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EVALUATION OF PASSIVE IMMUNE THERAPIES FOR THE ORAL TREATMENT OF CRYPTOSPORIDIOSIS

by

Vitaliano Antonio Cama-Lee

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Vitaliano A. Cama-Lee

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DEDICATION

To my wife Ynes Ortega and sons Vitaliano Roberto and Victor Antonio.

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With all my love,

Vita

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ABSTRACT

Cryptosporidium parvum is a ubiquitous parasite affecting a wide range of mammals. In immune competent hosts it causes profuse self-resolving diarrhea. In those with impaired immunity, diarrhea persists while the deficiency prevails because there are no effective therapies available. Previous studies reported the benefits of polyclonal antibodies for treating cryptosporidiosis. Data from preliminary studies supported the anti-parasitic efficacy of bovine and hen egg yolk polyclonal antibody preparations. Preliminary *in vivo* testing showed significant reductions in the parasite loads ($p<0.05$) of animals treated with yolk antibodies when compared to controls. Preparations of anti-*C. parvum* polyclonal antibodies from human breast milk, bovine colostrum and chicken egg yolks were obtained and tested *in vitro* for activity and potency and subsequently tested for efficacy in comparative *in vivo* studies. Hen egg yolk preparations were significantly more efficacious ($p<0.01$) and subsequently evaluated in human clinical trials. Analysis of data from the trial showed beneficial therapeutic value and also the need for improvements in the egg yolk formulations. Further work directed towards antibody concentration and lipid reductions were performed. The dilution of egg yolks with water precipitated the lipids and the lipid-reduced supernatant fluids were lyophilized resulting in an enhanced formulation with increased anti-cryptosporidial potency and about 90% reduction in the lipid contents. The efficacy of the enhanced preparations was also tested *in vivo* with parasite reductions in the order of 90%. Hen egg yolk antibodies could be a valuable component for treating or controlling enteric cryptosporidiosis.

CHAPTER 1: LITERATURE REVIEW, PROBLEM DEFINITION, AND DISSERTATION FORMAT

In this dissertation, work is presented towards the evaluation of three polyclonal antibody preparations for their therapeutic potential in treating enteric *Cryptosporidium parvum* infections. The three preparations tested were hyperimmune bovine colostrum, hyperimmune chicken egg yolks and pools of human breast milk samples collected in an endemic area.

I. Literature Review

A. General Information

Cryptosporidium parvum is an intracellular, extracytoplasmic, enteric-dwelling coccidian parasite that inhabits the brush-borders of the intestinal epithelium (enterocytes). Once thought to be only an animal pathogen affecting several species including calves, piglets, foals and lambs, it is now known to be an important cause of enterocolitis and diarrhea in humans.

B. *Cryptosporidium parvum* Enteric Disease

Cryptosporidiosis is an enteric disease affecting humans and many other mammals. Two distinct genotypes are reported: genotype 1 infecting only humans and genotype 2 infecting humans and other mammals (Peng et al, 1997, Sulaiman et al, 1998). In

immunocompetent hosts the disease is self-limiting and resolution is accompanied by cellular immune responses and antibody production to the various life-cycle stages of the organism. In immunodeficient humans, however, the disease can be considered life-threatening.

Healthy adults and animals usually have a short-term illness accompanied by watery diarrhea, abdominal cramps, malabsorption and weight loss. The very young are highly susceptible and may experience severe diarrheal illness. In adult humans, protracted diarrheal disease caused by *Cryptosporidium* has been reported in AIDS patients, individuals undergoing immunosuppressive treatment and hypogammaglobulinemic patients. Infection in such patients may escalate in severity with frequent and voluminous daily bowel movements accompanied by weight loss with stool oocyst shedding persisting for months or until the patient dies.

The patterns of incidence and pathways of spread for cryptosporidiosis are poorly understood and the symptoms of cryptosporidiosis have been observed to fluctuate in severity, with the immunocompromised being the most affected. Early reports from HIV-1 infected individuals noted that some patients appear to have an unrelenting diarrheal illness where symptoms do not go into remission, while others may have a short episode of diarrhea and then become asymptomatic (McGowan et al, 1993). This variation in symptoms may actually reflect the degree of immunosuppression in patients. In a retrospective clinical chart review, it was found that all patients with $CD4^+$ counts $\geq 180/mm^3$ (N = 8) had self-limited *Cryptosporidium* infections, which resolved

spontaneously within 4 weeks. Of 39 patients with $CD4^+$ counts <180/mm^3, five (13%) had self-limited disease and 34 (87%) had persistent disease (Flanigan et al, 1992).

C. Prevalence of the Disease

Before the use of antiretroviral therapies in developed countries, the reported prevalence of cryptosporidiosis in AIDS patients within the United States was close to 3% (Selik et al, 1987). *Cryptosporidium* prevalence rates of 15% and 16% have been recorded in U.S. studies aimed at evaluating AIDS patients with diarrhea (Laughon et al, 1988; Smith et al, 1988). Similar prevalence rates were recorded in Britain (Connolly et al, 1988). With the advent of combination anti-retroviral therapies, the current prevalence rates in immune-compromised populations are significantly lower than those previously reported (Clark, 1999). Human clinical trials to evaluate the potential efficacy of anti-cryptosporidial drugs in developed countries have practically disappeared due to the lack of enough AIDS patients suffering from cryptosporidiosis. This decrease in the prevalence of the disease within the AIDS population is not due to effective therapies against the parasite but to the improvements in the health status of the patients and increased CD4+ counts (Bobin et al, 1998).

In developing countries prevalence rates may frequently approach 40% in AIDS patients (Crawford et al, 1988) and 30% in children under 5 years living in endemic areas (Sterling et al, 1991). As the AIDS population grows and additional data are gathered,

particularly from late-stage HIV-infected persons, the prevalence of cryptosporidiosis is expected to increase in these countries.

D. Life Cycle of *Cryptosporidium parvum*

Briefly, infection begins following ingestion of the infectious oocyst which contains four naked sporozoites. Oocysts excyst within the intestine under the influence of host enzymes and release sporozoites. Each sporozoite is capable of infecting an intestinal epithelial cell, thereby beginning the asexual cycle of development. Sporozoites attach to the surface of epithelial cells and penetrate into the sub-membranous zone where they differentiate into trophozoites within a host cell-derived parasitophorous vacuole. This vacuole is in contact with the enterocyte cytoplasma via a membrane fold known as the feeding organelle. Even though the specific role of the feeding organelle is not clearly defined, this structure may be the key in the selective transport of nutrients to the parasite as well as limiting or excluding the transport of antiparasitic drugs, now proven ineffective in controlling *Cryptosporidium.*

Trophozoites reproduce asexually by merogony, producing eight Type I merozoites which when released from infected cells may initiate another cycle of merogony resulting in four Type II infectious merozoites. In immunocompromised hosts, the asexual cycle may be persistent. Alternatively, merozoites from Type II meronts may initiate the sexual cycle of development. Microgametocytes produce 12 to 16 microgametes which, on release from the host cells, seek out and fertilize macrogametocytes. The resulting zygote differentiates into an oocyst which is released

into the intestinal lumen. Fully sporulated thick-walled oocysts in varying stages of sporulation pass out of the host in feces. Thin-walled oocysts, which constitute a minority of oocysts produced oocysts, may spontaneously rupture in the host, ensuring persistent infection in immunocompromised hosts (Fayer et al, 1997).

E. Host parasite interaction: humoral and cellular responses

Several aspect of the host's immune response have been studied since the emergence of life threatening cryptosporidial infections in AIDS patients. Most of the information gathered on humoral responses described antibody reactivity against the parasite and compared antibody responses between different groups such as healthy and immunocompromised individuals, as well as infected versus non-infected animals. Since 1991 (Ungar et al, 1991) the knowledge of the role of antibodies in the infected host has evolved. The evolution in the conceptual role of antibodies is based on data generated from several studies using improved animal models in addition to recently available molecular techniques. Now, antibodies are no longer considered the key elements in disease clearance but a source of information in host-parasite interactions as well as valuable epidemiological markers.

Cellular immunity, now attributed with a significant role in the clearance of the disease, has been studied mainly in murine animal systems. There are reports of specific elements of the cellular response in relation to resolution of the disease, which have

increased the current understanding of the host parasite interaction and clearance of the disease.

E.1 Humoral responses: Role in disease eradication and serologic detection

Knowledge of the role of antibodies in the host-parasite interaction has evolved since the first serological studies. Serum antibodies to *Cryptosporidium* have been detected in both immunocompetent and immunocompromised individuals and in several animal species (Riggs 1997). Reports from several random serological studies in humans and animals have suggested that infection with this organism is common some time during life. Antibody responses in immunocompetent individuals show typical patterns of IgM and IgG production following infection. The latter may diminish within a few months or persist for a year or more. Elevated IgA and IgE responses also have been noted (Ungar et al, 1988).

Several epidemiological and clinical studies have demonstrated the presence of antibodies in serum from patients or animals exposed to the parasite. Data from sero-epidemiological studies aimed at evaluating antibody responses in endemic populations reported detectable titers for at least 2 years post documented clinical disease and Western Blot recognition of bands with molecular weights of 15-17 and 23 Kda (Reperant et al, 1994). For epidemiological purposes, antibodies against these antigens became valuable diagnostic tools. In further evaluations of the antibody responses, sera from convalescent patients were tested with a reported 88% Western Blot (WB) reactivity

for antigens with molecular weights of 6, 14 and 17KDa, suggesting the use of these antigens as indicators of parasite infection or exposure, but not active clinical disease (Frost et al, 1998).

Animal studies in mice designed to determine the role of humoral immunity demonstrated that there was no correlation between circulating IgM and IgG antibody titers and the severity of the disease, and B-cell deficient neonatal Balb/c mice did not differ on the time to onset, peak or duration of cryptosporidiosis when compared to immunocompetent Balb/c mice (Taghi-Kilani et al, 1990). Studies in ruminants, aimed to correlate antibody responses and parasite excretion, reported rises in sIgM and sIgA in association with declines in oocyst output, suggesting that antibodies may act at the luminal level by preventing the penetration of enterocytes by zoite stages. The authors of these studies conclude that the role of passive immunity in ruminants deserved further examination (Hill, 1990).

Studies performed in humans also evaluated the natural passive transfer of antibodies. Cryptosporidial infections have been demonstrated to be less prevalent in exclusively breast-fed infants than among non-breast fed infants, suggesting a possible protective role via the mother's milk. However, it was later demonstrated that the mechanism for reduced disease was not related to antibodies present in breast milk (Sterling et al, 1991), and that exclusively breast-fed children are healthier because of reduced exposure to pathogens and not to immune components present in breast milk.

Healthy adult human volunteers previously infected with 132 *C. parvum* oocysts were tested for IgG reactivity prior to and after a second infection, with doses ranging between 500 and 50,000 oocysts. The infectious dose 50 increased from 132 to 1880 oocysts, indicating that previous exposure conferred some degree of immunity against new challenges. Clinical disease was reported only in the volunteers receiving the higher infective doses. The analysis of the serological titers demonstrated that individuals with the higher antibody titers did not show increases in their values while those volunteers with low IgG responses had significant increments in their titers. Overall it was postulated that previous exposure with a small inoculum could confer some degree of protection from clinical disease (Chappell et al, 1999; Moss et al, 1998; Okhuysen et al, 1998b). The analysis of fecal IgA from these volunteers showed significantly higher anti-*Cryptosporidium* IgA titers in those with clinical symptomatology, with no IgG or IgM being detected in any of the samples, associating IgA reactivities with recent infections. (Dann et al, 2000).

These findings suggested that mucosal and systemic antibody responses played a role as markers of exposure but not disease eradication, and the need to determine the role of cellular immune responses.

E.2 Humoral responses: Role in identification of neutralizing antigens

Information obtained from serologic studies has aided in the identification of various *Cryptosporidium* protein fractions. The reported 14 and 17 KDa antigens are considered to be the same as the previously reported antigens of 15-17 KDa, suggesting their role as infection markers (McLauchlin et al, 1999). These antigens have the potential to identify exposure, in many times asymptomatic. The reactivity of serum samples from infected humans and experimentally infected animals showed Western Blot recognition of the 15-17 KDa antigens and gave clear indications that specific epitopes from those protein bands could be used as potential targets for immune therapies (Reperant. 1994). Another antigen frequently recognized by sera from most infected animals and humans is the 20-23 KDa protein (Ungar et al, 1988; Mead et al, 1988).

