory conditions and not necessarily represents what occurs *in vivo*. To facilitate the use of those proteins as markers for parasite differentiation in environmental or food samples, they must be present in the stage normally occurring in these samples: the infective stage “the oocyst”.

During the protein extraction step, the inclusion of the insoluble fraction in the IEF invokes smearing in the second dimension. Therefore, only the soluble fraction was analyzed. The identification of differentially expressed proteins by 2D-DIGE was possible with as low as 1.4 µg of *C. parvum* extracted proteins. At this concentration, lower numbers of spots in *C. parvum* MD isolate were identified. However, it was possible to observe some differences in
protein expression. The limited number of *C. parvum* MD samples analyzed could influence the greater *p*-values for the proteins of 78 and 72 kDa with pIs of 5.49 and 5.13 which seem to characterize the strain.

For the *C. parvum* IO and AZ isolates, 3 proteins differentially expressed (36 and 43 kDa with pIs of 6.6 and 6.36, respectively, for *C. parvum* AZ; and a 46 kDa with a pI of 6.91 for *C. parvum* IO) were useful in the identification of these isolates, and a group of proteins between MWs of 50 and 60 kDa (MWs of 52, 52, and 59 kDa with pIs of 5.72, 6.0, and 5.7) seem to characterize the *C. parvum* AZ as well, as it was observed in the DIGE images. A previous report mentioned that a 106 kDa protein and a family of 40 kDa protein were suitable for the differentiation between *C. parvum* isolates from Peru, Louisiana-Alabama, IOWA and Mexico (Mead et al., 1990). The pI value for the group of 40 kDa proteins was not reported. No proteins of 106 kDa and pIs between 5.5 and 6.2 were found significantly expressed for the isolates evaluated during this investigation. One protein of 105 kDa and pI of 6.83 which was different between *C. parvum* IO and AZ (*p* = 0.044) was the closest numerical match. However, the examination of the spots in the gels suggested they were probably not the same protein. Experimental conditions in this early work were definitively different, and therefore major comparisons can not be made.

Genetic differences were reported for *C. parvum* IO isolates propagated in calves and used in research experiments between 1989 and 2005 (Cama et al., 2006). The Arizona isolates obtained between 2004 and 2005 and between 1989 and 1996 showed different nucleotide substitutions in the gp60, a predictive telomeric-zinc protease (ZPT), a putative hydroxyproline-rich glycoprotein (DZ-HRGP), and a hypothetical predictive protein gene. The apparently original *C. parvum* IO isolate was localized in a clade where the 2004 and 2005 isolates were not
included. The *C. parvum* AZ isolates used in these experiments corresponded to those 2005 isolates. The differential protein expression suggested here for the *C. parvum* IO and *C. parvum* AZ isolates add additional evidence for the molecular differences between these two isolates. Further research may therefore be required to demonstrate biological implications of these findings. Future studies might also involve the identification of the corresponding genes for the proteins differentially-expressed reported in this work. Identification of target proteins in the parasite and the effect of their expression on isolate differentiation, especially with regards to parasite infectivity, must be elucidated to support the results of this research.
References


Table 2.1: Differentially expressed *C. parvum* Iowa (IO) vs. *C. parvum* Arizona (AZ) proteins detected by Two-Dimensional Difference Gel Electrophoresis (2D-DIGE)

<table>
<thead>
<tr>
<th>Spot N°</th>
<th>Student’s T-test p-value</th>
<th>Average Ratio*</th>
<th>Isoelectric Point</th>
<th>Molecular Weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>159</td>
<td>2.90E-05</td>
<td>-2.22</td>
<td>6.60</td>
<td>36000</td>
</tr>
<tr>
<td>161</td>
<td>3.20E-05</td>
<td>-1.73</td>
<td>6.36</td>
<td>43158</td>
</tr>
<tr>
<td>165</td>
<td>0.0003</td>
<td>-2.47</td>
<td>7.30</td>
<td>42182</td>
</tr>
<tr>
<td>93</td>
<td>0.0004</td>
<td>-6.34</td>
<td>5.70</td>
<td>59148</td>
</tr>
<tr>
<td>134</td>
<td>0.0011</td>
<td>-4.12</td>
<td>6.36</td>
<td>50396</td>
</tr>
<tr>
<td>79</td>
<td>0.0016</td>
<td>-2.32</td>
<td>6.15</td>
<td>65979</td>
</tr>
<tr>
<td>132</td>
<td>0.0018</td>
<td>-1.95</td>
<td>6.56</td>
<td>51301</td>
</tr>
<tr>
<td>138</td>
<td>0.0051</td>
<td>-2.48</td>
<td>5.10</td>
<td>49382</td>
</tr>
<tr>
<td>160</td>
<td>0.0085</td>
<td>-1.75</td>
<td>6.23</td>
<td>43158</td>
</tr>
<tr>
<td>170</td>
<td>0.0110</td>
<td>-1.59</td>
<td>6.27</td>
<td>40091</td>
</tr>
<tr>
<td>122</td>
<td>0.0120</td>
<td>-4.72</td>
<td>5.72</td>
<td>52355</td>
</tr>
<tr>
<td>40</td>
<td>0.0160</td>
<td>-4.34</td>
<td>5.73</td>
<td>76653</td>
</tr>
<tr>
<td>128</td>
<td>0.0170</td>
<td>-5.80</td>
<td>5.57</td>
<td>52222</td>
</tr>
<tr>
<td>124</td>
<td>0.0170</td>
<td>-1.69</td>
<td>6.76</td>
<td>52621</td>
</tr>
<tr>
<td>181</td>
<td>0.0190</td>
<td>-1.57</td>
<td>5.62</td>
<td>34773</td>
</tr>
<tr>
<td>155</td>
<td>0.0210</td>
<td>-1.83</td>
<td>4.76</td>
<td>44722</td>
</tr>
<tr>
<td>3</td>
<td>0.0210</td>
<td>-1.60</td>
<td>5.99</td>
<td>124555</td>
</tr>
<tr>
<td>50</td>
<td>0.0250</td>
<td>-3.84</td>
<td>5.73</td>
<td>73039</td>
</tr>
<tr>
<td>95</td>
<td>0.0250</td>
<td>-1.97</td>
<td>5.09</td>
<td>58699</td>
</tr>
<tr>
<td>129</td>
<td>0.0270</td>
<td>-12.05</td>
<td>6.66</td>
<td>52488</td>
</tr>
<tr>
<td>119</td>
<td>0.0290</td>
<td>-3.13</td>
<td>6.00</td>
<td>52355</td>
</tr>
<tr>
<td>149</td>
<td>0.0320</td>
<td>-1.55</td>
<td>7.84</td>
<td>47414</td>
</tr>
<tr>
<td>87</td>
<td>0.0350</td>
<td>-8.58</td>
<td>7.81</td>
<td>62549</td>
</tr>
<tr>
<td>18</td>
<td>0.0460</td>
<td>-2.76</td>
<td>7.01</td>
<td>93461</td>
</tr>
<tr>
<td>175</td>
<td>0.0460</td>
<td>-1.65</td>
<td>6.89</td>
<td>37911</td>
</tr>
<tr>
<td>56</td>
<td>0.0530</td>
<td>-1.61</td>
<td>6.91</td>
<td>71751</td>
</tr>
<tr>
<td>58</td>
<td>0.0530</td>
<td>-1.47</td>
<td>7.14</td>
<td>71934</td>
</tr>
<tr>
<td>154</td>
<td>0.0570</td>
<td>-3.20</td>
<td>6.77</td>
<td>44722</td>
</tr>
<tr>
<td>176</td>
<td>0.0590</td>
<td>-1.66</td>
<td>6.30</td>
<td>37432</td>
</tr>
<tr>
<td>192</td>
<td>0.0600</td>
<td>-3.54</td>
<td>7.83</td>
<td>34073</td>
</tr>
<tr>
<td>102</td>
<td>0.0740</td>
<td>-1.85</td>
<td>6.01</td>
<td>57225</td>
</tr>
<tr>
<td>127</td>
<td>0.0830</td>
<td>-2.61</td>
<td>5.47</td>
<td>51957</td>
</tr>
<tr>
<td>166</td>
<td>0.0880</td>
<td>-1.74</td>
<td>7.73</td>
<td>41438</td>
</tr>
<tr>
<td>146</td>
<td>0.0940</td>
<td>-1.61</td>
<td>7.68</td>
<td>47414</td>
</tr>
</tbody>
</table>

* Negative ratios indicate greater abundance of *C. parvum* Arizona proteins.
Table 2.2: Differentially expressed *C. parvum* Iowa (IO) vs. *C. parvum* Arizona (AZ) proteins detected by Two-Dimensional Difference Gel Electrophoresis (2D-DIGE)

<table>
<thead>
<tr>
<th>Spot Nº</th>
<th>Student’s T-test p-value</th>
<th>Average Ratio*</th>
<th>Isoelectric Point</th>
<th>Molecular Weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>0.002</td>
<td>2.65</td>
<td>5.44</td>
<td>78827</td>
</tr>
<tr>
<td>151</td>
<td>0.012</td>
<td>1.80</td>
<td>6.91</td>
<td>46107</td>
</tr>
<tr>
<td>84</td>
<td>0.016</td>
<td>1.96</td>
<td>6.69</td>
<td>62232</td>
</tr>
<tr>
<td>32</td>
<td>0.020</td>
<td>1.72</td>
<td>7.77</td>
<td>78228</td>
</tr>
<tr>
<td>142</td>
<td>0.025</td>
<td>1.41</td>
<td>5.39</td>
<td>48882</td>
</tr>
<tr>
<td>107</td>
<td>0.027</td>
<td>1.38</td>
<td>6.25</td>
<td>56359</td>
</tr>
<tr>
<td>61</td>
<td>0.028</td>
<td>1.63</td>
<td>6.54</td>
<td>71569</td>
</tr>
<tr>
<td>120</td>
<td>0.032</td>
<td>1.42</td>
<td>7.53</td>
<td>53159</td>
</tr>
<tr>
<td>108</td>
<td>0.039</td>
<td>1.46</td>
<td>6.34</td>
<td>56359</td>
</tr>
<tr>
<td>36</td>
<td>0.041</td>
<td>1.92</td>
<td>6.60</td>
<td>77634</td>
</tr>
<tr>
<td>9</td>
<td>0.044</td>
<td>1.44</td>
<td>6.83</td>
<td>104786</td>
</tr>
<tr>
<td>20</td>
<td>0.056</td>
<td>2.57</td>
<td>5.56</td>
<td>90423</td>
</tr>
<tr>
<td>21</td>
<td>0.068</td>
<td>2.28</td>
<td>5.37</td>
<td>87484</td>
</tr>
<tr>
<td>19</td>
<td>0.070</td>
<td>1.84</td>
<td>5.48</td>
<td>91813</td>
</tr>
<tr>
<td>30</td>
<td>0.073</td>
<td>2.84</td>
<td>5.56</td>
<td>78626</td>
</tr>
<tr>
<td>68</td>
<td>0.084</td>
<td>1.67</td>
<td>6.18</td>
<td>69773</td>
</tr>
<tr>
<td>49</td>
<td>0.098</td>
<td>2.63</td>
<td>5.39</td>
<td>73225</td>
</tr>
</tbody>
</table>

* Positive ratios indicate greater abundance of *C. parvum* Iowa proteins.
### Table 2.3: Differentially expressed *C. parvum* Moredum (MD) vs *C. parvum* Arizona (AZ) proteins detected by Two-Dimensional Difference Gel Electrophoresis (2D-DIGE)

<table>
<thead>
<tr>
<th>Spot No</th>
<th>Student’s T-test p-value</th>
<th>Average Ratio*</th>
<th>Isoelectric Point</th>
<th>Molecular Weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>159</td>
<td>0.0003</td>
<td>-21.29</td>
<td>6.60</td>
<td>36000</td>
</tr>
<tr>
<td>109</td>
<td>0.0018</td>
<td>-12.06</td>
<td>6.50</td>
<td>57341</td>
</tr>
<tr>
<td>151</td>
<td>0.0026</td>
<td>-11.88</td>
<td>6.91</td>
<td>46107</td>
</tr>
<tr>
<td>161</td>
<td>0.0028</td>
<td>-17.79</td>
<td>6.36</td>
<td>43158</td>
</tr>
<tr>
<td>107</td>
<td>0.0042</td>
<td>-7.89</td>
<td>6.25</td>
<td>56359</td>
</tr>
<tr>
<td>93</td>
<td>0.0061</td>
<td>-10.47</td>
<td>5.70</td>
<td>59148</td>
</tr>
<tr>
<td>165</td>
<td>0.0073</td>
<td>-4.35</td>
<td>7.30</td>
<td>42182</td>
</tr>
<tr>
<td>143</td>
<td>0.0078</td>
<td>-11.01</td>
<td>7.40</td>
<td>44168</td>
</tr>
<tr>
<td>91</td>
<td>0.0081</td>
<td>-8.23</td>
<td>5.53</td>
<td>59752</td>
</tr>
<tr>
<td>124</td>
<td>0.0092</td>
<td>-3.81</td>
<td>6.76</td>
<td>52621</td>
</tr>
<tr>
<td>145</td>
<td>0.0110</td>
<td>-4.76</td>
<td>7.05</td>
<td>46934</td>
</tr>
<tr>
<td>125</td>
<td>0.0130</td>
<td>-1.94</td>
<td>7.00</td>
<td>52488</td>
</tr>
<tr>
<td>17</td>
<td>0.0150</td>
<td>-6.70</td>
<td>6.85</td>
<td>99427</td>
</tr>
<tr>
<td>77</td>
<td>0.0170</td>
<td>-4.46</td>
<td>5.52</td>
<td>66653</td>
</tr>
<tr>
<td>12</td>
<td>0.0190</td>
<td>-6.06</td>
<td>7.04</td>
<td>98836</td>
</tr>
<tr>
<td>119</td>
<td>0.0280</td>
<td>-5.22</td>
<td>6.00</td>
<td>52355</td>
</tr>
<tr>
<td>102</td>
<td>0.0420</td>
<td>-3.81</td>
<td>6.01</td>
<td>57225</td>
</tr>
<tr>
<td>16</td>
<td>0.0430</td>
<td>-3.21</td>
<td>6.66</td>
<td>99592</td>
</tr>
<tr>
<td>90</td>
<td>0.0490</td>
<td>-4.41</td>
<td>5.46</td>
<td>59752</td>
</tr>
<tr>
<td>176</td>
<td>0.0510</td>
<td>-2.83</td>
<td>6.30</td>
<td>37432</td>
</tr>
<tr>
<td>134</td>
<td>0.0590</td>
<td>-4.73</td>
<td>6.36</td>
<td>50396</td>
</tr>
<tr>
<td>128</td>
<td>0.0610</td>
<td>-5.93</td>
<td>5.57</td>
<td>52222</td>
</tr>
<tr>
<td>186</td>
<td>0.0720</td>
<td>-2.40</td>
<td>7.15</td>
<td>32966</td>
</tr>
<tr>
<td>122</td>
<td>0.0810</td>
<td>-3.78</td>
<td>5.72</td>
<td>52355</td>
</tr>
<tr>
<td>138</td>
<td>0.0840</td>
<td>-3.59</td>
<td>5.10</td>
<td>49382</td>
</tr>
<tr>
<td>19</td>
<td>0.0930</td>
<td>-3.25</td>
<td>5.48</td>
<td>91813</td>
</tr>
<tr>
<td>78</td>
<td>0.0990</td>
<td>-2.41</td>
<td>5.61</td>
<td>66315</td>
</tr>
</tbody>
</table>