There are numerous additional reports on sporozoite and merozoite low molecular weight surface antigens, with different but very closely reported mobilities (Riggs et al, 1989; Tilley et al, 1991; 1991a; 1993; Whitmire and Harp, 1991; Lumb et al, 1989; Mead et al, 1989). These reported antigens were analyzed by a defined panel of monoclonal antibodies, also submitted to the CDC to be used as reference reagents. The conclusions from these evaluations were that the described antigens corresponded to two major surface antigens with estimated molecular weights of 17-18 KDa and of 27-28 KDa. The variations in reported sizes were attributed to the different electrophoretic conditions in which these antigens were separated, and reducing versus non-reducing conditions were also taken into account (Tilley and Upton, 1997). These antigens have been extensively

characterized and the 17-18 KDa antigen is now identified as the glycoprotein GP 15, and the 27-28 KDa antigen as P23. Both of these antigens are present on the surface of sporozoites and merozoites and are potential targets for immuno-neutralization of the parasite (Tilley et al, 1991).

Studies aimed at evaluating the therapeutic efficacy and characterization of several antibody preparations against *C. parvum* reported neutralization of the parasite when antibodies reacted with two surface antigens conserved in sporozoites and merozoites. The neutralization sensitive epitopes were located within GP15 and P23 (Tilley et al, 1991; Riggs et al, 1994). Evaluations using hyperimmune bovine colostrum described two additional neutralization-sensitive fractions not previously reported. One of those antigens is now identified as CSL (Riggs et al, 1997) and the other is a glycoprotein fraction with molecular weight greater than 900 KDa (Petersen et al, 1992).

The data gathered supports the notion that in addition to their diagnostic value, antibodies have allowed the identification of specific antigens associated with parasite neutralization which could be used as targets for passive immune therapies.

E.3 Cellular immune responses

Cellular responses have been studied in several animal models, mainly murine, and also in humans. Early reports on the immunology of the disease suggested a major role of the antibody responses, specifically sIgA in the resolution of *C. parvum* infections (Flanigan et al, 1994). Data generated with more refined animal models supported the

notion of a major role of cellular elements of the immune response in the infected host (McDonald and Bancroft, 1994), with reports describing that interferon gamma (IFN-γ) and $CD4^+$ cells are required for the host's control of the disease (Ungar et al, 1991). It has also been postulated that CD8+ cells probably have a significant role, while the presence or absence of tumor necrosis factor (TNF) had no impact on the outcome of infection. Cytokines from both Th1 and Th2 origin have been described to have a role in determining the outcome of parasite excretion and diarrheal disease. The CD40 receptor, important in the antibody class switch, has a reported role in the hosts defense mechanisms against *C. parvum*. Lack of CD40 receptors on the surface of enterocytes disrupts the CD40-CD8 interaction associated with induction of apoptosis and subsequent clearance of the parasitised cells (Cosyns et al, 1998).

E.3.a. Role of CD4+ cells

The studies on the effects of CD4+ counts started as a result of the observed lower counts in AIDS patients and the chronic life threatening presentation of cryptosporidial disease. Studies in experimentally infected bovine neonates showed significant increments in the populations of CD4+ and CD8+ intraepithelial lymphocytes (IEL) at the infection site, as wells as increased production of IFN-γ and interleukin 12 (IL-12) (Fayer et al, 1998; Wyatt et al, 1997; Theodos et al, 1997). The authors presented evidence of the changes in the cell populations in infected calves when compared to controls, and also reported no production of TNF in the infected animals. These findings

are similar to those described in *C. muris*, a species closely related to *C. parvum*, where it has been demonstrated that the presence of protective $CD4^+$ intraepithelial lymphocytes has a very strong correlation with the ability of the host to control enteric infection (McDonald et al, 1996).

In humans, a retrospective evaluation of medical records demonstrated a strong association between the CD4+ counts in AIDS patients and outcomes of infection. Individuals with CD4+ greater than 180 were significantly more likely to self resolve the disease than patients with lower CD4+ counts, where the disease was chronic and life threatening, usually leading to wasting syndrome or death (Flanigan et al, 1992). These findings have been corroborated with the use of highly active anti-retroviral therapies, also known as HAART, where combinations of anti-retroviral compounds are administered to AIDS patients resulting in significant elevations of CD4+. A study evaluating HAART and its impact on cryptosporidiosis demonstrated average elevations of CD4+ counts to $197/mm^3$, resulting in cessation of diarrhea and absence of parasite excretion in feces (Bobin et al, 1998). The reason for this effect may involve partial restoration of immune function due to inhibition of HIV replication, allowing incremental increases of CD4+ counts. It was concluded that CD4+ cells have a very important role in controlling *C. parvum* infections.

E.3.b. Cytokines

It is now generally accepted that IFN-γ has a very important role in the resolution of *C. parvum* infections (Ungar et al, 1991). Data from several studies have demonstrated that the presence or absence of IFN-γ drastically impacts the severity and length of the parasitism (Hayward et al, 2000; Kapel et al, 1996). Additional information obtained from several calf studies indicate that IFN-γ mediates the initial resistance to infection by an unidentified mechanism and has a limited role in mediating the final clearance of the disease (Fayer et al, 1998; Wyatt et al, 1997; Theodos et al, 1997) . This cytokine was described to be produced in intra epithelial lymphocytes (Culshaw et al, 1997). Studies in C57BL/6 mice have demonstrated that the onset of symptoms and severity of diarrhea were significantly more severe in mice with a targeted genetic disruption in the IFN-γ gene (GKO mice) than in regular C57BL/6 mice. In the GKO mice there was an overwhelming parasite infection resulting in high oocyst shedding, severe mucosal damage and death within 3 weeks. The control mice were induced to develop infection by a single injection of anti IFN-γ; these mice exhibited low parasite excretion and the disease was subsequently cleared within 30 days (Theodos et al, 1997).

There is limited but supporting information documenting the role of IFN-γ in human cryptosporidiosis. In studies performed with peripheral blood mononuclear lymphocytes (PBMC) from humans, both inmmunocompetent and immunosupressed, it was observed that stimulation of PBMC by *C .parvum* antigens and mitogens resulted in

production of IFN-γ, and it was concluded that resolution of infection was mainly associated with a Th1 response (Gomez Morales et al, 1996; 1999).

There is a case report of an HIV negative pediatric patient affected by severe chronic cryptosporidiosis. This patient did not present with any evident immunodefficiency. To elucidate if cytokine expression was associated with this severe case of cryptosporidiosis, samples of peripheral blood mononuclear cells were collected and exposed to a crude antigenic extract from *C. parvum* oocysts. The stimulated cells secreted interleukin 10 but not IFN-γ, while samples from a control patient had strong response to IFN-γ, suggesting that the absence of IFN-γ was responsible for the severity and chronicity of the disease (Gomez Morales et al, 1996).

Jejunal biopsies from human volunteers experimentally infected with *C. parvum* were examined by *in situ* hybridization for the expression of IFN-γ, indicating that there was an association between expression of this cytokine with prior sensitization and absence of parasite excretion (White et al, 2000). These results indicate a difference between the results from mouse and human studies which could be attributed to immunological differences between mouse models and healthy human adults. This reported pre-sensitization required for production of IFN-γ in humans may aid in understanding the difference in susceptibility between populations in endemic areas (mainly children under 5 years of age, Sterling et al, 1991) compared to populations with little to no previous exposure, where epidemic outbreaks affecting people of all ages have been documented (Anonymous, 1998; Frost et al, 2000).

Several interleukins have been reported to have roles in the immune process associated with enteric cryptosporidiosis and efforts are still underway to better define their roles in mediating resistance or eradication of the disease. In the case of IL-4, some reports indicated no significant levels of secreted IL-4 associated with infection when lymphocytes from mesenteric lymph nodes from healing mice were proliferated *in vitro*. In this report, the only cytokine detected in significant levels was IFN-γ (Theodos et al, 1997). In a different report the evaluation of gut-associated lymphoid tissue from infected mice provided strong evidence that IL-4 was important for the final clearance of *C. parvum* infections in adult mice (Aguirre et al, 1998). The potential discrepancy of these results could be explained by examining the study designs. In the first study, IL-4 was measured as a secreted product from cultured mesenteric lymph nodes from healing mice. In the second study, the basis for interpretation is in the depletion of IL-4 by the use of anti-IL4 monoclonal antibodies in adult infected mice. These cytokine depletion experiments have shown that IL-4 and IL-5 together were needed for decreases in parasite excretion, suggesting that IL-4 alone does not affect parasite shedding, but when complemented by IL-5, IL-4 appears to be an important factor in parasite control (Enriquez and Sterling 1993). Recently, adult IFN-γ knockout mice were used to gather information on the cytokine expression and specific lymphocyte proliferation associated with infection, providing support to the role of Th2 cytokines, specifically IL-5 in resolution of the infection (Smith et al, 2000).

The information available on the role of cellular immunity in *C. parvum* leads to the conclusion that no single element from the cellular response is solely responsible for resolution of the infection. There is consensus that CD4+ cells are required for clearance of the infection, that CD8+ and IFN-γ play very important roles, and that other cytokines including IL-4 and IL-5 also have relevant roles. It has to be noted that some of the reports with apparent conflictive information may actually complement each other (Urban et al, 1996). The source of the cells tested, study design, detection methodologies and the animal models used, besides other factors, must be placed into consideration when analysing the literature in this area.

II. Problem Definition

Enteric cryptosporidiosis causes diarrhea in immune competent individuals worldwide and life threatening diarrhea in AIDS patients who have no access to anti-retroviral therapies, mainly those in the developing world. Despite continued efforts from multiple research institutions and pharmaceutical companies, no product has been proven effective nor granted an indication for the treatment of enteric cryptosporidiosis in humans or animals. Overall, more than 100 antiparasitic, antimicrobial drugs and other small molecule compounds have been tested in humans and animal models, none with success (Blagburn and Soave, 1997).

The research efforts were initially slowed by the lack of *in vitro* propagation of the parasite, limiting the availability of infective stages for all aspects of biomedical research. The new *in vitro* methods available are generating data to better understand the microbial mechanisms of pathogenesis, chronicity of the disease in AIDS patients and screening of new drug candidates.

Anecdotical and pre-clinical data from studies using passive immune transfer suggest it's potential role as a viable alternative to traditional anti-parasitic drugs (Ungar et al 1990b; Tzipori et al, 1986; Nord et al, 1990). Passive immune therapies may mimic or replenish some immune system components that may aid the host in controlling the disease. When administered orally, these therapies are very safe and toxicity to the patients has been minimal to non-existent.

This dissertation will address the role of three anti-*C. parvum* polyclonal preparations in treating enteric cryptosporidiosis. It will generate information covering pre-clinical evaluations, product selection and will present and discuss data from the clinical evaluation of one of the products. The focus of this work is one of research and development with the potential for use by investigators to further the understanding of immune therapies for treating cryptosporidiosis.

A. Propagation of *Cryptosporidium parvum*

To date, one of the limitations for studying *C. parvum* has been the lack of an *in vitro* method for the propagation and development of the parasite throughout it's entire life cycle. Despite this, there have been drastic improvements in the cell culture techniques for the *in vitro* study of *C. parvum*. Cell culture methods expressing most intracellular life cycle stages have been accomplished (Upton, 1997). Over the past five years important information regarding parasite biology, and attachment and invasion of cells has been generated. One of the most recent and interesting findings was the report that Gal/GalNAc epitopes of glycoproteins on the epithelial apical membrane and Gal/GalNAc-specific sporozoite surface lectins are involved in the mechanism(s) of *C. parvum* attachment to intestinal and biliary epithelial cells, and that actin remodeling in host cells is required for *C. parvum* invasion (Chen et al, 2000). The *in vitro* systems available have also provided a mechanism for the preliminary screening of many candidate drugs for treating *C. parvum* infections (Woods and Upton, 1998). The *in vitro*

research may be partially limited by the availability of oocysts purified from other infected animals, but it has provided valuable information to better understand the pathobiology and biochemistry of the cell-parasite interaction.

Current procedures for the propagation of the complete life cycle of the parasite are limited to the infection of susceptible hosts, followed by the collection and purification of parasite infected feces. Two animal models are more frequently reported in the literature, the mouse model and the neonatal bovine model. In both models, animals are infected very early in life, develop infection and clinical symptoms and the infected feces are collected for parasite purification. The most common method for parasite purification is by means of centrifugation. Fecal material is centrifuged using solutions of specific gravities, and the partially purified oocysts are fully purified by separation using gradient solutions in high speed centrifuges. The oocysts are suspended and kept at 4°C until used (Arrowood and Donaldson, 1996). Preservation agents, such as potassium dichromate or Hanks balanced salt solution plus a mixture of antibiotics and antifungals are used to maintain sterility of the oocyst suspension (Arrowood and Sterling, 1987; Leek et al, 1979).

B. Evaluation of anti-cryptosporidial compounds: *In vitro* and *in vivo* testing

There is no defined standard methodology for the screening and testing of anti-cryptosporidial candidate compounds. The evaluation of a high proportion of candidate anticryptosporidial compounds has been performed almost exclusively in multiple animal

systems. When *in vitro* data is available, it is regarded as a very useful preliminary indication that should be followed by *in vivo* testing. *In vivo* models for cryptosporidiosis include the use of mouse models and occasionally, neonatal calves or piglets. There are several variations within the mouse model: the neonatal model, the adult immune-suppressed model and the immune-deficient adult model. Most mouse models have proven very useful, and despite the differences between them, they all provide basic pre-clinical data on the efficacy and safety of the candidate compounds.

The neonatal models are based on the susceptibility of neonates to develop enteric cryptosporidiosis with lesions that resemble the human pathology. Studies on the susceptibility dynamics demonstrated that Balb/c mice were one of the best strains for testing drugs with mice developing infection up to 14 days of age (Novak and Sterling, 1991). Other neonatal models are the ICR and CD-1 outbred mice (Blagburn et al, 1998a). The infection dynamics of the ICR Swiss mice demonstrated that age and weight were variables affecting the oocyst shedding and to minimize the impact of weight dependent variability of infection, ICR neonates should be infected on days 8-9 of age (Woods and Upton, 1998). Neonatal CD-1 mice were 100% susceptible to *C. parvum* infection when orally inoculated with at least 310 oocysts and are described as a suitable model for water disinfection studies (Finch et al, 1993). One of the advantages of neonatal models is the potential to test compounds in a large number of animals within a short period of time. A limitation is the time of susceptibility, which resembles acute cryptosporidiosis (Tarazona et al, 1998). Overall, neonatal mouse models can be

considered as the animal systems of choice for screening compounds that are active only in the lumen of the intestinal tract.

For compounds that work systemically as opposed to agents that are only active in the lumen in the gastrointestinal tract, animal models which develop chronic cryptosporidiosis are recommended (Mead et al, 1994). There are several chronic rodent models described in the literature (Ungar et al, 1990a; Mead et al, 1991, Tzipori et al 1995; Griffiths et al, 1998, Rasmussen et al, 1991; 1993). The two most commonly used are the severe combined immune deficient (SCID) and the immunosupressed C57BL adult mice. These models develop persistent diarrhea and extra intestinal infections. These model have the advantages of allowing long-term testing of compounds, evaluation of efficacy for hepatic cryptosporidiosis and chronic pathology. The disadvantages are mainly the logistical constraints of performing large scale testing of compounds, variability of oocyst shedding between mice, mortality associated with extraintestinal cryptosporidiosis, costs associated with the models and the time required for the mice to develop infections (at least two weeks).

Other models infrequently used include large mammals. The use of neonatal calves for the testing of anti-cryptosporidial compounds has been reported and preferably used when the primary indication for such candidate is bovine cryptosporidiosis (Fayer et al 1989; Perryman et al 1999, Sagodira et al, 1999b). Otherwise, this model is not commonly used as the preferred source of pre-clinical human or animal data.

C. Current Therapies

C.1.Chemotherapeutic compounds

Investigations into the treatment of cryptosporidiosis using a variety of chemotherapeutic agents have failed to demonstrate reliable preventative or treatment efficacy (Blagburn and Soave, 1997). The recommendations for treating acute cryptosporidial disease are limited to oral rehydration. Current recommendations for treating chronic *C. parvum* diarrhea vary, depending on the access patients may have to protease inhibitors. In the developing world, where access to these new antiretroviral therapies is limited to non existent, the recommended therapies include oral rehydration plus the administration of anti-diarrheal agents. If the patient deteriorates, therapy with paromomycin is recommended, at a dose of 500mg every six hours for 2 to 3 weeks, followed by maintenance therapy with 500mg twice daily (Hoepelman, 1996). Another compound with anecdotical success in the treatment of chronic diarrhea is nitazoxanide (NTZ) (Theodos et al, 1998). This compound was reported to show moderate success in a phase II clinical trial study conducted in the USA, however the FDA did not approve the indication of NTZ for treatment of *C. parvum* infections and requested additional studies for safety and efficacy. Despite not having regulatory approval, NTZ is available through many US AIDS patient networks (Sterling, 2000).

Since 1997, and specifically in developed countries, there has been a significant reduction in the number of AIDS patients affected by *Cryptosporidium*. This drastic

decline in the incidence of chronic disease in immune compromised patients is attributed to the use of anti retroviral compounds (Le et al, 1998). These drugs have no antiparasitic activities, and the beneficial effects are concomitant with the increases in CD4+ counts. The course of the disease is shortened and parasite excretion is reduced or eliminated (Maggi et al, 2000). A detailed clinical evaluations in a cohort of AIDS patients affected by *C. parvum* showed a significant increase in CD4+ counts after establishment of antiretroviral therapy, from 72 to 197/mm3. As a result of this restoration of immune function, diarrhea ceased and *Cryptosporidium* was no longer detected in the feces of the patients (Bobin et al, 1998). A prospective study enrolled AIDS patients suffering from either microsporidial or cryptosporidial diarrhea prior to starting anti retroviral therapy and followed the patients for at least 13 months after cessation of symptoms for both parasites. The short term analysis indicated resolution of the disease, with no parasites detected in fecal samples. However, the presence of high numbers of CD8+ cells and macrophage infiltration in the intestine, plus the short time to relapse in the event there was a decline in the CD4+ populations, suggested that the infections were controlled but not fully eradicated (Carr et al, 1998).

The search for a compound with therapeutic indications against *C. parvum* continues. Evaluations of paromomycin, nitazoxanide and carbazole compounds in neonatal mice confirmed the anecdotical successes previously described for these compounds (Blagburn et al, 1998b). Probiotic compounds are also being tested, and minimal beneficial effects have been reported using of *Lactobacillus acidophilus* and *L.*

reuteri as prophylactic agents. (Alak et al, 1997; 1999). Allicin, a nutraceutical compound and garlic derivative, has also been tested in the prevention of *C. parvum* infections in neonatal bovines. The results did not support any claims in the areas of therapeutic or prophylactic efficacy, showing no significant differences between the treatment and control groups (Olson et al, 1998).

C.2 Immune therapies

Multiple studies in animals have provided evidence that both polyclonal and monoclonal *C. parvum*-neutralizing antibodies may significantly reduce the severity of the disease caused by this parasite (Bjorneby et al, 1991; Riggs et al, 1994; Graczyk et al, 1998; 1999; 2000; Enriquez and Riggs, 1998; Riggs et al, 1997; Schaefer et al, 2000). Passive immunization is carried out by transferring elements of the immune system, generally antibodies, from an immunized donor to a non-immune recipient. Such a prophylactic or therapeutic strategy would provide immune protection to an individual who has not yet developed an effective autologous immune response to a threatened infection or existing disease, respectively. This approach may provide significant benefits to animals and humans suffering from cryptosporidial diarrhea until effective drugs are developed (Crabb, 1998).

C.2.a Passive transfer derived from infection exposure to *C. parvum*

Breast feeding in humans has been associated with prevention or amieloration of symptoms of enteric diseases (Naficy et al, 1999; Clemens et al, 1999; Scariati et al,

1997) in the case of cryptosporidiosis the benefits can be attributed to the breast feeding practice rather than specific immune components of the milk. Passive transfer of bile from experimentally infected rats was evaluated in nu/nu Balb/c mice in an attempt to determine the role of sIgA for the treatment of cryptosporidiosis. The histological examination of treated mice showed less severe pathological findings as well as fewer organisms per infected crypt compared to controls. Overall, these findings suggested a role for antibodies in control of the disease (Albert et al, 1994).

C.2.b Passive transfer of poly-specific hyperimmune bovine colostrum (HBC)

The lack of effectiveness of traditional small molecule compounds in treating *C. parvum* diarrhea has lead to the search of alternatives that mimic the host conditions of disease self resolution. The use of elements of the humoral immune response, specifically antibodies, is the next alternative. There are multiple reports on the use of polyclonal antibodies for treating and or preventing infectious diseases. The pre-clinical testing models are well described and understood and copious amounts of information have already been generated and published. Historically, antibodies have been used as antivenoms, as anti-rejection compounds and in the prevention of diseases (gamma globulin shots). There are reports of several passive transfer studies for treating cryptosporidiosis. The preparations tested in these studies varied from poly-specific polyclonal preparations to monoclonal antibodies, most of them with the same basic

scientific rationale but with different approaches and practical considerations (Craab, 1998).

Hyperimmune bovine colostrum was the first reported product used for successful oral passive transfer of antibodies aimed to treat chronic cryptosporidial diarrhea in a young immune deficient person (Tzipori et al, 1986). The beneficial effects of colostrum administration in AIDS patients suffering from protracted diarrhea was achieved by the continuous administration over a 1-week period of this preparation via nasogastric tube. In non controlled evaluations, HBC was administered on a compassionate basis to AIDS patients. HBC was administered to the affected patients over a four-day period, resulting in resolution of the diarrhea within a few days (Ungar et al, 1990b; Nord et al, 1990). Immune or hyperimmune bovine colostrum has been also tested for treating other infectious diseases of humans and other animals, as well as to enhance passive transfer to calves (Fayer et al, 1989).

Recent evaluations aimed at defining the prophylactic efficacy of HBC in healthy human volunteers reported a trend towards less diarrhea. These volunteers were administered HBC 30 minutes prior to and after the administration of a *C. parvum* inoculum, and three times daily thereafter for five consecutive days. The patients did not manifest significant differences in the frequency of bowel movements, but did have a 100-fold reduction in parasite excretion. These data demonstrated the potential of HBC in reducing the intensity of infection in humans (Okhuysen et al, 1998a).

HBC was also efficacious in preventing disease in experimentally infected calves, expanding the applications of the polyclonal therapies. A controlled treatment study evaluating HBC in neonatal calves showed partial protection against cryptosporidiosis in the treatment group. The specific anti-*Cryptosporidium* antibody titers in the HBC were very elevated for all isotypes (>1:200,000 for IgG, IgM and IgA), and the colostrum was well absorved by the calves. It was concluded that this preparation provided benefits to the calves (Fayer et al, 1989a) .

C.2.c Passive transfer of mono-specific preparations: HBC and monoclonal antibodies

Mono-specific antibody preparations against defined neutralization sensitive surface antigens of *C. parvum* have been generated, characterized and tested for *in vivo* efficacy. These mono-specific preparations include monoclonal and polyclonal antibodies raised against a specific antigen, either affinity purified or expressed as a recombinant antigen (Arrowood et al, 1989; Cho, 1993).

The well defined neutralization-sensitive P23 antigen has been the focus of several studies. Monoclonal anti P23 antibodies have been shown to neutralise the sporozoite by inactivating this stage and also by binding to neutralisation sensitive epitopes on the surface of the parasite. Panels of mouse monoclonal antibodies were evaluated in mice and demonstrated to be effective in neutralizing *C .parvum* in adult SCID mice (Riggs et al, 1997). Additional panels of monoclonal antibodies targeting P23 and two other neutralization sensitive epitopes identified as CSL and GP 25-200 have

shown enhanced anti-cryptosporidial efficacy (Schaefer et al, 2000). In addition to the panels tested, mouse sIgA monoclonal antibodies directed against P23 were orally administered to adult mice chronically infected and demonstrated significant therapeutic potential for treating cryptosporidiosis (Enriquez and Riggs, 1998). A recombinant P23 antigen was produced and identified as rC7. This recombinant P23 is very immunogenic and was tested in pregnant cows for the production of mono-specific HBC. These cows were immunized three times and the resulting colostrum was tested in neonatal calves receiving either HBC or sham colostrum. The calves were challenged with 10^7 oocyst and monitored for clinical disease. HBC generated by immunizations with rC7 conferred protection in the treated calves. The treated calves had no clinical disease and shed significantly fewer oocysts than controls, suggesting a very efficacious method for conferring protection to neonatal ruminants (Perryman et al, 1999) and the potential use of this same product in other mammals, including humans.