* Negative ratios indicate greater abundance of *C. parvum* Arizona proteins.
Table 2.4: Differentially expressed *C. parvum* Moredum (MD) vs *C. parvum* Arizona (AZ) proteins detected by Two-Dimensional Difference Gel Electrophoresis (2D-DIGE).

<table>
<thead>
<tr>
<th>Spot Nº</th>
<th>Student’s T-test p-value</th>
<th>Average Ratio*</th>
<th>Isoelectric Point</th>
<th>Molecular Weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>0.012</td>
<td>10.94</td>
<td>5.49</td>
<td>78427</td>
</tr>
<tr>
<td>55</td>
<td>0.089</td>
<td>25.62</td>
<td>5.13</td>
<td>71934</td>
</tr>
<tr>
<td>30</td>
<td>0.096</td>
<td>9.98</td>
<td>5.56</td>
<td>78626</td>
</tr>
</tbody>
</table>

* Positive ratios indicate greater abundance of *C. parvum* Moredum proteins.
Table 2.5: Differentially expressed *C. parvum* Moredum (MD) vs *C. parvum* Iowa (IO) proteins detected by Two-Dimensional Difference Gel Electrophoresis (2D-DIGE).

<table>
<thead>
<tr>
<th>Spot Nº</th>
<th>Student’s T-test p-value</th>
<th>Average Ratio*</th>
<th>Isoelectric Point</th>
<th>Molecular Weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>159</td>
<td>0.0013</td>
<td>-9.58</td>
<td>6.60</td>
<td>36000</td>
</tr>
<tr>
<td>151</td>
<td>0.0014</td>
<td>-21.34</td>
<td>6.91</td>
<td>46107</td>
</tr>
<tr>
<td>107</td>
<td>0.0021</td>
<td>-10.89</td>
<td>6.25</td>
<td>56359</td>
</tr>
<tr>
<td>109</td>
<td>0.0029</td>
<td>-9.13</td>
<td>6.50</td>
<td>57341</td>
</tr>
<tr>
<td>161</td>
<td>0.0058</td>
<td>-10.31</td>
<td>6.36</td>
<td>43158</td>
</tr>
<tr>
<td>143</td>
<td>0.0062</td>
<td>-12.68</td>
<td>7.40</td>
<td>44168</td>
</tr>
<tr>
<td>124</td>
<td>0.0120</td>
<td>-2.25</td>
<td>6.76</td>
<td>52621</td>
</tr>
<tr>
<td>125</td>
<td>0.0130</td>
<td>-1.75</td>
<td>7.00</td>
<td>52488</td>
</tr>
<tr>
<td>145</td>
<td>0.0150</td>
<td>-4.14</td>
<td>7.05</td>
<td>46934</td>
</tr>
<tr>
<td>17</td>
<td>0.0180</td>
<td>-6.25</td>
<td>6.85</td>
<td>99427</td>
</tr>
<tr>
<td>77</td>
<td>0.0190</td>
<td>-4.33</td>
<td>5.52</td>
<td>66653</td>
</tr>
<tr>
<td>12</td>
<td>0.0200</td>
<td>-5.66</td>
<td>7.04</td>
<td>98836</td>
</tr>
<tr>
<td>16</td>
<td>0.0440</td>
<td>-3.25</td>
<td>6.66</td>
<td>99592</td>
</tr>
<tr>
<td>19</td>
<td>0.0500</td>
<td>-5.97</td>
<td>5.48</td>
<td>91813</td>
</tr>
<tr>
<td>202</td>
<td>0.0510</td>
<td>-4.21</td>
<td>7.01</td>
<td>30006</td>
</tr>
<tr>
<td>91</td>
<td>0.0550</td>
<td>-5.57</td>
<td>5.53</td>
<td>59752</td>
</tr>
<tr>
<td>20</td>
<td>0.0590</td>
<td>-7.33</td>
<td>5.56</td>
<td>90423</td>
</tr>
<tr>
<td>186</td>
<td>0.0640</td>
<td>-2.74</td>
<td>7.15</td>
<td>32966</td>
</tr>
<tr>
<td>102</td>
<td>0.1000</td>
<td>-2.06</td>
<td>6.01</td>
<td>57225</td>
</tr>
</tbody>
</table>

* Negative ratios indicate greater abundance of *C. parvum* Iowa proteins.
Table 2.6: Differentially expressed *C. parvum* Moredum (MD) vs. *C. parvum* Iowa (IO) proteins detected by Two-Dimensional Difference Gel Electrophoresis (2D-DIGE)

<table>
<thead>
<tr>
<th>Spot Nº</th>
<th>Student’s T-test $p$-value</th>
<th>Average Ratio*</th>
<th>Isoelectric Point</th>
<th>Molecular Weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>0.093</td>
<td>15.43</td>
<td>5.13</td>
<td>71934</td>
</tr>
<tr>
<td>29</td>
<td>0.100</td>
<td>4.39</td>
<td>5.49</td>
<td>78427</td>
</tr>
</tbody>
</table>

* Positive ratios indicate greater abundance of *C. parvum* Moredum proteins.
Figure 2.1: Proteins of *Cryptosporidium parvum* Iowa (IO), Arizona (AZ), Moredum (MD) detected by Two-dimensional Difference Gel Electrophoresis (DIGE). *C. parvum* IO (A), AZ (B), and MD (C) proteins are Cy5 labeled (Red spots). Internal standard proteins are Cy3 labeled (Green spots).
Figure 2.2: Image of *Cryptosporidium parvum* internal standard in two-dimensional difference gel electrophoresis showing proteins differentially expressed between *C. parvum* isolates. *C. parvum* Arizona: Protein No. 159: Molecular weight (MW) = 36 kDa, Isoelectric point (pI) = 6.6, protein No. 161: MW = 43 kDa, pI = 6.36, protein No. 119: MW = 52 kDa, pI = 6.0, protein No. 122: MW = 52 kDa, pI = 5.7, protein No. 93: MW = 59 kDa, pI = 5.7. *C. parvum* Iowa: Protein No. 151: MW = 46 kDa, pI = 6.91. *C. parvum* Morehead: Protein No. 29: MW = 78 kDa, pI = 5.49, protein No. 55: MW = 72 kDa, pI = 5.49.
CHAPTER 3

CRYPTOSPORIDIUM PARVUM ANTIGENIC PROTEINS RECOGNIZED BY HUMAN SERA UNDER ACTIVE CRYPTOSPORIDIOSIS INFECTION

1Sanchez-Ingunza, Roxana and Ynés R. Ortega. To be submitted to Infection and Immunity.
Abstract

Cryptosporidium protein antigens useful for the identification of recent infections or acute cases of cryptosporidiosis were determined by one-dimensional and two-dimensional western blot analyses using human sera from Cryptosporidium-infected individuals. In one-dimensional analysis a C. parvum protein of 57 kDa reacted more strongly with acute human sera (p < 0.05) whereas the results of two-dimensional analysis suggest that C. parvum antigens of 43.4, 50.3, 50.3, 47.6, 64.7, and 50.3 kDa, with pIs of 5.4, 7.0, 7.2, 5.3, 6.6, and 6.7, respectively, may be used as markers of early Cryptosporidium infection. C. parvum antigens of 14, 16, 21 or 26 kDa detected in one-dimensional analysis, or 17.5 detected in two-dimensional analysis, reported previously in the literature, did not distinguish recent infection (p < 0.05). The role during Cryptosporidium infection of the antigenic proteins evaluated in this study remains to be elucidated. However, the results support the conclusion that they might be useful for diagnosis or for epidemiological studies in cryptosporidiosis.
Introduction

Components of cellular and humoral immune response have been identified in cryptosporidiosis. Epithelial cells are the only cells affected by the parasite *in vivo*, and they are stimulated to produce inflammatory molecules such as chemokines during *Cryptosporidium* infections. Neutrophils were observed in the infection area, the up-regulation of C-X-C chemokines, IL-8 and Groα, by *Cryptosporidium parvum* and the expression of pro-inflammatory molecules, such as the mucosal superoxide, are probably associated with their recruitment to the affected tissue (Laurent et al., 1997; Zadrozny et al., 2006). The role of neutrophils during infection is not clear; however, the presence of these cells in the epithelium was not associated with severity of *Cryptosporidium* infection, villus atrophy, or diarrhea in a neonatal piglet model (Zadrozny et al., 2006).

During *Cryptosporidium* infection, accumulation of toll-like receptors, TLR 2 and TLR 4 is observed in human cholangiocytes. However, *Cryptosporidium* attachment rate did not change when the expression of these receptors was blocked in the cells. An increase in the expression of IL-18 mRNA and HBD 2 antimicrobial peptide mediated by the activation of NF-κB pathway was also detected (Chen et al., 2005). IL-18 participated in the regulation of IL-4 and IL-13 (Th2 cytokines), and its role was evident when IL-18 was blocked by neutralizing antibodies causing the up-regulation of IL-4 and IL-3. Shifting to up-regulation of Th2 cytokines was also determined in the absence of IL-12 and IFN-γ with IL-18 (Tessema et al., 2009). The participation of IL-15 during infection was also evaluated. IL-15 caused cell lysis by the increase of the cytotoxic activity of NK cells (Dann et al., 2005). The expression of IFN-γ by IL-15 stimulated NK cells may be responsible for this effect (Hayward et al., 2000).
IFN-γ and TNFα mRNA were up-regulated after *C. parvum* infection in mice, and the expression of IFN-γ in re-challenging was significant (Tessema et al., 2009). In an experimental *Cryptosporidium* infection of sero-negative volunteers, IFN-γ mRNA was clearly detected in intestinal tissues when oocysts were not observed in the stools; however, AIDS patients with chronic cryptosporidiosis showed non-detectable IFN-γ. Therefore, decreased susceptibility in previous *Cryptosporidium* exposed individuals may be associated with the expression of IFN-γ in the absence of anti-*Cryptosporidium* antibodies (White et al., 2000). In a different study, the Th1 (IFN-γ, TNFα, IL2) or Th2 (IL4, IL10) cytokine response as well as the lymphoproliferative response seemed to be similar during cryptosporidiosis in HIV-positive patients and immunocompetent individuals (*p* > 0.05) (Kaushik et al., 2009).

T-cell lymphocytes are also involved in the immune response against *Cryptosporidium* infection (Hunter and Nichols, 2002). T-cells are essential for elimination of the parasite in mice (Heine et al., 1984) as well as in calves (Abrahamsen et al., 1997). The number of αβ⁺CD4⁺ and CD8⁺ T-cells, and γδ⁺ T-cells increased in the lamina propria and epithelium after *Cryptosporidium* infection in calves (Abrahamsen et al., 1997). In humans, the level of αβ⁻CD4⁺ T-cells increased in the intestinal mucosa in HIV-positive individuals after initiation of antiretroviral treatment, which correlated with the recovery from *Cryptosporidium* infection (Schmidt et al., 2001).

**Humoral immune response**

Many *Cryptosporidium parvum* antigens relevant to humoral immune response have been identified. The localization of *Cryptosporidium* antigens ranging from 40 to 210 kDa were localized in the micronemes of *C. parvum* sporozoites and merozoites, cytoplasm of trophozoites
and macrogametes, parasitophorous vacuoles of trophozoites, macrogametes and sporulated oocysts (Bonnin et al., 1991). The localization of *C. parvum* antigens in different parasite stages was also assessed by immunogold labeling of intestinal tissue from *C. parvum* infected mice and observed with transmission electron microscopy. Oocyst walls of *C. parvum* were heavily labeled by IgG human antibodies, but they were not labeled by IgM and trophozoites were the least reactive with human sera (Lee et al., 2009).

Calves, mice, piglets, and rabbits infected with *C. parvum* recognized different *C. parvum* antigens (Reperant et al., 1994). During the experimental infection, only calves and lambs showed clinical infection. The specific immune response was first observed between 4 and 15 days post-challenge, and the 15-17 and 23 kDa *C. parvum* antigens were suggested as early infection markers. In this study, IgA, IgM, and IgG calf sera recognized a 15-17 kDa antigen at day 8 post-infection (p.i.) which persisted for 30 days. Lamb IgA and IgG recognized antigens of 15 and 23 kDa from day 7 to 41 p.i. while IgM recognized a 18 kDa antigen from day 11 to day 20 p.i. In a different study, *C. parvum* antigens recognized by naturally-infected lambs were proteins of 15 and 17 kDa, several proteins between 30 to 67-69 kDa, and a double band of 94 kDa. The 23 kDa protein was not recognized by lamb sera, and sera reactivity against polypeptides of 15 to 17 kDa persisted for up to 4 weeks post-infection (Ortega-Mora et al., 1994). The 17 kDa *C. parvum* protein is glycosylphosphatidylinositol (GPI)-anchored, and the composition of this GPI anchor suggests that the protein plays a role in immunity response due to its resemblance to the dominant malarial GPI toxin (Priest et al., 2006b).

Asymptomatic adult cattle were also evaluated for their immune response against *C. parvum* antigenic proteins in western blots (Lorenzo et al., 1995). Sera from all the asymptomatic animals with only *C. parvum* oocysts in their feces recognized antigens between
56.5 and 69 kDa. Antigens from 17 to 20 kDa were recognized by 71% of those animals excreting only *C. parvum* oocysts and those excreting *C. parvum* and *Eimeria spp* oocysts in their feces. Further studies on the 17 and 23 kDa *C. parvum* antigens included the identification of epitopes common to the 23 kDa protein and the *C. parvum* proteins of 37, 49, 58, 68, 120, and 160 kDa in one-dimensional western blot using rabbit antiserum raised against the 23 kDa *C. parvum* antigen. All of these antigens, were localized in the membrane of sporozoites and merozoites in their anterior end as determined by immunoelectron microscopy (Lumb et al., 1989). Monoclonal antibodies (mAbs) which reacted with *C. parvum* proteins, such as p23, have been shown to reduce experimental *C. parvum* infection in mice (Perryman et al., 1996).

**Humoral immune response in humans**

Of a group of major protein bands in *Cryptosporidium* oocysts resolved by one-dimensional SDS-PAGE, antigenic proteins of 23 and 32 kDa from excysted sporozoites were recognized by human sera. In purified oocyst cell walls, the antigenic proteins detected were of 47.5, 55, 66 and 130 kDa. Of all the polypeptides identified in this work, those of 15.5, 32 and 47.5 kDa bands were suggested to be on the surface of the oocysts (Lumb et al., 1988). In AIDS patients, sera which were strongly positive in an ELISA test to detect IgA and IgM against *Cryptosporidium* did not react with a 23 kDa antigen in western blot analysis. In the same study five human sera from a group of stool-negative individuals were negative in ELISA but reacted with the 23 kDa *C. parvum* protein in western blot (Ungar and Nash, 1986).

In a study conducted on volunteers to determine the infection dose for *Cryptosporidium*, 72% were negative in ELISA for antibodies against crude *C. parvum* oocysts antigens before challenge, but they demonstrated reactivity against *C. parvum* proteins of 15, 17, and 27 kDa
(Moss et al., 1998). The presence of IgM antibodies to 27 kDa, and IgG to 17 kDa at day 0 correlated with protection against cryptosporidiosis symptoms and with lower counts of stool oocysts after challenge. Increased reactivity was more common for symptomatic volunteers, and whether that response observed before infection was specific for *Cryptosporidium* or not was not determined. Reactivity with *C. parvum* 15, 17 and 27 kDa was not previously observed in persons infected with *Giardia* or *Toxoplasma* (Priest et al., unpublished). In a different study, sera from AIDS patients showing clinical cryptosporidiosis recognized antigens of 15-17, 22-23, 30, 40, 53 and 58 kDa, and sera from immunocompetent persons recognized 14, 15-17, 22-23, 30 and 40 kDa antigens (Reperant et al., 1994). Infection persisted in AIDS patients even though a response against these antigens was observed, suggesting that other mechanisms such as cellular immunity might be required to clear the infection (Reperant et al., 1994).

Asymptomatic infections were associated with significantly higher post-infection levels of antibodies against the 27 kDa antigen compared to symptomatic infections, and the difference between the level of antibodies against the 17 kDa antigen was not significant between asymptomatic and symptomatic infections. No cross-reactivity with *Giardia* or *Cyclospora* infections was observed (Priest et al., 2006a).

A *C. parvum* Iowa (IO) antigen of 20 kDa was recognized by human anti-*Cryptosporidium* IgG and IgM antibodies as early as 10 days post-infection. Apparently re-exposure is necessary to maintain the level of reactivity against this antigen over a longer period of time, suggesting this antigen most likely correlates with recent *Cryptosporidium* infections (Mead et al., 1988).

In Peru and Venezuela the evaluation of humoral response in children and adults against *Cryptosporidium* antigens showed detectable IgG (19.8%) and IgM (15.5%) in ELISA,
suggesting recent *Cryptosporidium* infection, while 64% showed IgG response only, suggesting prior exposure. The humoral immune response in children increased between 2 and 3 years of age (Ungar et al., 1988). After a primary exposure to *C. parvum* in volunteers, an increase in serum IgM, even when oocysts were not observed in the stools, was detected. After a re-exposure one year later, the Ig isotypes detected were IgG and IgA, and no correlation between protection and development of antibodies at first exposure was observed in the re-challenge (Okhuysen et al., 1998b).

**Humoral immune response at the intestinal level**

Antibodies in feces of experimentally-infected calves reacted with a 11 kDa *C. parvum* antigen in one-dimensional western blot from day 7 post-infection (p.i.) to 16 weeks p.i. Antibodies to proteins of 15, 23 and 44 kDa were also detected later in the infection. Sera of these animals also reacted with proteins of 23 and 44 kDa. Serum IgG as well as fecal IgA rose during oocysts excretion, which coincided with the elimination of the parasite (Peeters et al., 1992). In a different study, flow cytometry was employed to detect IgA, IgG, and IgM against a 23 kDa *C. parvum* protein in feces. All the immunoglobulin isotypes were detected, and their levels declined after 2 months post-experimental infection. A response against a second exposure was short and characterized by IgM and IgA or Ig G2 (Wyatt et al., 2000).

**Protection against cryptosporidiosis**

The role of antibodies in protection against cryptosporidiosis is not fully understood. In a retrospective epidemiological study, HIV-positive patients did not necessarily have better
protection against diarrhea caused by *Cryptosporidium* when a strong humoral response against 15/17 or 27 kDa *C. parvum* antigen was observed by western blotting (Frost et al., 2005).

Several studies have been conducted on the evaluation of hyperimmune bovine colostrum against *Cryptosporidium* infection. Hyperimmune bovine colostrum has been reported as being protective in mice (Fayer et al., 1989). In addition, a *C. parvum* 15 kDa protein-DNA vaccine administered to goats was shown to be useful for the protection of kids against experimental *Cryptosporidium* infection when they were infected with *Cryptosporidium* after consuming colostrum (Sagodira et al., 1999). Also, hyperimmune colostrum obtained from cows vaccinated with a plasmid encoding *C. parvum* 15/60 kDa oocysts-sporozoite antigen was demonstrated to be protective in a mouse model (Jenkins et al., 1999).

Bovine colostrum antibodies recognized different proteins of *C. parvum* from different growth stages such as sporozoites, merozoites, meronts, microgametocytes, microgametes, and macrogamonts (Fayer et al., 1991; Tilley et al., 1990). IgA in colostrum recognized a 9 to 10 kDa protein while IgA, IgG₁, IgG₂ and IgM recognized a 14.5 to 16.5 kDa protein. A protein of >190 kDa in *C. parvum* sporozoites was identified by hyperimmune bovine colostrum, which reduced *Cryptosporidium* infection *in vivo*. This protein was named GP900, and the difference in molecular weight after de-glycosylation was attributed to the association of this protein to a large carbohydrate fraction. The protein was highly immunogenic and was the product of the expression of a single *Cryptosporidium* gene. The immunogenic GP900 was suggested to be used in immunocompetent persons to control *Cryptosporidium* infection (Petersen et al., 1992).

In a different study, bovine anti-*Cryptosporidium* immunoglobulin colostrum did not protect immunocompetent volunteers against *Cryptosporidium* infection (Okhuysen et al., 1998a).
Absorption of immunoglobulins from colostrum was evaluated in calves with cryptosporidiosis. The absorption efficiency and serum concentration of gamma globulins were directly associated with the number of Cryptosporidium oocyst shedding. Absorption efficiency was higher (46%) in calves with 0 or 1 shedding episode in a four week period after birth. Weight gain during this period was also associated with the gamma globulin level in serum at 24 h of birth \( (r = 0.48; \ p < 0.05) \) (Lopez et al., 1988). These results suggested that immunoglobulins from colostrum are associated with protection against cryptosporidiosis in calves. In children, the effect of breast feeding on cryptosporidiosis has not been clarified (Molbak et al., 1994). In a cohort study in Guinea-Bissau, breast feeding was found to be protective in children (Molbak et al., 1994; Nchito et al., 1998). During an epidemiological study in Zambia, breast-feeding was associated with an unexplained higher risk of cryptosporidiosis, which could be due to the low number of breast-fed children without diarrhea included in the study (Nchito et al., 1998). In Costa Rica, Cryptosporidium infection was not observed during the first year of age in children living in rural areas; however, children younger than one year of age in urban areas showed a 3% infection rate. The authors suggested that breast-feeding for longer time in rural children was a factor affecting this difference (Mata et al., 1984). The level of IgA antibodies against C. parvum in human breast milk was not associated with prevalence of Cryptosporidium in infants suggesting that increased levels of immunoglobulins in breast milk is not necessarily advantageous for decreasing the prevalence of cryptosporidiosis in breast-fed children (Sterling et al., 1991).

Monoclonal antibodies have been also evaluated for their protective effect against Cryptosporidium infection. For example, a monoclonal antibody (MAb5C3) which recognizes the C. parvum GP15 was observed to bind sporozoites, merozoites and middle-late meronts, and
it was protective in mice when used after an experimental challenge (Tilley et al., 1991). In a different study, comparison between the effectiveness to neutralize *C. parvum* sporozoite infectivity was done among monoclonal antibody (mAb) 18.44 which recognized a nonprotein *C. parvum* surface antigen, mAb 17.41 which recognized epitopes in the sporozoite antigens of 28, 55 and 98 kDa, hyperimmune bovine serum and hyperimmune bovine colostrum. In this study, greater protection against *Cryptosporidium* infection in mice was achieved when using hyperimmune bovine colostrum (Perryman et al., 1990).

Yolk from eggs produced by chickens immunized with *C. parvum* also reduced sporozoite infectivity. Morphological changes were observed in sporozoites when they were exposed to IgY antibodies. These antibodies were also shown to be protective in *C. parvum* infected mice; however, they did not clear the infection in these animals (Cama and Sterling, 1991; Kobayashi et al., 2004).

Recombinant proteins have been suggested for immunotherapy in cryptosporidiosis (Jenkins et al., 1993). For example, the *C. parvum* sporozoite surface proteins CP15 and CP60 kDa which are cross-reactive were used to generate a recombinant plasmid DNA encoding the rCP15/60 protein from *C. parvum*, which was then used to immunize sheep. A short-term immune response was observed in some animals after intra-mammary injection of this plasmid DNA (Jenkins et al., 1995).

**Present research interests**

Humoral as well as cell-mediated immunity appear to be necessary to clear *Cryptosporidium* infection. Peptides are the only antigens recognized by both host-defense systems, and those *C. parvum* antigenic proteins recognized in acute infections may be relevant
for parasite infectivity. Some *C. parvum* antigenic proteins have been suggested as early infection markers (Mead et al., 1988; Reperant et al., 1994); however, sera reactivity in confirmed human cases of cryptosporidiosis as well as protection against infection were not necessarily associated with these proteins.

In a previous work (Sanchez and Ortega, unpublished), sera from a person with multiple *Cryptosporidium* infections collected over two years and analyzed by one-dimensional western blot (Figure 3.1) reacted in the first and second infection with *C. parvum* Iowa proteins between 30 and 80 kDa while antigenic proteins between 14 and 30 kDa were detected after the third infection. This finding suggested that proteins other than *C. parvum* antigens of 17, 21 or 27 kDa may be more relevant in detecting recent exposure to *Cryptosporidium*. Therefore, the present study is focused on the determination of *Cryptosporidium* protein antigens recognized in acute human cases of cryptosporidiosis. These proteins may be subsequently employed to design new molecular epidemiological tools for population screening, and they may also be evaluated as potential therapeutic targets or vaccines to control cryptosporidiosis in humans.

**Objective**

This study was conducted for the purpose of identifying antigenic proteins which are recognized by human anti-*C. parvum* antibodies from recently-infected individuals.
Material and Methods

_Cryptosporidium parvum_ Iowa (IO) isolate antigenic proteins were analyzed with human sera in one-dimensional and two-dimensional immunoelectrotransference assays. Proteins were separated by molecular weight for one-dimensional analysis and by isoelectric focusing and molecular weight for two-dimensional analysis. Statistical significance in antigenic protein intensities between groups was determined, and proteins of interest were selected based on their increased reactivity with human sera from confirmed acute cases of cryptosporidiosis.

**Strain**

A _C. parvum_ IO isolate obtained from The Sterling Parasitology Laboratory, Department of Veterinary Science and Microbiology in Tucson, Arizona in 2009 was used in the experiments. Oocysts were provided in an antibiotic solution containing 0.01% Tween 20, 100 U of penicillin, and 100 μg of gentamicin per ml, and were maintained at 4°C until analyzed.

**Human sera**

Human sera used in these experiments were from individuals excreting and not excreting _Cryptosporidium_ oocysts. These de-identified samples have been archived at the Parasitology Laboratory at the Center for Food Safety, University of Georgia, Griffin, GA.