A recombinant P15 antigen was extensively studied for the selection of the expression system (Iochmann et al, 1999), the immunogenicity of nasal vaccinations (Sagodira et al, 1999a) and the protective efficacy of the colostral antibodies generated in goats for the protection of their neonatal kids from *C. parvum* challenge. A plasmid DNA vaccine expressing the recombinant P15 antigen was used to nasally immunize pregnant goats and evaluate the weight losses of kids from immunized and non-immunized goats. There were significant differences in favour of the kids from immunized goats (Sagodira et al, 1999b). This passive transfer approach, based on the recombinant expression of an

already defined neutralizing antigen, gives information towards the development of mucosal vaccines aimed to enrich the naturally produced colostrum with direct benefit to the offspring. The potential use of this approach for inducing mucosal immunity needs further evaluation and could become a viable alternative for those in need of anti-cryptosporidial medications.

A third recombinant antigen recently tested was the P15/60 protein. The P15 antigen is neutralization sensitive and is derived from a 60 KDa precursor found in the cytoplasm of the parasite. The P60 precursor is cleaved into two proteins (15 and 44 KDa) that localize on the surface of zoite stages. The DNA used in this recombinant antigen encoded for fractions common to both the P60 precursor and the P15 KDa surface antigens. This antigen was expressed in a plasmid system and used to perform intra mammary gland immunizations of pre-parturient cows. The colostrum was tested in adult C57BL/6NCr mice, with treatments being administered 12 hours prior to challenge with viable *C. parvum* oocysts and at 12 hour intervals thereafter for 3 consecutive days. The final analysis indicated a partial protection from *C. parvum* infections, with reductions in the excretion of parasites, but not in clearance of the disease (Jenkins et al, 1999).

C.2.d Passive transfer using hyperimmune hen egg yolks

Another polyclonal strategy is the use of eggs from hens hyperimmunized with *C. parvum* antigens. Egg yolk antibodies were tested *in vivo* in neonate Balb/c mice for

efficacy against cryptosporidial infections, showing statistical significant differences when compared to the non antigen immunized controls (Cama and Sterling, 1991). The immunoglobulin component of hen egg yolks from hyperimmunized chickens are typically referred to as HEY antibody. There are several references in the literature describing the potential use of HEY in the treatment of infectious diseases. The development of tolerance to HEY antibody prior to therapeutic administration was achieved by repeated oral ingestion of egg yolk and was followed by enteral administration of HEY antibody to a selected bacterial antigen (Peralta et al, 1994; Carlander et al, 2000). Snake antivenoms that were an injected form of HEY antibody were described as an option to mammalian antivenoms (Almeida et al, 1998). Preparations for the oral treatment of *Clostridium difficile* infections were tested in hamsters. These preparations contained high antibody titers that neutralized specific *Clostridium difficile* toxins (Kink et al, 1998). Another study reported the used of HEY for preventing rotaviral diarrhea in mice (Bartz et al, 1980). Hyperimmune egg yolk antibodies have also been used topically; two studies reported protection of rats against dental caries by feeding them HEY antibody raised against *Streptococcus mutans* (Hamada et al, 1991; Otake et al, 1995).

C.3 Active immunizations

Active immunoprophylaxis, such as immunization that results in a specific reaction of the immune system against the immunogen (e.g., vaccination) or administration of a chemical (e.g., immunoadjuvant) which systemically activates immune cells, has proven ineffective in controlling cryptosporidiosis (Harp et al, 1998; de Graaf et al, 1999).

Other approaches, such as gene therapy to reconstitute a functional immune system is still problematic and may not be applicable for this parasitic disease. Gene therapy is not yet a practical solution even in disorders where a single enzyme defect (e.g., adenosine deaminase) or infectious agent (e.g., a member of the lentivirus family) has been identified as the sole etiologic agent of a disease. A powerful example is immunodeficiency as a symptom.

In the case of cryptosporidiosis, the feasibility of developing a vaccine, and the groups in need of protection: children in the developing world, AIDS patients in the developing world and domestic large mammal neonates, make the passive transfer approach a valuable alternative (de Graaf et al, 1999).

Summarising, it could be stated that all preparations tested for passive immune transfer showed varying degrees of therapeutic or prophylactic potential. To determine the real therapeutic potential of each proposed indication, the study design shall consider testing of the candidates in the species where the product will be ultimately used. Data from the HBC generated against whole *C. parvum* antigens showed benefits for the

calves and human patients, but did not show significantly better results when compared to the other polyclonal or monoclonal preparations. Although there are additional factors to consider, such as frequency and length of treatment, the reports reviewed indicate the potential therapeutic applications for HBC, HEY and human IgA antibodies for treating enteric cryptosporidiosis. In this study, human breast milk per se will not represent a potential candidate for a therapeutic product. It will be tested to generate information on the efficacy of anti-*C. parvum* secretory IgA antibodies in enteric cryptosporidial disease. Mono-specific antibodies, either monoclonal or polyclonal and directed against specific antigens with reported neutralizing characteristics, could be considered as next generation products from the poly-specific polyclonal preparations, either alone or in combination therapies.

III. Dissertation Format

The area of therapies and prevention of cryptosporidial infections, both in humans and animals is vast, with multiple parallel ongoing efforts. There will be opinions that will support the use of passive immune therapies for enteric cryptosporidiosis, while others will maintain that the use of traditional antibiotic or antiparasitic compound of systemic use are better candidates. Based on the information currently available, passive immune therapy seems the alternative that can immediately benefit those suffering from the disease. Chapters 2-5 describe the efforts at the laboratory and pre-clinical level including the *in vivo* efficacy evaluation for three polyclonal preparations. Chapter 6 summarises data obtained from the human clinical trial evaluation of one of the polyclonal approaches in patients affected by chronic cryptosporidiosis. Chapter 7 reflects the research initiatives to be followed based on the results described in the previous chapters.

Overall, it is the intention to provide a basic example on how veterinary science knowledge could be used in an innovative way. The efforts required for this dissertation required the merger of veterinary medicine, pathobiology, parasitology, immunology, epidemiology and basic medical knowledge in order to generate and test polyclonal preparations for oral applications. The information generated at the laboratory level and at the user (clinical) level shall be used in an interactive way, with the intention of enhancing the odds of developing an effective preparation towards treating enteric cryptosporidiosis.

This study will evaluate the potential of bovine, chicken and human polyclonal antibodies for the treatment and or prevention of cryptosporidial diarrhea, with the intention of identifying a polyclonal preparation that can provide an effective, safe and practical method for controlling enteric *C. parvum* infections.

CHAPTER 2: PRODUCTION AND *IN VITRO* EVALUATION OF ANTI-*C. PARVUM* HYPERIMMUNE BOVINE COLOSTRUM

I. Introduction

Previous studies in mice and anecdotical therapeutic studies in people with immune deficiencies strongly suggest that orally administered antibodies are beneficial to infected animals and patients (Tzipori et al, 1986; Nord et al, 1990; Ungar et al 1990b; Fayer et al, 1989; Cama and Sterling, 1991; Riggs et al, 1994; Okhuysen et al, 1998a). This chapter describes the work towards the generation and evaluation of hyperimmune bovine colostrum against *C. parvum*.

II. Methods for the Generation of Hyperimmune Bovine Colostrum

A. Immunization groups

Forty five 5-month pregnant Holstein cows (Campbell Avenue Farm, University of Arizona), were selected and assigned to three groups: Oocyst group receiving oocyst antigen, Sporozoite group (sporozoite antigen) and Adjuvant group (adjuvant alone). All cows had completed at least one lactation year and none had more than five. This criterion was used to minimize unexpected events more often associated with younger cows.

B. Antigen preparation

B.1. Oocyst purification

Cryptosporidium parvum oocysts were generated by infection and amplification of a viable inoculum through passage in neonatal calves. Calves were infected on day 1 of age with 10^8 purified *C. parvum* oocysts. Starting on day 5 post-infection, the fecal material was collected daily for the next 7 days, preserved in 5% potassium dichromate, and transported to the laboratory where the material was stored at 4°C until processed. The purification of *C. parvum* oocysts started by sieving the material through stainless steel screens with a final mesh size of 63 μm porosity. Recovery of *C. parvum* oocysts utilized 2 sequential discontinuous sucrose gradients. These discontinuous gradients were prepared from Sheather's solution diluted with phosphate buffered saline (0.025 M). The 1:2 solution had a specific gravity of 1.103 and the 1:4 solution a specific gravity of 1.064. The 1:2 solution was layered below the 1:4 solution, then the sieved feces were placed on top of the 1:4 solution and the tubes centrifuged at 1975 x *g* for 30 minutes. Oocysts were recovered from the interface of the sucrose solutions, diluted with 0.85% saline solution and pelleted. The pellets were resuspended in 2.5% potassium dichromate, dispensed over new sucrose gradients (secondary purification) and centrifuged as before.

Percoll (Pharmacia, NJ) solutions of 1.091 g/mL were prepared by mixing 9 parts Percoll, 1 part 10x Alsever's, and 9 parts 1x Alsever's. Oocysts from secondary purification were layered over Percoll solutions and centrifuged at 22,000 x *g* for 30 minutes at room temperature. The layer containing oocysts was recovered, washed in

0.085% saline, and stored in potassium dichromate at 4°C until it was used for antigen preparation (Arrowood and Sterling, 1987).

B.2. Antigen preparation

Percoll-cleaned oocysts of the Iowa isolate of *C. parvum*, a bovine isolate originally obtained from Dr. Harley Moon of Iowa State University, were used for the generation of the antigens needed. The oocysts were washed exhaustively to remove potassium dichromate, resuspended in sterile phosphate-buffered saline (PBS), and counted on a hemacytometer.

The microbiological quality of the antigen was tested by culturing antigen samples for bacterial aerobes and coliforms. Petri-films (3M, MN) were used for these evaluations. Antigen batches with positive coliforms results were discarded. Antigen batches with aerobe counts exceeding 10/ml were discarded.

B.3. Whole oocyst antigen

The sonicator (Branson, CT) was pre-cleaned by two 30-second cycles in 95% ethanol. Whole oocysts were fragmented by five 3-minute cycles of sonication at 20 MHz or until approximately 90% of the oocysts were disrupted. All sonication steps were performed with the oocyst preparation placed in an ice bath. The mixture was allowed to cool for 3-5 minutes between sonication cycles. The protein concentration of the antigen preparations were determined using the BCA mini protein assay (Pierce

Chemical, CA) following the manufacturers instructions. On average, about 6.54 mg of antigen was obtained from 2.36 x 10^9 oocysts.

To verify consistency between batches of antigen, samples of subsequent batches were compared by SDS-PAGE. Briefly, the *C. parvum* antigen was electrophoresed on 5-15% gradient polyacrylamide gels at 30 mAmps constant current and stained with Coomassie blue. Protein bands from the different batches were compared.

B.4. *C. parvum* sporozoite antigen

Percoll purified oocysts were collected by filtration through a 1um size exclusion polycarbonate membrane (Nucleopore, CA). The oocysts were rinsed with 0.025M PBS in a volume at least 10 times the volume potassium dichromate. The rinsed oocysts were then subjected to excystation. Oocysts were incubated at 37°C with the addition of a bile salts and sodium thiosulfate. After 15 minutes of incubation, and every 5 minutes thereafter, samples were collected and observed under phase contrast microscopy for determining the percent of excystation. Once the percentage of excysted oocysts reached 80%, or there was no significant increase in the percentage, the excystation process was stopped. The sample was cooled to 4°C and layered over a Percoll gradient similar to the procedure described for oocyst purification. The excysted oocysts and sporozoites were layered over Percoll solutions and centrifuged at 22,000 x *g* for 30 minutes. Three bands were observed post centrifugation, each containing oocyst shells, unexcysted oocysts and sporozoites. The layer containing sporozoites was recovered from the Percoll tubes,

gently washed in physiological saline (0.085% NaCl), aliquoted and stored at -20°C. Determination of the protein concentration by the BCA (Pierce Chemicals, CA) method was done after the sample had been frozen for at least 24 hours. The protein concentration of sporozoite antigen was 1.803 mg/ml per 10^9 excysted oocysts.