**Reagents**

Thiourea, urea and 87% glycerol were acquired from GE Healthcare Biosciences (Pittsburgh, PA); CHAPS, ASB-14, and DTT were purchased from Sigma Aldrich (St. Louis,
MO); tris base was obtained from Fisher Scientific (Pittsburgh, PA); iodoacetamide, SDS, and Bio-lyte® 3-10 ampholytes were purchased from Bio-Rad (Hercules, CA).

**Protein extraction**

*Cryptosporidium parvum* oocysts were enumerated with a Neubauer hemacytometer (VWR Scientific, Suwanee, GA), and a fraction of $10^7$ oocysts was used in the following steps. Oocysts were washed five times in 1 ml of distilled sterile water by suspension and centrifugation at 6,000 ×g for 3 min each time. Washed oocysts were sonicated twenty times in 300 µl of sterile water for $3 \pm 0.5$ sec at level 5 using a Sonic Dismembrator Model 100 (Fisher Scientific, Pittsburgh, PA). Each sample volume was subsequently reduced to 20 µl in a Speed-Vac (Savant Instruments, Holbrook, NY) at 30°C, and protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions.

**One-dimensional analysis**

*Cryptosporidium* antigen was diluted to a final volume of 200 µl with sample buffer supplemented with 6% v/v glycerol, 38% w/v DTT and 10% w/v SDS. The final *C. parvum* protein concentration was 0.05 µg/µl. The protein solution was loaded full-length onto 12% polyacrylamide gels and electrophoresed in a Mini-Protean 3 Cell electrophoresis unit (Bio-Rad, Hercules, CA) along with a high molecular weight pre-stained protein marker (Bio-Rad, Hercules, CA) for 1 h at 200 V. After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) for 2 h at 1 Amp using a Mini Trans-Blot Cell (Bio-Rad, Hercules, CA). Strips of 2-3 mm thickness were subsequently cut from the nitrocellulose membranes and incubated overnight with 1:50 dilutions of human sera. Next, strips were
incubated for 1 h with a 1:500 goat anti-human IgA, IgM, IgG coupled to horseradish peroxidase (KPL Inc, Gaithersburg, MD) followed by colorimetric detection using 3-3’ diaminobenzidine tetrahydrochloride dehydrate (Aldrich Chemical Company, St. Louis, MO) and hydrogen peroxide (Sigma Aldrich, St. Louis, MO).

Two-dimensional analysis

Extracted proteins from sonicated oocysts were diluted to 100 μl in protein solubilization buffer (2M thiourea, 7M urea, 2% w/v CHAPS, 2% w/v ASB14, 15 mM Tris) and were kept in this buffer at room temperature overnight. Proteins were then cleaned of salts, lipids, phenolics, nucleic acids, and charged detergents by using the 2D clean-up kit (Amersham Biosciences, Pittsburgh, PA) and resuspended in 125 μl of IPG buffer (2M thiourea, 7M urea, 2% w/v CHAPS, 2% w/v ASB14, 0.018M DTT, and 0.2% v/v Bio-lyte® 3-10 ampholytes). Samples were subsequently centrifuged at 12,000 ×g for 5 min, and the supernatant was then used in the isoelectric focusing (IEF).

Seven-cm-long strips of 3-10 immobilized pH gradient (IPG) (Amersham Biosciences, Pittsburgh, PA) were loaded with 125 μl of sample and actively rehydrated at 50 V for 12 h, followed by isoelectric focusing using a Bio-Rad Protean IEF Cell for a total of 16 kVh (ramped to 500 V for 5 min, held at 500 V for 55 min, ramped to 1000 V for 5 min, held at 1000 V for 55 min, ramped to 2000 V for 5 min, held to 2000 V for 55 min, ramped to 4000 V for 1 h, held at 4000 V for 2 h 30 min). The cysteine sulfhydryls were reduced and carbamidomethylated while the proteins were equilibrated by 10 min incubation of the focused strips in equilibration buffer (1.5M tris, 6M urea, 87% v/v glycerol, 10% w/v SDS) supplemented with 1% w/v DTT followed by incubation in equilibration buffer supplemented with 2.5% w/v iodoacetamide for 10 min.
Second dimension SDS-PAGE was run on 12% polyacrylamide gels at 200 V for 40 min by using a Bio-Rad Mini-Protean 3 Cell electrophoresis unit.

After electrophoresis, proteins were transferred to Bio-Rad nitrocellulose membranes for 2 h at 1 Amp using a Bio-Rad Mini Trans-Blot Cell. The nitrocellulose blots were incubated overnight with 1:50 human sera dilutions. Blots were incubated for one hour with 1:500 goat anti-human IgA, IgM, and IgG coupled to KPL horseradish peroxidase, and colorimetric detection was then conducted using 3-3’ diaminobenzidine tetrahydrochloride dehydrate and hydrogen peroxide.

Experimental design and data analysis

Two experimental groups were compared. The experimental Group 1 consisted of 13 human serum samples from individuals with cryptosporidiosis confirmed by stool examination. Samples were collected during the first week after stools were confirmed positive for Cryptosporidium oocysts. The experimental Group 2 included 7 human serum samples collected from individuals without any previous history of cryptosporidiosis and negative for Cryptosporidium oocysts in the stools. One blank sample which did not contain human sera was also included in each analysis as a control.

Sera were used for the determination of C. parvum antigenic proteins in one-dimensional and two-dimensional immunoelectrotransference assays, images were captured with a Nikon Coolpix 5200 digital camera (Nikon Inc., Melville, NY) and subsequently processed as follows: The one-dimensional images were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD), and the two-dimensional images were analyzed with Kodak Molecular Imaging software (Carestream Health, Woodbridge, CT). Protein molecular weights and isoelectric
points were estimated by comparison with a low molecular weight protein standard (Bio-Rad, Hercules, CA) and by using the Immobiline DryStrip pH 3-10 gradient graph (Amersham Biosciences, Pittsburgh, PA).

The intensities for one spot in all nitrocellulose membranes, identified as “a” in Figure 3.2, were normalized to the intensity of the same spot in a nitrocellulose membrane showing antigens detected by IgY. This facilitated the comparison between spot intensities in all membranes. Student’s T test was employed to compare similar spots between experimental groups, and significant differences were determined at $p < 0.05$.

**Results**

Protein concentration for $10^7$ *C. parvum* oocysts was estimated at 14 µg. The soluble fraction was used in one-dimensional and two-dimensional analyses. Blank samples exhibited some bands or spots with light intensities, and these intensities were subtracted from those samples with the same bands and spots.

**One-dimensional analysis**

Sera from Groups 1 and 2 recognized antigenic proteins ranging from 14 to over 79 kDa. The image of *C. parvum* IO antigenic bands is shown in Figure 3.2. Intensity averages for 15 distinct bands were compared between experimental groups, and the statistical results are depicted in Table 3.1. Sera reactivity against antigenic *C. parvum* proteins of 79, 57, 33 and 26 kDa was found to be significantly different between groups ($p < 0.05$). Greater reactivity was observed for a 57 kDa band in Group 1 while proteins constituting bands of 79, 33, and 26 kDa
were more reactive with human sera from Group 2. Figure 3.3 shows the localization of the 57 kDa protein band.

**Two-dimensional analysis**

Antigenic proteins ranged from 14 to 167 kDa with isoelectric points (pIs) ranging from 4.4 to 10. Fourteen *C. parvum* IO antigenic proteins from 36 to 70 kDa detected by two-dimensional western blot were used for determining differences in the immune response between individuals with acute cryptosporidiosis (Group 1) and individuals without previous exposure to *Cryptosporidium*.

The spots analyzed for the two-dimensional western blot assay are shown in Figure 3.4. As was observed in the one-dimensional analysis, human sera from Group 2 also reacted with *C. parvum* proteins, and a representative image of the *C. parvum* antigens recognized by human sera from this group is displayed in Figure 3.5. Table 3.2 shows the molecular weights (MWs), pIs for the antigenic spots of interest, and the comparison between groups. Human sera from Group 1 reacted more strongly than human sera from Group 2 to six *C. parvum* antigenic proteins (*p* < 0.05). In order of significance, these proteins were those identified with numbers 8 (43.4 kDa, 5.4 pl), 2 (50.3 kDa, 7.0 pl), 3 (50.3 kDa, 7.2 pl), 7 (47.6 kDa, 5.3 pl), 13 (64.7 kDa, 6.6 pl), and 1 (50.3 kDa, 6.7 pl) in Figures 3.4 and 3.5.

**Discussion**

*Cryptosporidium parvum* IO antigenic proteins were detected by one-dimensional and two-dimensional western blots by using human sera from acute cases of cryptosporidiosis.
Antigenic proteins of interest were selected from those *C. parvum* polypeptides, which reacted more strongly with these sera when comparing them to sera from non-infected individuals. A paired-comparison between pre- and post-*Cryptosporidium* infection human sera could facilitate the detection of early-recognized *Cryptosporidium* antigens. Unfortunately, samples representing these two groups are not easily obtained from naturally infected individuals.

The *Cryptosporidium* species, which were detected in stools of individuals from Group 1 are not known. However, the antigenic proteins detected as indicators of recent cryptosporidiosis infection are most probably common antigens recognized by *Cryptosporidium* species that are pathogenic to humans.

Sera from individuals that do not react to *Cryptosporidium* antigens are unusual since most people are exposed to the parasite sometime early in their lives (Newman et al., 1994; O'Donoghue, 1995). Reactivity to *C. parvum* proteins in sera from persons not excreting oocysts was also observed, and it was possible to determine differences between the two experimental groups in the present study apparently due to the higher quality of protein separation during two-dimensional analysis.

Previous studies suggested *C. parvum* antigenic proteins of 15-17, 20 and 23 kDa may be indicators of early infection in animals or humans (Mead et al., 1988; Reperant et al., 1994). In humans, IgG and IgM recognized a 20 kDa *C. parvum* antigen as early as 10 days post-infection (p.i.) (Mead et al., 1988). The results in the present work did not show significant differences favoring Group 1 for bands of 14, 16, 21 or 26 kDa in one-dimensional analysis and neither for a 17.5 *C. parvum* antigenic protein in two-dimensional analysis (*p* < 0.05). There was a significant difference in reactivity against proteins of 26 kDa, this being the group of non-infected individuals who had the greatest response. The reduced number of samples evaluated may
explain this finding. On the other hand, the similar level of reactivity against the 14, 16, and 21 kDa antigens between experimental groups may suggest that those proteins are not necessarily good indicators of ongoing Cryptosporidium infection.

A C. parvum protein of 57 kDa reacted stronger with acute human sera in one-dimensional analysis. This was the only protein which was significantly recognized by infected individuals. Antigenic proteins of nearly the same molecular weight have been detected in other reports. For example, 55 and 66 kDa C. parvum proteins from purified oocysts cell wall were detected by human sera from acute and convalescent cases of cryptosporidiosis (Lumb et al., 1988). Proteins of 53 and 58 kDa have been faintly recognized by human sera from AIDS patients with clinical cryptosporidiosis, and only one out of ten individuals did not recognize these bands (Reperant et al., 1994). The 57 kDa antigenic protein determined in the present study was previously identified by serum samples from an individual who had repeated Cryptosporidium infections (figure 3.1, Sanchez and Ortega, unpublished). This protein was recognized by human sera before any C. parvum protein smaller than 30 kDa was identified.

C. parvum antigenic proteins of 79, 33 and 26 kDa were found at levels significantly different between groups (p < 0.05), and stronger reactivity was observed with human sera from Group 2. In a previous study, a 32 kDa antigenic protein was identified by human sera from acute and convalescent cases of cryptosporidiosis, and serum from one acute case did not recognized this polypeptide (Lumb et al., 1988). From the results of the present study it is not possible to determine the significance of this finding during an active Cryptosporidium infection.

The analysis of spots in the two-dimensional western blots focused on proteins between 36 and 70 kDa. Antigenic proteins of 43.4, 50.3, 50.3, 47.6, 64.7, and 50.3 kDa with pIs of 5.4, 7.0, 7.2, 5.3, 6.6, and 6.7, respectively, reacted more strongly with human sera from
Cryptosporidium infected individuals than with sera from non-infected individuals ($p < 0.05$). The sera reactivity against the 17 kDa C. parvum protein was not significantly different between experimental groups, thereby corroborating the results from the one-dimensional analysis.

In 1991, Tilley and Upton identified a C. parvum antigen of 15 kDa with pI between 4.15 to 4.25 in sporozoites as well as in merozoites (Tilley and Upton, 1991). In the present study, an antigenic C. parvum protein of approximately 17 kDa with a pI of 4.4, which may correspond to the 15 kDa protein of Tilley and Upton, was detected in sera from both experimental groups.

Hemaglutination (lectin) activity was observed for C. parvum proteins of 15, 20, 22, 24, 40 (doublet), and 60 kDa in a previous study (Thea et al., 1992). The carbohydrate moiety implicated in cell-to-cell interaction was not determined. However, the study suggested that these C. parvum proteins may participate in host-cell invasion (Thea et al., 1992). In the present study, two antigenic proteins in one-dimensional analysis with 36 and 41 kDa observed as a doublet, that probably corresponded to 43 and 48 kDa proteins with pIs of 5.4 and 5.3, respectively, were observed in two-dimensional analysis. These antigens were significantly recognized by sera from the C. parvum infected individuals. Therefore, if these proteins are associated with lectins, they could very well play an important role in the parasite host-cell invasion.

Finally, human sera from both experimental groups recognized many C. parvum IO antigens suggesting that prior exposure may have occurred. Therefore, presence of antibodies did not necessarily correlate with protection against infection, as was previously reported (Okhuysen et al., 1998b; Reperant et al., 1994). Other components of the immune system, such as components of cellular immunity, may be required to efficiently clear Cryptosporidium
infection, and additional work is essential to determine the role during clinical cryptosporidiosis of the antigenic proteins detected in this study.
References


Sagodira, S., Buzoni-Gatel, D., Iochmann, S., Naciri, M., Bout, D., 1999, Protection of kids against Cryptosporidium parvum infection after immunization of dams with CP15-DNA. Vaccine 17, 2346-2355.


Tessema, T.S., Schwamb, B., Lochner, M., Forster, I., Jakobi, V., Petry, F., 2009, Dynamics of gut mucosal and systemic Th1/Th2 cytokine responses in interferon-gamma and
interleukin-12p40 knock out mice during primary and challenge Cryptosporidium parvum infection. Immunobiology 214, 454-466.


Table 3.1: Student’s T test results for the comparison between Group 1 (Cryptosporidium infected individuals) vs. Group 2 (non-infected individuals) of antigenic bands observed in one-dimensional western blot analysis

<table>
<thead>
<tr>
<th>MW</th>
<th>Group 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Group 2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>( t )</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>79</td>
<td>1292.60</td>
<td>2477.06</td>
<td>-3.23</td>
<td>0.005</td>
</tr>
<tr>
<td>76</td>
<td>1498.74</td>
<td>1562.47</td>
<td>-0.25</td>
<td>0.800</td>
</tr>
<tr>
<td>57</td>
<td>2749.32</td>
<td>1695.24</td>
<td>2.78</td>
<td>0.012</td>
</tr>
<tr>
<td>52</td>
<td>3146.56</td>
<td>3874.77</td>
<td>-1.40</td>
<td>0.180</td>
</tr>
<tr>
<td>44</td>
<td>1483.80</td>
<td>1595.25</td>
<td>-0.52</td>
<td>0.610</td>
</tr>
<tr>
<td>41</td>
<td>3525.30</td>
<td>4069.80</td>
<td>-1.31</td>
<td>0.210</td>
</tr>
<tr>
<td>36</td>
<td>2247.19</td>
<td>2305.56</td>
<td>-0.14</td>
<td>0.590</td>
</tr>
<tr>
<td>33</td>
<td>2114.85</td>
<td>4362.34</td>
<td>-4.34</td>
<td>0.0004</td>
</tr>
<tr>
<td>32.4</td>
<td>745.380</td>
<td>1022.46</td>
<td>-1.77</td>
<td>0.094</td>
</tr>
<tr>
<td>32</td>
<td>1518.49</td>
<td>1547.84</td>
<td>-0.07</td>
<td>0.940</td>
</tr>
<tr>
<td>26</td>
<td>1263.89</td>
<td>2472.54</td>
<td>-2.83</td>
<td>0.011</td>
</tr>
<tr>
<td>21</td>
<td>2326.73</td>
<td>1889.84</td>
<td>0.93</td>
<td>0.370</td>
</tr>
<tr>
<td>16</td>
<td>5602.23</td>
<td>3814.56</td>
<td>1.11</td>
<td>0.280</td>
</tr>
<tr>
<td>14</td>
<td>4206.60</td>
<td>3230.36</td>
<td>1.31</td>
<td>0.210</td>
</tr>
<tr>
<td>10</td>
<td>2330.93</td>
<td>1740.96</td>
<td>0.95</td>
<td>0.360</td>
</tr>
</tbody>
</table>

MW: Molecular weight in kDa.

<sup>a</sup>Band area: pixel<sup>^2</sup>
Table 3.2: Student’s T test results for the comparison between Group 1 (*Cryptosporidium* infected individuals) vs. Group 2 (non-infected individuals) of protein spots of interest resolved by two-dimensional western blot

<table>
<thead>
<tr>
<th>Spot N°</th>
<th>MW</th>
<th>pI</th>
<th>Group 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Group 2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>t</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50.3</td>
<td>6.7</td>
<td>-1071.81</td>
<td>-390.10</td>
<td>-2.17</td>
<td>0.043</td>
</tr>
<tr>
<td>2</td>
<td>50.3</td>
<td>7.0</td>
<td>-1716.40</td>
<td>-190.37</td>
<td>-2.52</td>
<td>0.022</td>
</tr>
<tr>
<td>3</td>
<td>50.3</td>
<td>7.2</td>
<td>-1627.07</td>
<td>9.68</td>
<td>-2.49</td>
<td>0.023</td>
</tr>
<tr>
<td>4</td>
<td>50.3</td>
<td>7.4</td>
<td>-634.20</td>
<td>-1754.07</td>
<td>1.67</td>
<td>0.110</td>
</tr>
<tr>
<td>5</td>
<td>50.3</td>
<td>7.7</td>
<td>-1011.47</td>
<td>-1974.67</td>
<td>1.39</td>
<td>0.180</td>
</tr>
<tr>
<td>6</td>
<td>50.3</td>
<td>7.8</td>
<td>-1290.59</td>
<td>-2219.59</td>
<td>1.14</td>
<td>0.270</td>
</tr>
<tr>
<td>7</td>
<td>47.6</td>
<td>5.3</td>
<td>-1326.26</td>
<td>-308.02</td>
<td>-2.31</td>
<td>0.033</td>
</tr>
<tr>
<td>8</td>
<td>43.4</td>
<td>5.4</td>
<td>-2659.90</td>
<td>-209.94</td>
<td>-3.34</td>
<td>0.004</td>
</tr>
<tr>
<td>9</td>
<td>58.3</td>
<td>5.6</td>
<td>-3049.54</td>
<td>-821.64</td>
<td>-1.91</td>
<td>0.072</td>
</tr>
<tr>
<td>10</td>
<td>57.5</td>
<td>6.4</td>
<td>-1716.66</td>
<td>205.09</td>
<td>-2.01</td>
<td>0.059</td>
</tr>
<tr>
<td>11</td>
<td>64.7</td>
<td>6.2</td>
<td>-1228.58</td>
<td>-270.43</td>
<td>-1.63</td>
<td>0.120</td>
</tr>
<tr>
<td>12</td>
<td>64.7</td>
<td>6.4</td>
<td>-1490.39</td>
<td>-251.87</td>
<td>-1.66</td>
<td>0.110</td>
</tr>
<tr>
<td>13</td>
<td>64.7</td>
<td>6.6</td>
<td>-1063.46</td>
<td>-192.48</td>
<td>-2.25</td>
<td>0.037</td>
</tr>
<tr>
<td>14</td>
<td>17.5</td>
<td>4.4</td>
<td>-21307.4</td>
<td>-6946.43</td>
<td>-1.99</td>
<td>0.062</td>
</tr>
</tbody>
</table>

MW: Molecular weight in kDa. pI: Isoelectric point.

<sup>a</sup>Pixel intensities
Figure 3.1: *Cryptosporidium parvum* Iowa antigens recognized by human sera collected after 3 consecutive *Cryptosporidium* infections. Numbers indicate infection event.

Method: One-dimensional western blotting. MW: Molecular weight in kDa. (+): *C. parvum* antigens recognized by egg yolk from hyperimmunized hens. (-): blank.
Figure 3.2: *Cryptosporidium parvum* Iowa isolate antigenic bands detected by human sera in one-dimensional western blotting. Proteins resolved in 12% polyacrylamide gels. From 1 to 13 (A): Antigenic protein bands recognized by human sera from *Cryptosporidium* infected individuals (Group 1). From 14 to 20 (B): Antigenic protein bands recognized by human sera from non-infected individuals (Group 2). MW: Molecular weight in kDa.
Figure 3.3: *Cryptosporidium parvum* Iowa isolate antigenic bands detected by human sera in one-dimensional western blotting and resolved in 10% polyacrylamide gels. A: Antigenic protein bands recognized by human sera from *Cryptosporidium* infected individuals. B: Antigenic protein bands recognized by human sera from non-infected individuals. Arrow indicates band of 57 kDa.
Figure 3.4: Cryptosporidium parvum antigenic proteins used in the statistical comparison between experimental groups. Method: Two-dimensional western blotting. Proteins resolved in 12% polyacrylamide gels. A: Antigenic proteins detected by human sera from infected individuals (Group 1). B: Antigenic proteins detected by egg yolk from hyperimmunized hens.
Figure 3.5: *Cryptosporidium parvum* antigenic proteins recognized by human sera from non-infected individuals (Group 2). Method: Two-dimensional western blotting. Proteins resolved in 12% polyacrylamide gel. Numbers indicate those proteins which were used for statistical comparison between experimental groups.
CHAPTER 4
DETERMINATION OF CRYPTOSPORIDIUM PARVUM ANTIGENIC PEPTIDES FROM PROTEINS ASSOCIATED WITH HUMORAL IMMUNE RESPONSE IN HUMANS DURING ACUTE CRYPTOSPORIDIOSIS

\[1\] Sanchez-Ingunza, Roxana and Ynes R. Ortega. To be submitted to Infection and Immunity.
Abstract

*Cryptosporidium parvum* proteins previously recognized as antigenic by reactivity with human IgA, IgG, and IgM in sera from individuals with clinical cryptosporidiosis were identified by Tandem Mass Spectrometry (MS/MS). Antigenic peptides were predicted and their uniqueness as *C. parvum* antigenic peptides was evaluated by bioinformatics analysis. A *Cryptosporidium* serine/threonine phosphatase, an actin protein, a hypothetical protein, and a chaperone protein-related were detected. This is the first report confirming the existence of a *Cryptosporidium* serine/threonine phosphatase that is also involved in humoral immune response against cryptosporidiosis. Two antigenic peptides for the phosphoprotein phosphatase, one peptide for actin, three for hypothetical protein Chro 70534, and one for the chaperone protein-related were suggested as theoretically specific for *Cryptosporidium* based on their lowest E-values and highest scores in the alignment analysis with the non-redundant protein sequences from the NCBI database. Further studies are required to determine whether these antigenic peptides are useful in diagnosis, or suitable for therapeutic or vaccine interventions.
Introduction

*Cryptosporidium parvum* proteins and peptides recognized by human sera from infected persons might be useful to detect cases of human cryptosporidiosis as well as to detect *Cryptosporidium* in environmental or contaminated food samples. These molecules may be good candidates for vaccines or anti-*Cryptosporidium* drug development.

Several factors should be considered when antigenic peptides are determined from antigenic proteins. Antigenic peptides generated from an antigenic protein may have different conformations in solution from the ones they have in the intact protein; therefore antibodies raised against the original product might react weakly to those antigenic peptides. However, antigenic peptides can be used to generate monoclonal antibodies (mAbs), polyclonal antibodies, or monospecific antiserum which cross-react efficiently with the original protein. These antibodies can be used to distinguish very closely related proteins or to recognize proteins from the same family, as well as in diagnosis. The immunogenic capacity of those peptides cannot be predicted because it depends also on host characteristics such as host immunoglobulin repertoire, self-tolerance or various mechanisms of host immunity. Peptides to be used as vaccines require that the antibodies raised against them are able to neutralize the parasite and protect the host (Van Regenmortel, 2001).

*C. parvum* antigenic proteins may be identified by scanning *C. parvum* expression libraries, using polyclonal antisera. For example, proteins of 20, 35 and 40 kDa were detected by screening these libraries and were evaluated with human sera (Tosini et al., 1999). The polypeptides of 35 and 40 kDa were recognized by human sera from infected individuals; however, human sera reacted with the 20 kDa peptide independently of a *C. parvum* infection
(Tosini et al., 1999). In another study, fourteen sporozoite immunogenic proteins were identified from a collection of recombinant peptides by scanning 94 randomly collected clones with a rabbit anti-sporozoite serum (Trasarti et al., 2007). Bioinformatics analysis on the selected peptides (30-35 amino acids in length) showed that three proteins had conserved domains common to several organisms; three proteins had enzymatic function; three were probably involved in cytoskeleton assembly or cell motility; two proteins showed a myosin domain; one contained a region similar to the F-actin cross-linking gelation factor from *Dyctiostelium discoideum*; one was an RNA-dependant helicase and one was not associated with any particular molecular trait. Interestingly, two proteins were apparently restricted to the genera *Cryptosporidium* (Trasarti et al., 2007). In a different study, mAbs C6B6 and 7D10 against a 23 kDa *C. parvum* protein which showed to be protective for mice were used to identify antigenic peptides from a cDNA library prepared from *C. parvum* sporozoites (Perryman et al., 1996).

Antigenic proteins have been used to design recombinant proteins useful in diagnosis. For instance, a recombinant p23 used in dot blot analysis detected positive cases of cryptosporidiosis in calves and cows (Shayan et al., 2008). A recombinant Cp23 (designed from a 27 kDa *C. parvum* protein) was also used in ELISA to evaluate its capability to detect *Cryptosporidium* infection in HIV-positive persons during a case-control study, and the results showed that the IgG response against this protein between cases and controls after diagnosis of cryptosporidiosis was significantly different (1334 vs. 329 U/µl, respectively, \( p = 0.01 \)) (Eisenberg et al., 2001). Recombinant antigenic proteins have been also suggested as a means to differentiate *Cryptosporidium* species. For example, a 41 kDa *C. parvum* protein considered as probably specific for *C. parvum* when it was compared with *C. meleagris*, *C. baileyi*, and *C. serpentis* was used to design a recombinant protein (rCP41). In this specific example, ELISA
antibody titers against this recombinant did not increase after natural exposure of calves to \textit{C. parvum} (Jenkins et al., 1999). Recombinants designed from \textit{C. parvum} antigenic proteins, such as p23, were also used to screen libraries of human antibodies genes (Tomlinson I and J libraries) and detect recombinant anti-\textit{Cryptosporidium} antibodies (rAbs). The use of these libraries facilitated the identification of human antibodies against \textit{C. parvum} with minimum or non cross-reactivity with antibodies from other species (Boulter-Bitzer et al., 2009).