B.5. Adjuvant

The adjuvant for this study was Ribigen Adjuvant (Ribi Immunochemicals, MT) formulated for bovine species. It contained the mitogen STM which is a proteinaceous fraction of a mutant *Salmonella typhimurium* and the metabolizable lipid LES. To prepare the antigen-adjuvant mixture, 3 volumes of the respective antigen in sterile 0.025M PBS (3.75ml) were added to 1.25 ml of Ribigen. The mixture was vortexed until a milky emulsion was formed and loaded into sterile disposable syringes. Each dose (1.0 ml) contained 25 μg of antigen and 100 μg of STM. All prepared material were stored in portable coolers with ice packs until used at the farm, where they were warmed up and vortexed prior to use.

C. Immunization overview

All cows were immunized at 4, 3, 2, and 1 months prior to their scheduled delivery dates. For each immunization the cows were injected with 1ml of the respective antigen-adjuvant mixture in 4 different sites, 0.25 ml per site. The first two immunizations were injected on both sides of the shoulder and hind areas while the third consisted of one single dose of 1ml (25 μg of antigen) injected intravenously into the tail vein. The last immunization (4 sites) was injected subcutaneously around the supra-mammary lymph nodes. All cows received 1 ml of the emulsion, containing either 25 μg of whole *C. parvum* antigen (oocyst group), 25 μg of sporozoite antigen or no antigen. The immunizing emulsion was equally divided and injected into the designated immunization sites.

D. *In vitro* monitoring of the immune responses

D.1 Colostrum collection

To ensure collection of colostrum, cows within 3 weeks of their due fresh date were monitored on a daily basis for signs of delivery. Upon delivery, calves were immediately transferred to pens and were fed previously collected high quality colostrum. The dam was checked and kept in the delivery pen until the next milking time. At this time, and before cows from other pens were pushed to the milking area, the cow in the study was taken to the parlor to ensure she was always in the first group to be milked. Colostrum for this study was always collected at the beginning of the milking session,

when the milking system was clean and just sanitized. This procedure was performed to minimize microbial contamination of the colostrum.

Colostrum was first collected into the system's measuring vessels, then transferred into sanitized vacuum bottles (Nalgene) and immediately transferred to the laboratory in refrigerated coolers. Once in the laboratory, colostrum was transferred from the vacuum bottles into sterile 1gallon jugs and frozen to –20°C until used. Samples for laboratory testing were collected into sterile 50ml tubes and stored frozen until tested. Colostrum was collected at the first three milkings: delivery time, 12, and 24 hours post delivery. Samples of milk, about 150 ml per sample, were collected at 7, 14, 21, 28, 60, 90, 120 and 150 days post delivery.

D.2. Serological samples

Blood samples were collected from the tail vein on months 5, 6, 7, and 8 of gestation, on the delivery day, and on months 1, 2, 3, 4 and 5 post-delivery. Blood samples were collected in plastic tubes and transferred to the laboratory. For serum separation purposes, the blood samples were incubated at 37°C for 2 hours, then incubated at 4°C for another 2 hours and centrifuged at 3000 rpm (1925 x g) for 10 minutes. The serum was collected and aliquots were stored frozen in 15ml sterile tubes and 1ml sterile micro-centrifuge tubes until tested.

D.3. Testing

Immunological testing was performed on the collected samples by an immuno fluorescent assay, ELISA and Western Blot. The potency of HBC was titrated using excysted sporozoites. Ten-well slides were pre-coated with 1% poly-L-Lysine (Sigma Chemical Co., MO) and allowed to air dry. Sporozoites purified as described above were diluted to 50,000 sporozoites/ml and 10 ul of this preparation were applied per well. Slides were allowed to air dry and stored at -20°C until used. Fifteen two-fold serial dilutions of HBC starting at 1:2 were made and applied at 10ul/well. Goat anti-bovine IgG FITC (KPL, MD) diluted 1:250 was used to detect bound HBC antibodies. Slides were read in a epifluorescent microscope using 450-490 nm dichroic mirror excitor filters.

To determine the specific *C. parvum* antigen recognition by antibodies from HBC, samples were evaluated by Western Blot (Appendix 2). Briefly, sonicated oocysts were boiled in SDS-PAGE reducing sample buffer, electrophoresed in 10% acrylamide gels and the separated *C. parvum* fractions were electro-transferred onto nitrocellulose membranes. Blotted membrane strips were probed with HBC antibodies and the reaction was visualized by the use of a goat anti-bovine IgG HRPO followed by TMB chromogen (KPL, MD).

ELISA was used to quantitate antibody responses against *C. parvum* and the procedure is described in Appendix 1. ELISA values were entered into electronic databases and the data was statistically analyzed using two tailed Student's T test.

III Results

A. Immunization groups and immunizations

No unexpected adverse reactions associated with the immunizations were detected during this study. No local inflammatory reactions were detected by palpation at the injection sites. Slight swelling of the regional lymph nodes was detected in about 10% of the cows. Production of colostrum and milk in the study group was not affected, with no significant statistical differences between the study cows and other cows in the herd. From all forty-five cows assigned to the study, one cow from the Adjuvant group (#274) had delivery complications and died before any colostrum samples could be collected (Table 1).

B. Sampling and evaluation of the immune potency of the samples

Colostrum samples were collected from 44 cows: 13 (30%) were on the second lactation, 16 (36%) on the third, 11 (25%) on the fourth and 4 (9%) on the fifth lactation. No statistically significant differences were detected in the mass of colostrum per collection between any of the three production groups (t-test, $p>0.2$) (Table 2). When the production values were stratified by lactation year, no trends nor statistically significant differences were detected (t-test, $p>0.2$), demonstrating that this was a fairly homogeneous group. Age as defined by year of lactation had no negative impact on the performance of the cows.

C. *In vitro* activities:

ELISA activities from serum samples showed a marked increase in the anti-*C. parvum* activities from animals in the Oocyst and Sporozoite groups, peaking in values around the delivery time and showing a constant and gradual decrease in values over the next 5 months.

The serum samples were tested at two different dilutions: 1/1350 and 1/4050. Values obtained from samples diluted 1/1350 showed the Adjuvant group with a baseline value of 0.087, with no increases detected at 1 month after the first immunization (OD=0.029) or 1 month after completion of immunizations (OD=0.025). On the groups receiving oocyst or sporozoite antigen, the baseline OD values were 0.121 and 0.265 respectively. Significant increases in the OD values were detected at 1 month after the prime immunization (OD values of 0.700 and 0.766 respectively). Both groups had peak ELISA values at 1 month after the fourth and last immunization, with ODs of 1.477 and 1.489 respectively. The peak values in serum coincided with the expected delivery times, increasing the opportunity for the formation of colostrum rich in anti-*C. parvum* antibodies. These values had a gradual decrease and by the time of the last sampling, 5 months post delivery and 6 months after the last immunization, the OD activities were 0.439 in the oocyst group and 0.843 in the sporozoite group (Figure 1). The data collected from samples tested at a 1/4050 dilution showed the same pattern, with smaller OD values as expected for more diluted samples. The Adjuvant group had OD values of 0.004 at baseline, 0.013 at 1 month post prime immunization and 0.018 by delivery time. The Oocyst group had OD activities of 0.0238 at

baseline sampling, increasing to 0.340 one month after the first immunization and peaking at 1.170 by delivery time and 0.194 at the time of the last sampling. Similarly, the Sporozoite group had values of 0.179 at baseline, 0.387 at 1 month after the first immunization, 1.229 by delivery time and decreased to 0.37 by 5 months post delivery (Figure 2).

The colostrum samples were also tested at two different dilutions 1/1350 and 1/4050, with the latter being a better indicator of differences. Values obtained from samples diluted 1/1350 showed the Adjuvant group with values of 0.322, 0.195 and 0.106 for the first, second and third colostrum collection. On the groups receiving oocyst or sporozoite antigen, the average OD values were 1.61 and 1.149 for the first colostrum collected, 1.316 and 0.893 for the second collection and 1.022 and 0.468 for the third collection respectively. As with serum samples, the data from the ELISA testing of colostrum samples at the 1/4050 dilution resembled the results from the 1/1350 dilution. The OD values at this dilution for the first, second and third colostrum collection were 0.103, 0.054 and 0.034 for the Adjuvant group; 0.974, 0.672 and 0.49 for the Oocyst group and, 0.779, 0.554 and 0.382 for the Sporozoite group.

Differences in the OD values were significant between the three colostrum samples collected in the Oocyst and sporozoite groups when compared to the Adjuvant group. These differences were more significant at the first collection, with significance values of $p<0.0000001$. In the second colostrum collection, the differences were still very significant ($p<0.0002$). Data from the third colostrum collection showed significant differences in the

OD values between the Oocyst and Adjuvant groups ($p<0.0001$). The differences in the average OD values between the Sporozoite group and the Adjuvant group were significant, but not as defined as for the previous two collections ($p<0.05$, where $p=0.013$). When analyzing the values between the Oocyst and Sporozoite groups, there were statistically significant differences in all three colostrum collections, with significance values of $p=0.013$ for the first colostrum, $p=0.049$ for the second colostrum collected and $p=0.013$ for the third colostrum (Figure 3).

The data from the milk samples collected on days 7, 14, 21, 28, 60, 90, 120 and 150 post delivery, showed OD values very close to zero. When pooling this ELISA data into two groups: colostrum and milk, the average OD values of colostral samples per group were 0.210 for the Adjuvant group, 1.316 for the Oocyst group and 0.837 for the Sporozoite group. The average OD values for the milk samples collected were 0.015 for the Adjuvant group, 0.027 for the Oocyst group and 0.001 for the Sporozoite group. The statistical analysis showed significant differences in the OD values of all colostral samples when compared to milk samples, with significance values of $p=3.6E-20$ for the Adjuvant group, $p=1.85E-45$ for the Oocyst group and $p=4.27E-19$ for the sporozoite group (Figures 4 and 5).

Samples of colostrum were also titrated by immunofluorescent assay (IFA). Briefly, excysted oocysts were coated onto 10 well slides which were pre-coated with 1% poly-L-Lysine. The antigen coated slides were allowed to air dry and stored frozen until used. Colostrum samples from all three groups were serially diluted and 10 uL of each dilution per

well were applied. The slides were incubated 15 minutes in a 37°C humid chamber, and the slides were rinsed 3 times with 0.025 PBS-0.1% lauryl sulfate (tween 20) wash solution. A fluorescent labeled antibody (affinity purified goat anti-bovine IgG FITC, Kirkegard and Perry Laboratories, MD) diluted at 1:100 was used as the secondary antibody and 10uL of this preparation was added per well. The slides were incubated and subsequently rinsed as in the first step. The excess of wash buffer was removed and coverslips were mounted on the slides using glycerol. The slides were read in an epifluorescent microscope. The IFA titers for samples of the Adjuvant group were 1,024, while samples from the Oocyst and Sporozoite groups had values greater than 32,768 (Figure 6).

Western blot analysis demonstrated reactivity with at least 21 different antigenic bands (194, 172, 147, 125, 113, 104.5, 89, 73.2, 70.3, 61.5, 50, 44, 35.1, 33, 28.5, 26, 23, 22, 19, 17.3, and 15 KDa) , including *C. parvum* fractions of 44, 23 and 15 KDa (Photo 1). The SDS-PAGE analysis of three batches of antigen showed band homology between all preparations (Photo 2).

IV Discussion

The data analysis indicates that generation of hyperimmune bovine colostrum was feasible, and the use of Ribigen as an adjuvant was successful. Freund's adjuvant system, a very popular adjuvant utilized for the generation of polyclonal antibodies cannot be used in dairy cows. This is due to the presence of *M. tuberculosis* antigens as part of the Freund's complete adjuvant, which may engender immune responses that will result in positive TB skin tests.

The statistical analysis indicates that for any further testing, colostrum from the first milking would be the material of choice. The second colostrum still had very significant activities. The third colostrum had lower activities and would be avoided for *in vivo* testing. Anti-*C. parvum* activities were not detected in any of the milk samples, regardless of significant serum values of circulating antibodies. This is justified by the fact that prior to delivery, there is a specific passage and concentration of circulating antibodies, mainly of the IgG type, into the alveoli of the mammary gland. Upon delivery, the antibodies concentrated in the udder are excreted into the first secretions of the dam, the colostrum. Since this high concentration process only happens prior to delivery and not post delivery, insignificant amounts of specific antibodies would be expected in the milk samples.