The analysis of host-parasite interaction may aid in the identification of valuable antigenic proteins which could be considered as targets for interventions during parasite infection. First, the mechanism of attachment and host-cell invasion must be analyzed even though it has not yet been fully elucidated. The apical complex organelles are believed to actively participate during infection. Proteins such as gp40 and GP900 were observed in sporozoite surface, apical complex, and in a trail left behind the sporozoites when they were allowed to glide on poly-L lysine coated slides after excystation. These proteins were glycosylated, contained \(\alpha\)-linked GalNac residues, and were suggested to participate in attachment and/or invasion. The \(\alpha\)-linked GalNac residues may mediate attachment by direct interaction with host-carbohydrate binding proteins which are not defined yet. The O-glycans of gp40 and GP900 did not contain sialic acid, and their O-glycosylation contributes to the adhesion characteristics of these mucins (Cevallos et al., 2000). GP900 was also localized in micronemes (structures comprising the apical complex) of sporozoites and merozoites. This N-glycosylated protein has transmembrane and proline-threonine-serine (PTS) domains which suggest membrane localization and support previous observations related to its participation in cell adhesion (Barnes et al., 1998). The relevance of this protein was demonstrated when host cell invasion was inhibited by antibodies directed to GP900 (Barnes et al., 1998).
Other proteins implicated in cell adhesion are 15 and 45 kDa *C. parvum* proteins. The 15 kDa *C. parvum* protein (Cp15), presumably the product of a proteolytic cleavage of a 60 kDa protein (gp60), as well as gp45, were localized on the surface of sporozoites and were recognized by antibodies and lectins. Both proteins had mucin-like O-linked glycosylation sites, and the gp45 also had a N-glycosylation site near its C terminus (Strong et al., 2000). Regarding timing of protein expression during host-cell invasion, the Cp15 mRNA of *C. parvum* was observed as early as 2 h post-infection (p.i.) in HCT-8 cell culture; Cp17 mRNA of *C. parvum* was detectable 8 h p.i., with a maximum detection between 26 h and 50 h p.i.; *C. parvum* p23 mRNA signal was stronger between 14 h and 50 h p.i.; and the GP900 mRNA was stronger at 14 h p.i.; which is the time when the apical complex organelles have been formed in merozoites. All of these mRNAs were detected in sporozoites as well as in merozoites (Jakobi and Petry, 2006).

Changes in the dynamics of protein expression in *Cryptosporidium* during invasion were assessed by using rabbit antiserum against *C. parvum* sporozoite, a mAb (4E9) which recognized the carbohydrate moiety of GP900 and gp40 in sporozoite (used to label microneme proteins), and a rabbit polyclonal antibody against CP2 (used for labeling sporozoite membrane). Sporozoites were exposed to 4 and 37°C during excystation in the absence of host-cells. Immediately after excystation a faint reaction was observed at the apical region of sporozoites with these fluorescent antibodies at 37°C. Proteins were detected in the supernatant fluid, but fewer dense granules and microneme proteins were observed in the apical region of the sporozoites compared to those sporozoites exposed to 4°C. Rhoptry was detected in few sporozoites by electron microscopy. The infection rate decreased when sporozoites were exposed to 37°C compared to those exposed to 4 and 18°C for 0 to 24 h before exposing them to cholangiocytes. Therefore, early release of apical complex proteins may affect parasite invasion.
The apparent discharge of proteins from apical organelles was mediated by cytoskeleton remodeling in the sporozoites and the concentration of intracellular Ca\textsuperscript{2+}. The inhibition of \textit{C. parvum} actin and tubulin polymerization by colchicine and the application of the intracellular chelator of Ca\textsuperscript{2+} BAPTA-AM reduced the invasion rate of cholangiocytes. Blocking of the apical complex discharge decreased sporozoite gliding motility; however, host-cell attachment was not inhibited (Chen et al., 2004b). Other proteins such as actin-related proteins, characterized by an actin fold domain which participated in cell division, have been determined as unique for apicomplexans. The actin-like protein-1 (ALP1), for example, is an actin-related protein identified in \textit{T. gondii} forming large protein complexes when inactive, but monomers participated in parasite cell division (Gordon et al., 2009).

\textit{Cdc42} and \textit{RhoA}, members of the Rho family of small guanosine triphosphatases (GTPases), were found in the host-parasite interface at the dense band which separates the parasite from the host cytoplasm during host-cell invasion. These molecules were not found in the parasite itself, and only \textit{Cdc42} was activated during parasite infection. \textit{Cdc42} was proven to invoke actin accumulation at the host-cell cytoplasm, membrane protrusion, dense-band and parasitophorous vacuole formation; however, the mutation of \textit{Cdc42} in the host-cell did not completely block invasion, and this suggested that other mechanisms were also involved in successful \textit{Cryptosporidium} infection (Chen et al., 2004a).

\textit{C. parvum} also induced the accumulation and activation by phosphorylation of phosphatidylinositol 3- kinase (PI3K) at the cell-parasite interface during invasion. Fabrin, a guanine nucleotide exchange factor specific for \textit{Cdc42}, is also recruited and seems to be required for \textit{Cdc42} accumulation and its further activation. The inhibition of PI3K reduced the host-cell actin remodeling and \textit{C. parvum} invasion of biliary epithelia; however, \textit{C. parvum} sporozoite
attachment did not depend on this mechanism. A significant increase in invasion was observed when cells were transfected with PI3K-p85 (active subunit). The sporozoite attachment may be involved in the activation of PI3K via tyrosine phosphorylation of its p85 subunit (Chen et al., 2004c).

A short description of protozoan phosphatases follows due to the relevance of these enzymes during host-cell and parasite interaction, and their structural diversity, especially in their catalytic domains, which make them good targets for therapeutics without interfering with the host-cell biology (Andreeva and Kutuzov, 2008). Phosphatases are involved in diverse physiological processes such as regulation of transduction pathways causing modifications on protein expression patterns (Garcia et al., 1999). There are two groups of phosphatases: protein serine/threonine phosphatases and protein tyrosine phosphatases. Protein serine/threonine phosphatases, which dephosphorylate serine and threonine residues, are encoded by two gene families, PPP and PPM. PP1, PP2A and PP2B are comprised in the first group while PP2C and pyruvate dehydrogenase phosphatase are included in the second group (Garcia et al., 1999; Kutuzov and Andreeva, 2008). The protein serine/threonine phosphatase 1 (PP1) in Toxoplasma gondii, for example, controls phosphorylation of several parasite proteins, and phosphatase inhibitors reduced T. gondii invasiveness in 50% (Delorme et al., 2002). PP1 may have phosphatase activity over some proteins in Toxoplasma, and their inhibition may cause hyperphosphorylation of proteins mediated by kinases or PP1 may control kinase activities, thereby causing reduction in protein phosphorylation (Delorme et al., 2002). In C. parvum, 8 PPP and 10 PPM phosphatases have been predicted from the genome sequence. However, their role in Cryptosporidium biology has not been described (Garcia et al., 1999; Kutuzov and Andreeva, 2008).
Protein tyrosine phosphatases which dephosphorylate phosphotyrosyl residues are grouped into three classes: Protein tyrosine phosphatases (PTPs), Cdc25, and low molecular weight phosphatases (LMW-PTPs). *C. parvum* has 6 predicted proteins with tyrosine phosphatase domain and, as well as in the case of serine/threonine phosphatases, their biological functions remain unknown. Ecto-phosphatases on the surface of parasitic protozoa which may dephosphorylate host proteins as well as Shelphs phosphatases in apicomplexans have also been predicted from the genome (Andreeva and Kutuzov, 2008).

Recently, a membrane-bound acid phosphatase activity has been reported in *C. parvum* oocysts, this enzyme might belong to the group of tyrosine phosphatases and not to the group of serine/threonine phosphatases because its activity was not inhibited by okadaic acid and calyculin A. Anti-human placental PTPase antibody recognized a 30 and 31 kDa proteins in *C. parvum* oocysts (Aguirre-Garcia and Okhuysen, 2007). Once again, information about its role in the parasite metabolism and/or host-cell invasion has not yet been determined.

Regarding the host-cell response, the expression profile of human genes in HCT-8 cells infected with *C. parvum* compared to that of non-infected cells was also evaluated. Genes differentially expressed at 24 h p.i. were detected by microarray analysis. Of the total, 125 genes were up-regulated and 98 genes were down-regulated. Several genes involved in heat shock response were up-regulated, and a mechanism to recruit γδ T-cells to the infected tissue was suggested. IL-18 was down-regulated therefore implying a Th1 response during *C. parvum* infection, and several genes involve in apoptosis were up-regulated or down-regulated (Deng et al., 2004). *C. parvum* has been proven to down-regulate infected biliary epithelial cell apoptosis presumably to facilitate parasite propagation (Chen et al., 2001).
In the present study, *Cryptosporidium* proteins previously detected by human sera from positive cases of cryptosporidiosis (Chapter 3) were identified by mass spectrometry. Antigenic peptides, which might be useful as molecular targets for diagnosis, outbreak investigations, and control of cryptosporidiosis, were determined from those antigenic proteins.

**Objective**

The aim of this study was to identify *C. parvum* antigenic proteins and peptides recognized by sera antibodies against *Cryptosporidium* from humans under acute infection.

**Material and Methods**

**Antigenic proteins**

Three *C. parvum* Iowa (IO) proteins were analyzed in this study. These *C. parvum* polypeptides were previously recognized by human sera from *Cryptosporidium* in two-dimensional western blotting (Figure 4.1). Protein 1 was identified by IgG, IgM, and IgA in sera from *Cryptosporidium* infected individuals, and proteins 2 and 3 were recognized by IgG, IgM, and IgA in sera from infected individuals as well as in sera of non-infected individuals. Protein 1 was significantly recognized by human sera from infected individuals ($p = 0.0036$). The estimated molecular weights (MWs) were 43.4, 58.3, and 57.5 kDa for protein 1, 2, and 3, respectively. The isoelectric points (pIs) were 5.4, 5.6, and 6.4 for protein 1, 2, and 3, respectively.
Strain

A *C. parvum* IO isolate obtained from The Sterling Parasitology Laboratory, Department of Veterinary Science and Microbiology in Tucson, Arizona in 2009 was used to obtain *Cryptosporidium* proteins for separation in two-dimensional analysis. Oocysts were provided in an antibiotic solution containing 0.01% Tween 20, 100 U of penicillin, and 100 µg of gentamicin per ml, and maintained at 4ºC until analyzed.

Reagents

Thiourea, urea and 87% glycerol were acquired from GE Healthcare Biosciences (Pittsburgh, PA); CHAPS, ASB-14, and DTT were purchased from Sigma Aldrich (St. Louis, MO); tris base was obtained from Fisher Scientific (Pittsburgh, PA); iodoacetamide, SDS, and Bio-lyte® 3-10 ampholytes were purchased from Bio-Rad (Hercules, CA).

Protein extraction

*Cryptosporidium parvum* oocysts were enumerated with a Neubauer hemacytometer (VWR Scientific, Suwanee, GA), and a fraction of $10^7$ oocysts was used in the following steps. Oocysts were washed five times in 1 ml of distilled sterile water by suspension and centrifugation at 6000 ×g for 3 min each time. Washed oocysts were sonicated twenty times in 300 µl of sterile water for 3 ± 0.5 seconds at level 5 using a Sonic Dismembrator Model 100 (Fisher Scientific, Pittsburgh, PA). Each sample volume was subsequently reduced to 20 µl in a Speed-Vac (Savant Instruments, Holbrook, NY) at 30ºC, and protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions.
Two-dimensional separation

Extracted proteins from sonicated oocysts were diluted to 100 μl in protein solubilization buffer (2M Thiourea, 7M Urea, 2% w/v CHAPS, 2% w/v ASB14, 15 mM Tris) and were maintained in this buffer at room temperature overnight. Proteins were then cleansed of salts, lipids, phenolics, nucleic acids, and charged detergents by using the 2D clean-up kit (Amersham Biosciences, Pittsburg, PA) and resuspended in 125 μl of IPG buffer (2M thiourea, 7M urea, 2% w/v CHAPS, 2% w/v ASB14, 0.018M DTT, and 0.2% v/v Bio-lyte® 3-10 ampholytes). Samples were subsequently centrifuged at 12000 ×g for 5 min, and the supernatant fluid was then used in isoelectric focusing (IEF).

Seven-cm-long strips of 3-10 immobilized pH gradient (IPG) (Amersham Biosciences, Pittsburgh, PA) were loaded with 125 μl of sample and actively rehydrated at 50 V for 12 h, followed by isoelectric focusing using a Bio-Rad Protean IEF Cell (Bio-Rad, Hercules, CA) for a total of 16 kVh (ramp to 500 V for 5 min, hold at 500 V for 55 min, ramp to 1000 V for 5 min, hold at 1000 V for 55 min, ramp to 2000 V for 5 min, hold to 2000 V for 55 min, ramp to 4000 V for 1 h, hold at 4000 V for 2 h 30 min). The cysteine sulfhydryls were reduced and carbamidomethylated while the proteins were equilibrated by 10 min incubation of the focused strips in equilibration buffer (1.5M tris, 6M urea, 87% v/v glycerol, 10% w/v SDS) supplemented with 1% w/v DTT followed by incubation in equilibration buffer supplemented with 2.5% w/v iodoacetamide for 10 min. Second dimension SDS-PAGE was run on 12% polyacrylamide gels at 200 V for 40 min by using a Bio-Rad Mini-Protean 3 Cell electrophoresis unit (Bio-Rad, Hercules, CA).
Protein staining

Gels from the two-dimensional analysis were washed in ultra-pure water before staining with Gel Code™ Blue Safe stain (Thermo Scientific, Rockford, IL) for about 3 h. Destaining was conducted overnight in ultra-pure water. Protein spots of interest were excised from the gels and kept at 4°C until analyzed by mass spectrometry.

Mass Spectrometry

Protein spots of interest were sent to the Centers for Disease Control and Prevention (CDC) in Atlanta, GA, to be analyzed by Mass Spectrometry (MS). The instrument used for Mass Spectrometry analysis was a quadrupole-time of flight (Q-TOF) Premier (Waters, Milford, MA) equipped with a nanoAcquity UPLC™ system (Waters, Milford, MA). A brief description of the methods used is as follows: Gel slices were dried out in a speed vacuum centrifuge and 5 µg trypsin (Promega, Madison, WI) with 25 ul of a tryptic digestion buffer (50 mM NH₄CO₃, 1 mM CaCl₂, pH = 8.5) were added. Samples were incubated overnight at 37°C, and volumes were subsequently adjusted to 40 ul with 0.1% formic acid. After sonication for 2-3 min and centrifugation for 10 min at 13000 ×g, supernatant fluids were discarded and samples resuspended in 0.1% formic acid up to 50 ul. This solution was directly applied to a nanoACQUITY UPLC BEH C18 Column (Waters, Milford, MA) for liquid chromatography (LC) separation at a flow rate of 1.2 ml/min. Fragmentation data were collected from the m/z range 50-1990 during 50 min. The ProteinLynx Global SERVER™ (PLGS) v2.3 software (Waters, Milford, MA) was used to analyze MS data and obtain statistically-validated peptide and protein identifications by scanning the Swiss-Prot Database. The detection of two or more peptides in a protein indicated a significant match.
Antigenic peptides determination

Once identified, antigenic peptides were predicted with the Kolaskar and Tongaonkar method (Kolaskar and Tongaonkar, 1990). This method is based on the occurrence of amino acid residues in experimentally known segmental epitopes and it is 75% accurate. The Basic Local Alignment Search Tool for protein queries BLASTP™ (NCBI, Bethesda, MD) was employed for determining protein sequences closely related to the selected *C. parvum* antigenic proteins. E-values lower than 1e-50 were considered for these sequences to be selected for further alignment analysis. Related proteins from other parasitic protozoa (*Cryptosporidium* spp., *Plasmodium* spp., *Theileria* spp., *Babesia* spp., *Toxoplasma* spp., *Trypanosoma* spp., *Leishmania* spp., *Entamoeba* spp.), *Homo sapiens* and *Gregarina* spp. were aligned with the sequences of *C. parvum* antigenic proteins and analyzed for specific *C. parvum* antigenic peptides by using the Vector NTI™ Advance 11 software (Invitrogen, Carlsbad, CA). Specific selected peptides were subsequently aligned with all non-redundant protein sequences by using the NCBI BLASTP algorithm, and those peptides with significant protein sequences hits associated with *Cryptosporidium* were reported. The Constraint-based Multiple Alignment Tool Cobalt™ (NCBI, Bethesda, MD) was used to construct the phylogenetic trees by the Neighbor Joining method with a maximum sequence difference of 85%.

Results

Mass Spectrometry

Antigenic protein number and identification, protein database accession numbers, microorganism species of origin, estimated molecular weights (MWs) and isoelectric points
(pIs), coverage, and number of peptides identified by mass spectrometry are reported in Table 4.1. Two significant hits were detected for protein N° 1, and they corresponded to a phosphoprotein phosphatase-related protein in *C. hominis* (Accession N° EAL36686) and in *C. parvum* IO (Accession N° XP_001388227). The gene sequence encoding for this protein was annotated in chromosome 2 in the published genomes of *C. parvum* and *C. hominis* (Abrahamsen et al., 2004; Xu et al., 2004). This sequence contains a tetratricopeptide repeat (TPR) domain from a protein phosphatase 5 (PPP5) region, and a protein phosphatase 2A (PP2Ac) homologous catalytic domain.

The antigenic protein N° 2 was identified as actin from *C. parvum* (Accession N° P26183). The gene encoding this protein was localized in chromosome 5 (Abrahamsen et al., 2004). This protein was first identified in 1992 by Kim *et al* (Kim et al., 1992), and the genomic prediction was confirmed by Sanderson *et al* in 2008 (Sanderson et al., 2008). *Cryptosporidium* actin is localized in the cytoplasm and cytoskeleton, and contains ATP-, gelsolin and profilin binding sites.

Two significant hits were obtained for protein N° 3: A hypothetical protein Chro 70534 (Accession N° EAL37510) and a chaperone-related protein (Accession N° EAL35079) from *C. hominis*. The hypothetical protein Chro 70534 from *C. hominis* was predicted from the genome of *C. hominis* published in 2004 by Xu *et al* (Xu et al., 2004). The gene sequence for this protein was located in chromosome 7 and contains a PA14 domain. The chaperone related-protein from *C. hominis* was also predicted from the genome of *C. hominis*; the sequence was located at chromosome 3 and contains an hscB region (Xu et al., 2004).
Antigenic peptides

The list of antigenic peptides identified by the method of Kolaskar and Tongaonkar (Kolaskar and Tongaonkar, 1990) are indicated in Table 4.2. In the alignment of related proteins (Figures 4.2, 4.3, 4.4, and 4.5), 26 sequences were analyzed for the phosphoprotein phosphatase proteins (Spot N° 1, Accession N° EAL36686, XP_001388227), 43 sequences for the actin protein (Spot N° 2, Accession N° P26183), 14 sequences for the hypothetical protein Chro 70534 (Spot N° 3, Accession N° EAL37510), and 5 sequences for the chaperone protein (Spot N° 3, Accession N° EAL35079). The antigenic peptides selected as unique for Cryptosporidium after the alignment (Table 4.2) were indicated as N° 2, 6, 7, and 8 for the phosphoprotein phosphatase protein; N° 9, 11, and 14 for the actin protein; N° 5, 6, 10, 12, 13, 16, 19, 21, 23, 25 for the hypothetical protein Chro 70534; and all of the antigenic peptides identified for the chaperone protein. Best scores were obtained for antigenic peptides N° 2 and 8 for the phosphoprotein phosphatase (E-values < e-05), both sequences observed in C. hominis as well as C. parvum. Phosphoprotein phosphatase peptide N° 6 obtained E-values of 212 and 0.23 for the C. hominis and C. parvum sequences respectively. Peptide N° 9 for the actin protein obtained the best scores (E-values from 0.002 to 0.17) for Cryptosporidium matches among the three peptides selected; peptide N° 14 was identified in non-Cryptosporidium sequences. Best scores (E-value < e-04) were obtained for the antigenic peptides N° 5, 12, and 22 for the hypothetical protein Chro 70534 (Accession N° EAL37510); peptides N° 10, 21 and 25 were observed also in non-related Cryptosporidium sequences. Best scores were obtained for the antigenic peptides N° 1 and 7 (E-values < e-08) for the chaperone related-protein.

Phylogenetic trees for the selected antigenic proteins are indicated in Figure 4.2, 4.3, 4.4, and 4.5. The Cryptosporidium sequences for phosphoprotein phosphatase and the hypothetical
protein Chro 70534 formed a clade separated from sequences from other apicomplexans, and *C. parvum* and *C. hominis* were more closely related to each other compared to *C. muris*. The *Cryptosporidium* actin sequences were conserved among *Cryptosporidium* species; however, they do not necessarily cluster in one clade. Few protein sequences were available for the analysis of the *Cryptosporidium* chaperone related-protein.

**Discussion**

This study identified *Cryptosporidium* antigenic proteins recognized by human sera from persons infected with this parasite and experiencing clinical symptoms. These proteins were determined previously (Chapter 3), and sera reactivity was significantly increased for one of those antigens in infected individuals. Antigenic peptides predicted from these polypeptides which may be used in diagnosis, epidemiological investigations, or may be suitable for therapeutic or vaccine interventions were also suggested.

Antigenic *Cryptosporidium* proteins have been used in attempts to differentiate infected from non-infected individuals, and the results have not been consistent (Eisenberg et al., 2001; Tosini et al., 1999). *Cryptosporidium* proteins such as Cp15, GP900, or gp40 were suggested as participants in host-cell attachment (Barnes et al., 1998; Cevallos et al., 2000; Strong et al., 2000), and the host-immune response observed against these proteins as well as protection against infection when these antigens are blocked or neutralized by specific antibodies supported this observation. In this study, new *Cryptosporidium* antigenic proteins were identified, of which, one could be used to positively-diagnose *Cryptosporidium*-infected individuals.
The phosphoprotein phosphatase-related protein identified in this study contains domains which clearly indicate that this *Cryptosporidium* protein is a serine/threonine phosphatase from the PP5 subfamily. PPP phosphatases are used by apicomplexans for invasion, and protein phosphatases may also act as tyrosyl phosphatases *in vivo* by association with other molecules (Cayla et al., 1993; Kutuzov and Andreeva, 2008). PP2A phosphatases each consist of a catalytic subunit and a regulatory subunit, and a second regulatory subunit is observed in trimeric holoenzymes (Garcia et al., 1999). In *Theileria* and *Leishmania* the recruitment of host PP2A may impair the host-immune response (Kutuzov and Andreeva, 2008). In *Giardia* a PP2Ac was localized in the cytoskeleton and cyst wall. This protein participated in encystation as well as parasite excystation by regulation of motility, adhesion, cell division and dephosphorylation of cyst wall proteins during excystation (Lauwaet et al., 2007). The PP5 is characterized by an N-terminal tetratricopeptide (TPR) repeat domain, and it has been associated with a chaperone HSP90 in *Plasmodium* which may protect the parasite from toxic effects (Kutuzov and Andreeva, 2008). From the results of this study, active participation in host-cell invasion of the identified *Cryptosporidium* serine/threonine phosphatase may be implied considering the significant recognition of this protein by the host immune system during active infection. This is the first report confirming the existence of a *Cryptosporidium* serine/threonine phosphatase which is also involved in humoral immune response against the parasite. Protein sequence allowed the identification of two antigenic peptides (N° 2 and 8) which may be used to identify *C. parvum* and *C. hominis* by reaction with mono-specific or monoclonal antibodies designed against these peptides. The phylogenetic distance observed suggests that the protein as well as the antigenic peptides might be highly specific for this parasite, especially for *C. parvum* and *C.*
Further investigation is required to determine the capability of these antigenic peptides in detecting acute cases of cryptosporidiosis.

The *C. parvum* actin protein was initially suggested for therapeutic intervention since many cell mechanisms associated with parasite survival, such as gliding locomotion and host-cell invasion, involve molecules relevant for microorganism motility (Kim et al., 1992). The binding sites indicated for the *Cryptosporidium* actin identified in this work characterize actins. The Ca$^{2+}$-dependent gelsolin protein blocks growth of F-actin while profilin alters the conformation of G-actin to promote ATP/ADP exchange and therefore actin polymerization (Singh et al., 1996). Binding sites for these two actin-associated proteins were determined for the *Cryptosporidium* actin. Actin might participate in *Cryptosporidium* cytoskeleton remodeling which mediates the discharge of proteins from apical organelles required for host-cell invasion (Chen et al., 2004b). Other molecules, such as phosphatidylinositol 3-kinase (PI3K) and Cdc42, have been related to actin functionality in the cells (Chen et al., 2004a; Chen et al., 2004c; Singh et al., 1996). Activation by phosphorylation of PI3K at the cell-parasite interface during *Cryptosporidium* host-cell invasion and the final activation of Cdc42 invoked actin accumulation at the host-cell cytoplasm, membrane protrusion, dense-band, and parasitophorous vacuole formation while inhibition of PI3K reduced host-cell invasion (Chen et al., 2004a; Chen et al., 2004c). The PI3K has two subunits, the 85 kDa subunit which is tyrosine phosphorylated, and the 110 kDa subunit. The 110 kDa subunit has a Mn$^{2+}$-dependent protein kinase activity, which phosphorylates the 85 kDa subunit, and a Mg$^{2+}$-dependent lipid kinase activity which phosphorylates phosphatidylinositol. Profilin and profilin/actin bind to the p85 subunit, and profilin and gelsolin mediate phosphorylation of phosphatidylinositol by PI3K. Binding of phosphorylated phosphatyl inositol to profilin may cause the dissociation of profilin or gelsin
from actin and subsequent actin polymerization (Singh et al., 1996). All of these molecules have not been detected in Cryptosporidium yet, and the elucidation of the cell mechanisms involving actin or actin-related molecules might aid in the understanding of the parasite behavior during host-cell invasion and might open possibilities for interventions to block parasite infection. Other novel cytoplasmic proteins involved in cytoskeleton remodeling and motility have also been suggested for Cryptosporidium when sporozoite-antisera recognized antigenic peptides from a collection of recombinant peptides; however, their specific role in parasite biology is not clear either (Trasarti et al., 2007). One predicted antigenic peptide (N° 9) might be useful to identify Cryptosporidium spp.; however, due to the highly conserved sequence of Cryptosporidium actin, pathogenic as well as non-pathogenic species may be recognized in diagnostic tests with antibodies directed against this peptide. Because this protein was recognized by human sera from infected and non-infected individuals, screening tests for sera reactivity using this antigenic peptide may indicate exposure to Cryptosporidium; however, test specificity must be evaluated first, and other molecules would be required to identify exposure to Cryptosporidium pathogenic species.