Differences in the antibody activities from the colostrum collected from the groups immunized with Oocyst and Sporozoite antigens, showed a significant difference favoring the Oocyst immunized cows. This higher titer could be due to the fact that most

of the testing antigens used were oocyst based. IFA titration of the samples however, showed similar activities in the reactivity of both colostra against oocyst and sporozoites. For the *in vivo* testing, samples from either group would be considered. Additionally, data supports the use of either whole oocyst or purified sporozoite antigens for future immunizations. Based on the non-significant differences detected, oocyst antigen may used interchangeably with sporozoite antigens. In order to expose the animals to be hyperimmunized with as many antigens as possible that mimic natural exposure, whole oocyst antigen were used in future evaluations.

Photo 1. Western blot of hyperimmune bovine colostrum (HBC) and and Hyperimmune Hen egg yolks (HEY)

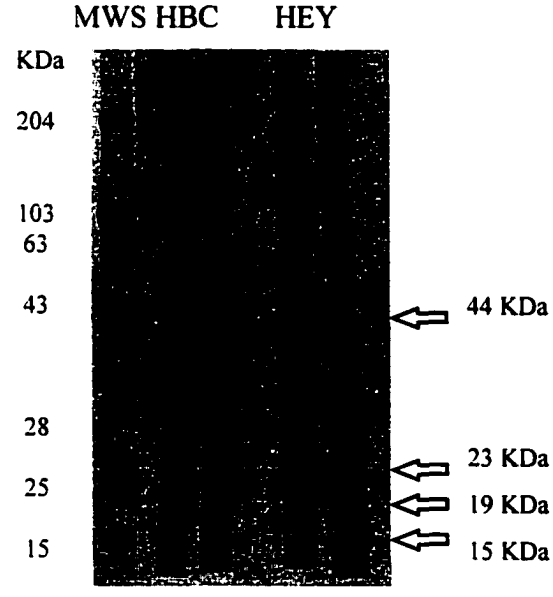


Photo 2. SDS-PAGE (5-15%) of 3 batches of *C. parvum* antigen

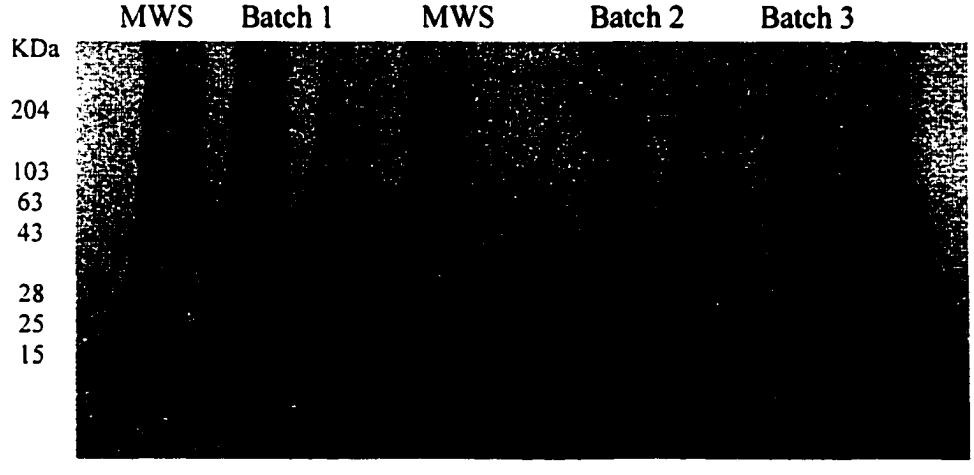


Table 1. Immunization and Sample Collection Schedule

cow	group	first	second	third	fourth	day 0	day 7	day 14	day 21	28	60	90	120	150
95	adjuvant	10/2	11/1	12/1	12/31	1/30	2/6	2/13	2/20	2/27	3/31	4/30	5/30	6/29
116	adjuvant	12/21	1/20	2/19	3/21	4/20	4/27	5/4	5/11	5/18	6/19	7/19	8/18	9/17
152	adjuvant	11/17	12/17	1/16	2/15	3/17	3/24	3/31	4/7	4/14	5/16	6/15	7/15	8/14
156	adjuvant	12/28	1/27	2/26	3/28	4/27	5/4	5/11	5/18	5/25	6/26	7/26	8/25	9/24
206	adjuvant	12/28	1/27	2/26	3/28	4/27	5/4	5/11	5/18	5/25	6/26	7/26	8/25	9/24
232	adjuvant	12/28	1/27	2/26	3/28	4/27	5/4	5/11	5/18	5/25	6/26	7/26	8/25	9/24
233	adjuvant	12/8	1/7	2/6	3/8	4/7	4/14	4/21	4/28	5/5	6/6	7/6	8/5	9/4
279	adjuvant	11/22	12/22	1/21	2/20	3/22	3/29	4/5	4/12	4/19	5/21	6/20	7/20	8/19
289	adjuvant	12/8	1/7	2/6	3/8	4/7	4/14	4/21	4/28	5/5	6/6	7/6	8/5	9/4
1113	adjuvant	9/29	10/29	11/28	12/28	1/27	2/3	2/10	2/17	2/24	3/28	4/27	5/27	6/26
1183	adjuvant	11/17	12/17	1/16	2/15	3/17	3/24	3/31	4/7	4/14	5/16	6/15	7/15	8/14
1192	adjuvant	11/22	12/22	1/21	2/20	3/22	3/29	4/5	4/12	4/19	5/21	6/20	7/20	8/19
3317	adjuvant	2/26	3/28	4/27	5/27	6/26	7/3	7/10	7/17	7/24	8/25	9/24	10/24	11/23
274	adjuvant	3/12	4/11	5/11	6/10	7/10 dead								
240	adjuvant	3/19	4/18	5/18	6/17	7/17	7/24	7/31	8/7	8/14	9/15	10/15	11/14	12/14
163	oocyst	11/1	12/1	12/31	1/30	3/1	3/8	3/15	3/22	3/29	4/30	5/30	6/29	7/29
173	oocyst	11/1	12/1	12/31	1/30	3/1	3/8	3/15	3/22	3/29	4/30	5/30	6/29	7/29
174	oocyst	11/5	12/5	1/4	2/3	3/5	3/12	3/19	3/26	4/2	5/4	6/3	7/3	8/2
176	oocyst	5/7	6/6	7/6	8/5	9/4	9/11	9/18	9/25	10/2	11/3	12/3	1/2	2/1
183	oocyst	10/9	11/8	12/8	1/7	2/6	2/13	2/20	2/27	3/6	4/7	5/7	6/6	7/6
252	oocyst	12/10	1/9	2/8	3/10	4/9	4/16	4/23	4/30	5/7	6/8	7/8	8/7	9/6
285	oocyst	5/5	6/4	7/4	8/3	9/2	9/9	9/16	9/23	9/30	11/1	12/1	12/31	1/30

Table 1. Immunization and Sample Collection Schedule

1191	oocyst	5/5	6/4	7/4	8/3	9/2	9/9	9/16	9/23	9/30	11/1	12/1	12/31	1/30
2838	oocyst	10/18	11/17	12/17	1/16	2/15	2/22	3/1	3/8	3/15	4/16	5/16	6/15	7/15
3007	oocyst	12/17	1/16	2/15	3/17	4/16	4/23	4/30	5/7	5/14	6/15	7/15	8/14	9/13
1201	oocyst	6/27	7/27	8/26	9/25	10/25	11/1	11/8	11/15	11/22	12/24	1/23	2/22	3/24
291	oocyst	6/28	7/28	8/27	9/26	10/26	11/2	11/9	11/16	11/23	12/25	1/24	2/23	3/25
307	oocyst	7/2	8/1	8/31	9/30	10/30	11/6	11/13	11/20	11/27	12/29	1/28	2/27	3/29
1104	oocyst	7/6	8/5	9/4	10/4	11/3	11/10	11/17	11/24	12/1	1/2	2/1	3/3	4/2
42	sporoz.	11/16	12/16	1/15	2/14	3/16	3/23	3/30	4/6	4/13	5/15	6/14	7/14	8/13
119	sporoz.	1/11	2/10	3/12	4/11	5/11	5/18	5/25	6/1	6/8	7/10	8/9	9/8	10/8
172	sporoz.	5/14	6/13	7/13	8/12	9/11	9/18	9/25	10/2	10/9	11/10	12/10	1/9	2/8
259	sporoz.	10/29	11/28	12/28	1/27	2/26	3/5	3/12	3/19	3/26	4/27	5/27	6/26	7/26
271	sporoz.	5/25	6/24	7/24	8/23	9/22	9/29	10/6	10/13	10/20	11/21	12/21	1/20	2/19
1116	sporoz.	5/25	6/24	7/24	8/23	9/22	9/29	10/6	10/13	10/20	11/21	12/21	1/20	2/19
1127	sporoz.	11/13	12/13	1/12	2/11	3/13	3/20	3/27	4/3	4/10	5/12	6/11	7/11	8/10
1140	sporoz.	11/10	12/10	1/9	2/8	3/10	3/17	3/24	3/31	4/7	5/9	6/8	7/8	8/7
1165	sporoz.	1/11	2/10	3/12	4/11	5/11	5/18	5/25	6/1	6/8	7/10	8/9	9/8	10/8
6001	sporoz.	10/24	11/23	12/23	1/22	2/21	2/28	3/7	3/14	3/21	4/22	5/22	6/21	7/21
6002	sporoz.	11/15	12/15	1/14	2/13	3/15	3/22	3/29	4/5	4/12	5/14	6/13	7/13	8/12
6787	sporoz.	10/20	11/19	12/19	1/18	2/17	2/24	3/3	3/10	3/17	4/18	5/18	6/17	7/17
230	sporoz.	6/5	7/5	8/4	9/3	10/3	10/10	10/17	10/24	10/31	12/2	1/1	1/31	3/2
284	sporoz.	6/15	7/15	8/14	9/13	10/13	10/20	10/27	11/3	11/10	12/12	1/11	2/10	3/12
1131	sporoz.	6/26	7/26	8/25	9/24	10/24	10/31	11/7	11/14	11/21	12/23	1/22	2/21	3/23

Table 2: Pounds of Colostrum per Collection

cow #	group	first	second	third
95	adjuvant	22	11	15
206	adjuvant	35	19	4
1113	adjuvant	8	9	1
1192	adjuvant	30	10	17.3
3317	adjuvant	17.2	14	21.3
156	adjuvant	7.4	7.1	7
240	adjuvant	19	0	16.5
289	adjuvant	11.3	10.1	13.4
279	adjuvant	14.1	11.3	22
1183	adjuvant	18.1	4.2	10.1
116	adjuvant	25	6.4	17.4
152	adjuvant	10.8	10	22.5
232	adjuvant	31.5	6	2
233	adjuvant	40	14	25.7
2838	oocyst	22	11	18
252	oocyst	26	13	18
163	oocyst	31	21	32
1181	oocyst	34	2	21
174	oocyst	30.9	22	35
1191	oocyst	13.7	5.1	12
176	oocyst	38	11	18
3007	oocyst	15	15	18
173	oocyst	21	10	6
183	oocyst	49	15	21
285	oocyst	7	6.5	13.5

cow #	group	first	second	third
1116	sporozoite	14	9	13.3
6002	sporozoite	12	13	13
42	sporozoite	45	19	20
6787	sporozoite	25	7	9
172	sporozoite	16.5	15	24
1165	sporozoite	12	44	3.8
6001	sporozoite	23	25	22
230	sporozoite	11.2	2	13.2
271	sporozoite	31.5	10.1	17.1
119	sporozoite	42	3.5	5
1127	sporozoite	9	5	8
259	sporozoite	13.5	13	24
292	sporozoite	17	5.1	17
1140	sporozoite	45	18	23

STATISTICS P value	1st	2nd	3rd
Adjuvant vs Oocyst	0.235	0.256	0.114
Adjuvant vs Sporozoite	0.666	0.216	0.668
Oocyst vs Sporozoite	0.294	0.804	0.148
Adjuvant mean	20.671	9.436	13.943
Oocyst mean	26.145	11.964	19.318
Sporozoite mean	22.621	13.479	15.171

Figure 1:Serum Activities

Average of immunized groups
ELISA 450 nm (dilution 1/1350)