A carbohydrate binding function or participation in metabolism was suggested for those proteins containing PA14 domain such as the hypothetical protein Chro 70534 identified in this study (Rigden et al., 2004). This protein was also identified by Sanderson et al under the name of CpFNPA, extracellular protein with a signal peptide, FN2 domain, and an anthrax toxin-N-terminus like domain of C. parvum IO II (Accession N° XP_628628.1 or cgd7_4810) (Sanderson et al., 2008). Glycosidases, glycosyltransferases, proteases, amidases, adhesines, bacterial toxins and other molecules shared this PA14 domain, and binding of this domain induces the activation of a toxic activity in bacteria such as Bacillus anthracis (Rigden et al., 2004). The FN2 or
fibronectin domain may participate in cell adhesion and maintenance of cytoskeleton in human cells (Dean et al., 1987). The hypothetical protein Chro 70534 may therefore participate in cell adhesion, and consequently the recognition by the host-immune system is highly probable and supported by the antibody reactivity observed in this study for Cryptosporidium-infected people. Antigenic peptides suggested for the hypothetical protein Chro 70534 (N°5, 12, and 22) might be also useful for the identification of C. parvum and C. hominis and characterization of the immune response against these species. This protein was identified along with the chaperone related-protein of C. hominis from the same excised spot in the two-dimensional polyacrylamide gel analyzed in the present study. The hscB (heat shock cognate protein B) region in this polypeptide has been proven to function as a co-chaperone with chaperone hscA in iron-sulphur proteins in humans, and may participate in protein folding (Bitto et al., 2008). Few protein sequences from related molecules in parasitic protozoa were found for the alignment analysis and construction of the phylogenetic tree. Based on the current available sequences, this antigenic polypeptide seems to be Cryptosporidium specific, especially for C. hominis and C. parvum, as observed in the phylogenetic analysis and from the high distance between Cryptosporidium and other genera. The information obtained from this study should be expanded by complementary work directed to clearly define the role of each of the identified antigenic proteins and to test the suitability of their antigenic peptides for diagnosis, therapeutics or as vaccine molecules.
References


Cevallos, A.M., Bhat, N., Verdon, R., Hamer, D.H., Stein, B., Tzipori, S., Pereira, M.E.,

Chen, X.M., Huang, B.Q., Splinter, P.L., Orth, J.D., Billadeau, D.D., McNiven, M.A.,

Paya, C.V., LaRusso, N.F., 2001, Cryptosporidium parvum activates nuclear factor kappaB in biliary epithelia preventing epithelial cell apoptosis. Gastroenterology 120, 1774-1783.

LaRusso, N.F., 2004b, Apical organelle discharge by Cryptosporidium parvum is temperature, cytoskeleton, and intracellular calcium dependent and required for host cell invasion. Infect Immun 72, 6806-6816.


Trasarti, E., Pizzi, E., Pozio, E., Tosini, F., 2007, The immunological selection of recombinant peptides from Cryptosporidium parvum reveals 14 proteins expressed at the sporozoite stage, 7 of which are conserved in other apicomplexa. Mol Biochem Parasitol 152, 159-169.


### Table 4.1: Identification of *Cryptosporidium parvum* proteins by Tandem Mass Spectrometry or MS / MS

<table>
<thead>
<tr>
<th>Protein N°</th>
<th>Accession Number</th>
<th>Description</th>
<th>MW kDa</th>
<th>pI</th>
<th>Peptides N°</th>
<th>% Coverage (Score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EAL36686</td>
<td>Phosphoprotein phosphatase-related. <em>Cryptosporidium hominis</em> Phosphoprotein phosphatase-related. <em>Cryptosporidium parvum</em></td>
<td>59.6</td>
<td>6.30</td>
<td>7</td>
<td>13.9</td>
</tr>
<tr>
<td>1</td>
<td>XP_001388227</td>
<td>Phosphoprotein phosphatase-related. <em>Cryptosporidium parvum</em></td>
<td>59.6</td>
<td>6.30</td>
<td>3</td>
<td>9.3</td>
</tr>
<tr>
<td>2</td>
<td>P26183</td>
<td>Actin. <em>Cryptosporidium parvum</em> Hypothetical protein</td>
<td>42.2</td>
<td>5.05</td>
<td>16</td>
<td>51.9</td>
</tr>
<tr>
<td>3</td>
<td>EAL37510</td>
<td>Chro 70534. <em>Cryptosporidium hominis</em> Chaperone protein-related. <em>Cryptosporidium hominis</em></td>
<td>87.0</td>
<td>6.97</td>
<td>5</td>
<td>6.2</td>
</tr>
<tr>
<td>3</td>
<td>EAL35079</td>
<td>Chaperone protein-related. <em>Cryptosporidium hominis</em></td>
<td>28.3</td>
<td>8.95</td>
<td>2</td>
<td>12.7</td>
</tr>
</tbody>
</table>

MW: molecular weight in kDa. pI: Isoelectric point
Table 4.2: Antigenic peptides identified by the method of Kolaskar and Tongaonkar from *Cryptosporidium parvum* proteins identified in this study.

<table>
<thead>
<tr>
<th>Accession N° and protein name</th>
<th>Antigenic Peptide N°</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAL36686</td>
<td>1</td>
<td>EAIEYTLAIKT</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td><strong>KNLHIYYSNRALCHIR</strong></td>
</tr>
<tr>
<td>Spot N° 1</td>
<td>3</td>
<td>ESIKCCPSFSKA</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>AYFNLLKYSLA</td>
</tr>
<tr>
<td>Phosphoprotein phosphatase-related.</td>
<td>5</td>
<td>QSKIQICTKLK</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>APGFAVSN</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>SGPHYKPLVDHSDK</td>
</tr>
<tr>
<td><em>Cryptosporidium hominis</em></td>
<td>8</td>
<td><strong>SREFVIPKVPDASLIRE</strong></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>FVTIELDF</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>HRKYAYMIVYDLIQVLKEASKPLVRI</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>EHTVCGD</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>HGQFFDLLNI</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>GLPSVNN</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>DRGSFSVEVILVLFTLKIMPYHVHLA</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>FEGEVVLKDYDSGLYDLISEAFCLPLAHVINDKVFVVFVHGGLCSE</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>DNVKLSDIEQLYSRCPE</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>FMSSSLWS</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>SPRGVGC</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>LDYIIRSHEVKQEGYVSDHGKDCITVFSAPNY</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>AFIKIEY</td>
</tr>
<tr>
<td>XP_001388227.1</td>
<td>6</td>
<td>DFSAPGFVVS</td>
</tr>
<tr>
<td>Spot N° 1</td>
<td></td>
<td>N</td>
</tr>
<tr>
<td>(C. parvum) *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P26183</td>
<td>1</td>
<td>TQALVVD</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>DAPRCVFPSTIVGR</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>QKDCYVG</td>
</tr>
<tr>
<td>Spot N° 2</td>
<td>4</td>
<td>GILTLKYPHEGI</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>NELRVAPEEHVLLT</td>
</tr>
<tr>
<td>Actin</td>
<td>6</td>
<td>TFNVPAMYVINQAVSLYA</td>
</tr>
<tr>
<td><em>Cryptosporidium parvum</em></td>
<td>7</td>
<td>TGIVLDS</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>DGVSTVPIYEYGAIPHAIRMLDLAG</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td><strong>LTDFLMKILHD</strong></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>KEKLCYIAL</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>LPDGHVITV</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>RFRCPEALFQPG</td>
</tr>
<tr>
<td>Accession N° and protein name</td>
<td>Antigenic Peptide N°</td>
<td>Sequence</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>----------------------</td>
<td>----------</td>
</tr>
<tr>
<td>P26183</td>
<td>13</td>
<td>SIMKCDL</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>LYANIVLS</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>ELTSLAP</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>MKIKVVAPP</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>RKYSVWIGGSILSSLST</td>
</tr>
<tr>
<td>EAL37510</td>
<td>1</td>
<td>AHANLQVLH</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CKYGPACNYRIVKEVVFE</td>
</tr>
<tr>
<td>Spot N° 3</td>
<td>3</td>
<td>KYSHLES</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>KFKGVFKGLSFILLAGN</td>
</tr>
<tr>
<td>Hypothetical protein Chro 70534</td>
<td>5</td>
<td>APTSQQLQEALAQYR</td>
</tr>
<tr>
<td>Cryptosporidium hominis</td>
<td>6</td>
<td>VDGRILCSAVFVQD</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>TYTDCTI</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>REWCVYVEPVQAS</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>WNYCLPVTNYY</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>RLTDLLE</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>NSTCGSQQSIVSS</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>NIESLIQKGQHCLN</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>SITKVDVLK</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>ENCELLPGYED</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>GRYYFSIEADCARFLFLGGRAIIVDR</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>SERAVPLLL</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>HDGPVKVTSVGAELVG</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>YRIRVFHSSHY</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>IQEQVIPSSY</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>QPLKLSGVPVFYEI</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>FIADLPAG</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>HNMIKVSVPNQPCTM</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>IAAVTDKLVPVT</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>TMKVAQVTI</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>TDISLKV</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>KPFILFFK</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>CSDKLLETDAVESVKKSS</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>IIIKFKQPVETDFQYIPL</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>WPASLTMYIKQ</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>EKFTLAHTASLNAHKYMLQYPYPLTAKEVKIEVSQMFVD</td>
</tr>
</tbody>
</table>
Table 4.2 (cont): Antigenic peptides identified by the method of Kolaskar and Tongaonkar from *Cryptosporidium parvum* proteins identified in this study

<table>
<thead>
<tr>
<th>Accession N° and protein name</th>
<th>Antigenic Peptide N°</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAL35079 Spot N° 3 Chaperone</td>
<td>1</td>
<td>NEKLLVSRILISKLAPNYKILVSCFKQFPSSYLLI</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>NKKSVDLSY</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>ISIHIKQ</td>
</tr>
<tr>
<td>Chaperone protein- related.</td>
<td>4</td>
<td>FERALLLISL</td>
</tr>
<tr>
<td><em>Cryptosporidium hominis</em></td>
<td>5</td>
<td>DTIPVSSSLLEQ</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>KINHCIK</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>SITKILSILKELRIYQKLSD</td>
</tr>
</tbody>
</table>

* 20 antigenic peptides were identified for this protein; however, all of them but the N° 6 had the same sequence as those for the protein identified as EAL36686.

Highlighted sequences indicate suggested immunogenic peptides for *Cryptosporidium* identification.
Figure 4.1: *Cryptosporidium parvum* proteins selected for identification by Mass Spectrometry. Proteins were resolved in 12 % polyacrylamide gel in two-dimensional gel electrophoresis and stained with Coomassie Blue (Total protein concentration = 14 µg). Spot N°1: 43.4 kDa protein (Isoelectric point-pI = 5.4). Spot N°2: 58.3 kDa protein (pI = 5.6). Spot N°3: 57.5 kDa protein (pI = 6.4).
Figure 4.2: Neighbor joining phylogenetic tree for the *Cryptosporidium* phosphoprotein phosphatase-related protein. Maximum sequence difference = 0.85.
Figure 4.3: Neighbor joining phylogenetic tree for the *Cryptosporidium* actin protein. Maximum sequence difference = 0.85.
Figure 4.4: Neighbor joining phylogenetic tree for the Cryptosporidium hypothetical protein Chro 70534. Maximum sequence difference = 0.85.
Figure 4.5: Neighbor joining phylogenetic tree for the *Cryptosporidium* chaperone protein-related. Maximum sequence difference = 0.85
CHAPTER 5

CONCLUSIONS

The comparison of protein expression profiles of Cryptosporidium parvum Iowa (IO), C. parvum Arizona (AZ) and C. parvum Moredum (MD) by two-dimensional difference gel electrophoresis (2D-DIGE) demonstrated that these isolates are not only different at the genomic level, as it was reported previously, but also that they are different in their protein repertoire. These results suggest that metabolic mechanisms and/or structural parasite architecture involving those isolate-specific proteins might also differ among isolates.

The protein concentration required for these experiments could be reduced at least 100-fold times with successful results. This is a significant achievement, taking into account the difficulties encountered to procure Cryptosporidium specimens especially for proteomic studies concerning closely-related organisms.

Even though, anti-Cryptosporidium human sera antibodies were detected in infected as well as non-infected individuals, it was possible to determine increased reactivity for six antigenic proteins in persons suffering from clinical manifestations related to cryptosporidiosis and with the parasite detectable in their stools. These findings are relevant considering that Cryptosporidium is widely-spread in nature, and exposure is very common, and information about specific immune response under natural exposure to pathogenic strains is at present very limited.

C. parvum antigenic proteins of 15-17, 20 and 23 kDa, previously suggested as indicators of early infection in animals or humans, were not significantly recognized by human sera from
infected individuals when compared with the non-infected group in this study. Current epidemiological, diagnostic or research methods may therefore now include the six antigenic proteins identified in this study for evaluation in the future.

Directed-immune response and protection against infection have also been reported for Cryptosporidium proteins such as Cp15, GP900 or gp40 and their neutralizing synthetic or natural produced antibodies, respectively. This work expanded the information concerning Cryptosporidium antigenic proteins likely-involved in host-parasite interaction by the successful identification of a Cryptosporidium serine/threonine phosphatase significantly recognized by human sera from infected individuals. This is the first report confirming the existence of a Cryptosporidium serine/threonine phosphatase which was predicted from the genome sequence of C. parvum and C. hominis and for which there was not any previous information regarding its existence or relevance to parasite biology.

Antigenic peptides predicted from the Cryptosporidium antigenic proteins, especially for the Cryptosporidium serine/threonine phosphatase should be henceforth evaluated for diagnostic or epidemiological tests. Some antigenic peptides were suitable to distinguish C. parvum and C. hominis from other apicomplexans. The C. parvum actin protein, the hypothetical protein Chro70534, and the chaperone related-protein were also recognized by antibodies in human sera, therefore complementary work should be directed to clearly define their role in parasite-host cell interaction. Finally, those antigenic proteins identified in this work (by molecular weight, isoelectric point and mass spectrometry) were also observed in the protein profile of C. parvum Arizona isolate evaluated by two-dimensional difference gel electrophoresis. Therefore, antigenic peptides could perhaps recognize not only the Iowa strain of C. parvum but others such
the Arizona isolate; however, further investigation is required to confirm similarities among these proteins.