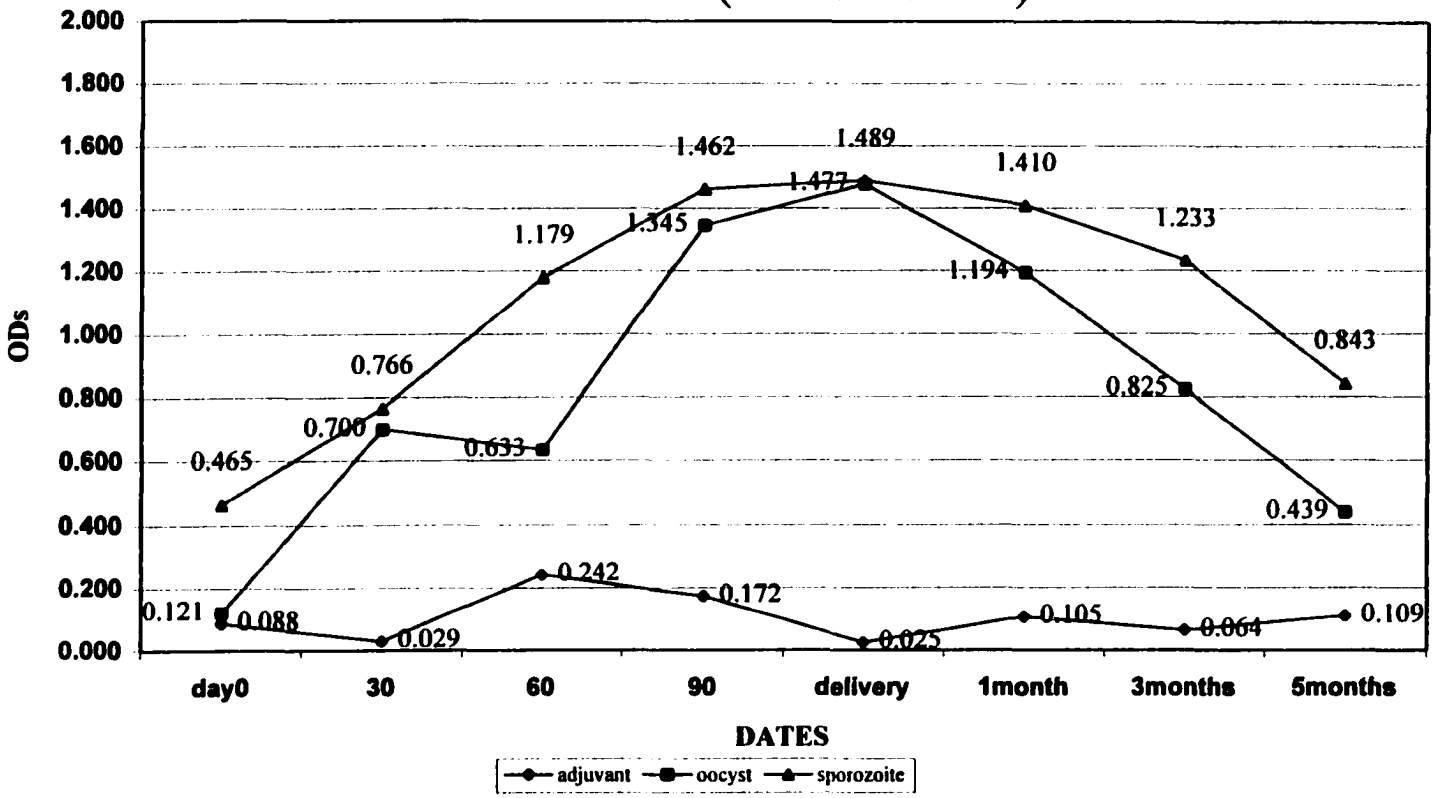


Figure 2:Serum Activities

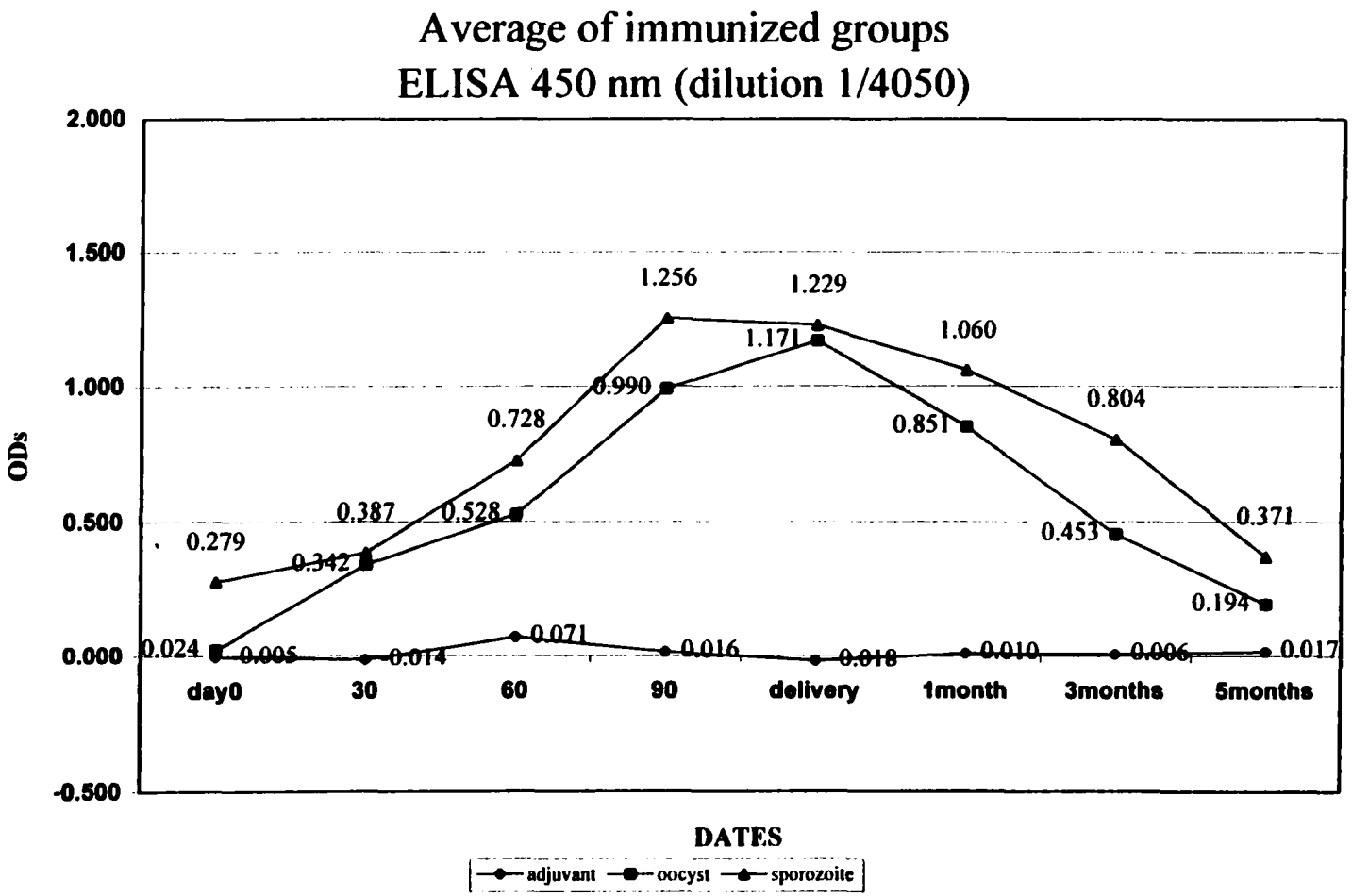


Figure 3:Colostrum Activities

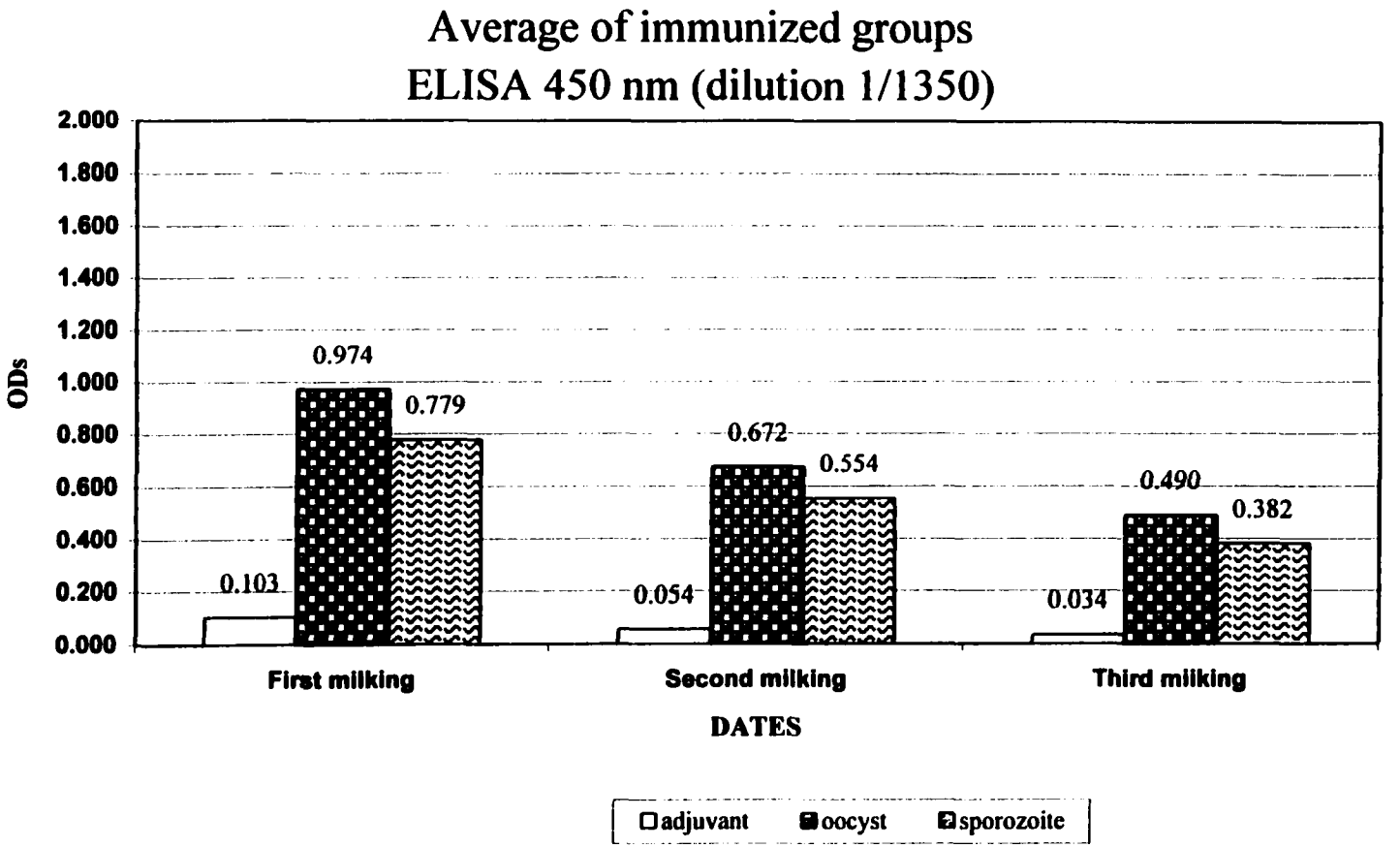


Figure 4:Colostrum and Milk Activities

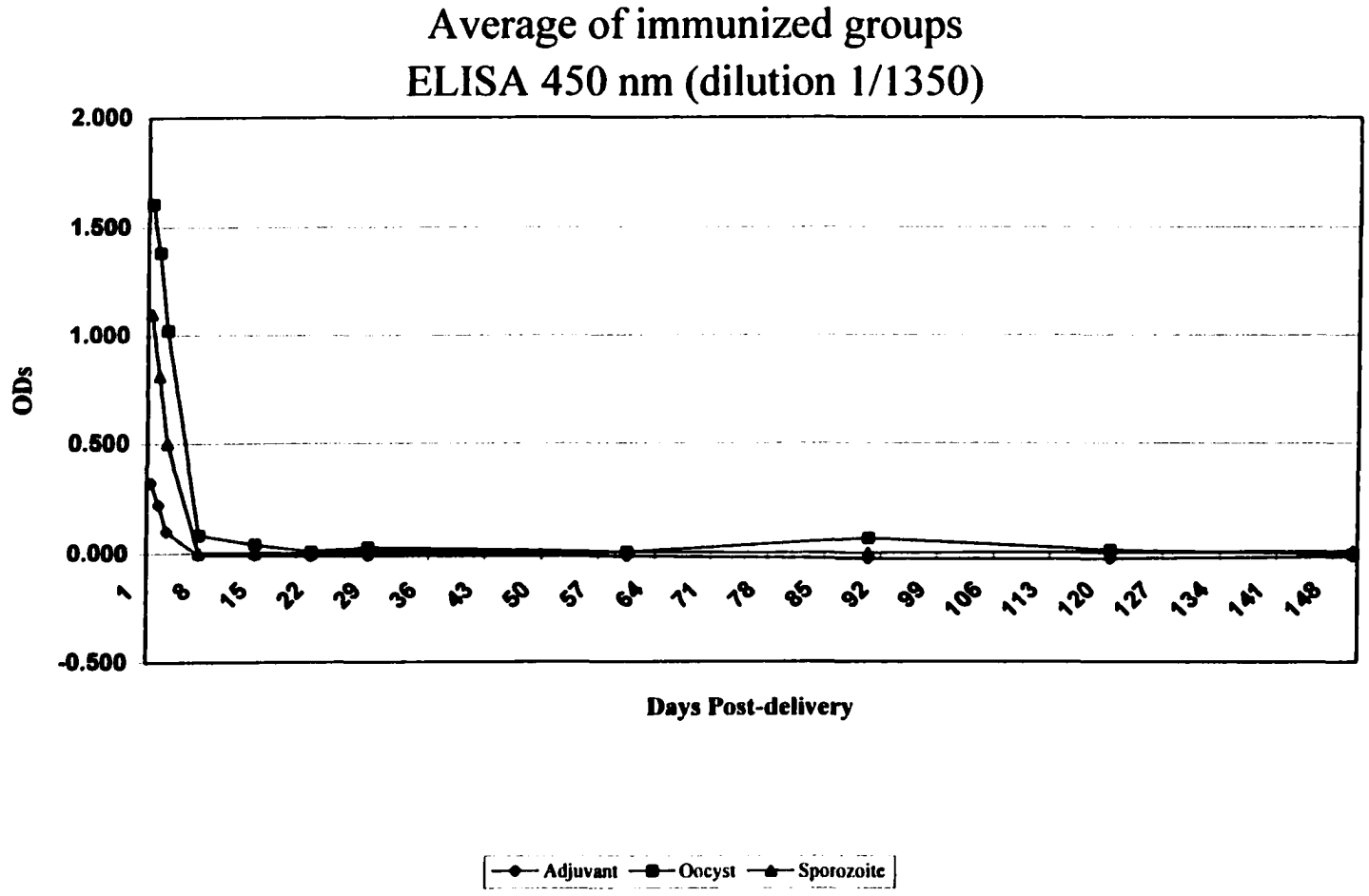


Figure 5:Colostrum and Milk Activities

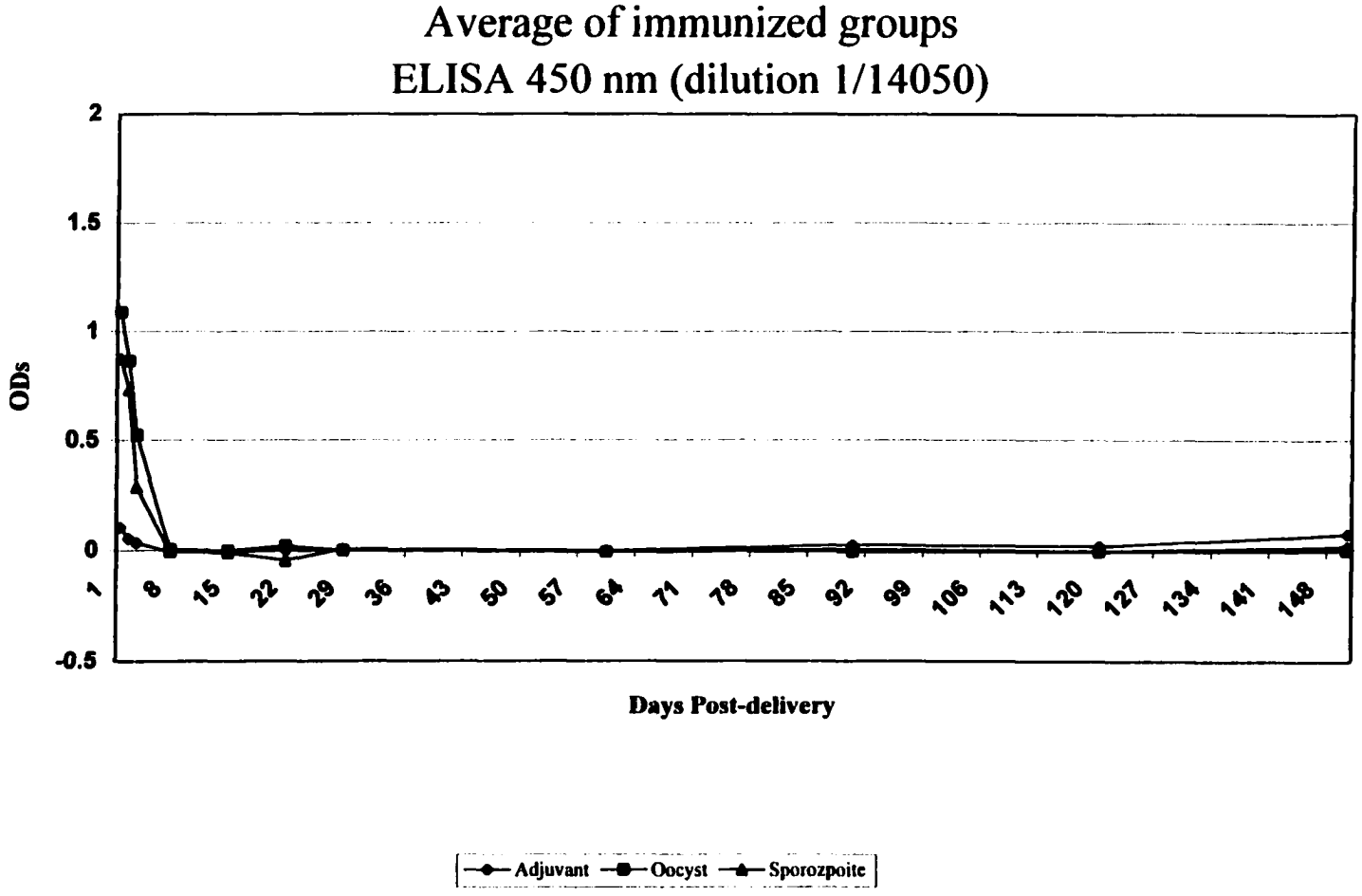
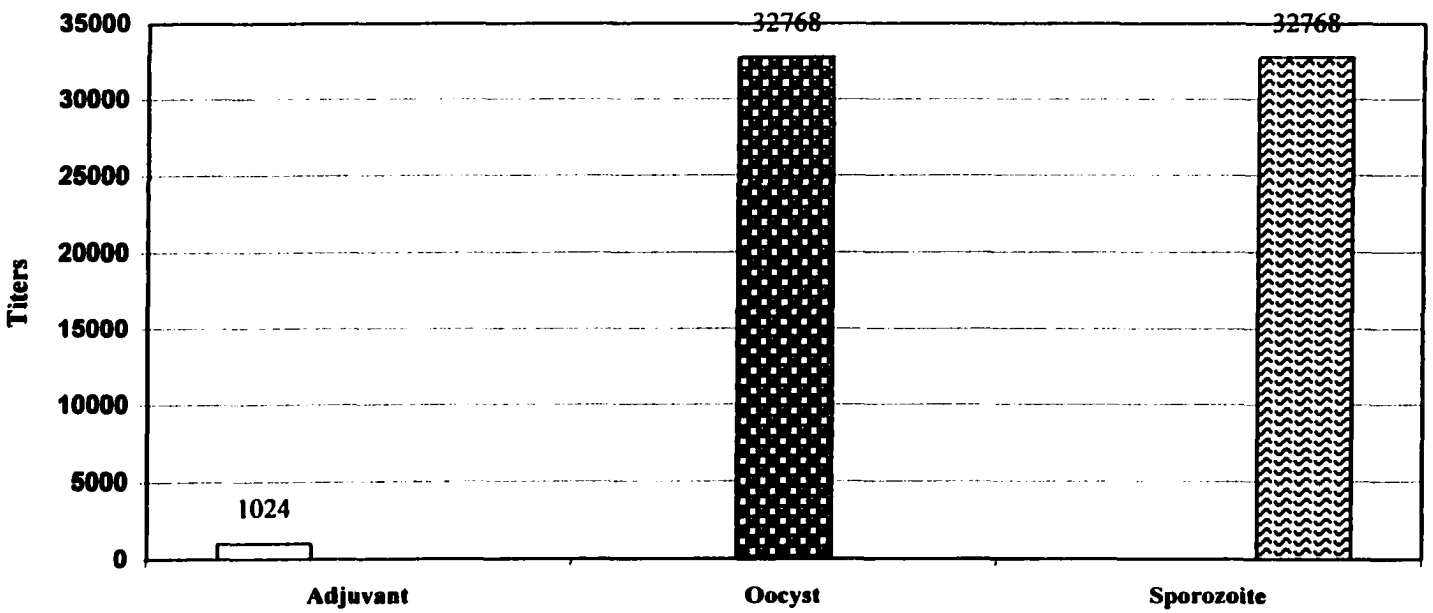


Figure 6:Colostrum IFA Titers



CHAPTER 3: PRODUCTION AND *IN VITRO* EVALUATION OF ANTI *C. parvum* HYPERIMMUNE CHICKEN EGG YOLKS

I. Introduction

In the absence of effective chemotherapy to combat cryptosporidiosis, new approaches for treatment and prophylaxis are being sought. There is preliminary evidence to support the contention that appropriate immunotherapeutic modalities of therapy and prophylaxis will provide relief to affected patients. An immune approach for the treatment of cryptosporidiosis involving the use of hyperimmune bovine colostrum (HBC) was previously evaluated in anecdotal compassionate trials in AIDS patients (Ungar et al, 1990b). This treatment, administered to the affected patients over a four-day period, resulted in resolution of the diarrhea within a few days. Practical limitations for the production, collection and storage of HBC placed constraints on the large scale application of this material. This would especially be true in developing countries. An alternative approach to the production of a hyperimmune product suitable for passive transfer is the potential use of hyperimmunized hens. Eggs laid by hens hyperimmunized with *Cryptosporidum* antigens have high anti-*Cryptosporidium* activities (similar to HBC). Furthermore, egg production is constant and large scale production and storage is feasible. A significant parasite reduction was achieved using hyperimmune yolks in a *Cryptosporidium* infected neonatal mouse model (Cama and Sterling, 1991).

Previously, anti-*Cryptosporidium* antibodies were generated in cows using 25 μg/immunization of whole *C. parvum* oocyst or sporozoite antigens for the generation of HBC (Chapter 2). Based on the results gathered from the HBC work, a pilot study was carried out to evaluate the feasibility of producing anti-*C. parvum* hyperimmune hen egg yolks (HEY) with therapeutic activities against *C. parvum*. In that study, whole *C. parvum* antigen at a concentration of 25 μg/hen/immunization was emulsified in Freund's adjuvant and used to generate HEY. The activities of HEY, both *in vitro* and *in vivo*, suggested the therapeutic potential of using HEY for the treatment of enteric cryptosporidiosis.

The information presented in this chapter describes the evaluation of four different adjuvants and three antigen concentrations for the generation of HEY. The objective of this work was to determine which adjuvant-antigen combination along with which concentration of antigen would generate the best immune response.

II. Methods

A. Animals, facilities and procedures

A.1. Hens

One hundred and two 16-week-old white Leghorn pullets were purchased from Stanford Farms (College Station, TX). The hens were housed at a dedicated farm and were certified to be *Salmonella* free. The farm was operated under the all-in/all-out principle, meaning keeping a uniform flock, hens of a single type and age, and that upon instalment, no hen could be introduced or re-introduced into the farm. No other birds or mammals were raised or kept in this building. About one week after arrival, all hens were weighed and given I.D. numbers using plastic leg bands. Also, all hens were bled prior to immunization by venipuncture of the alar vein. This initial bleeding established a baseline value for serum anti-*Cryptosporidium* antibody levels.

A.2. Location

The farm was located at the West Campus Agricultural Center, 0.5 miles west from Interstate 10. The chicken house was located approximately 150 yards west of the Arizona Veterinary Diagnostics Laboratory facilities. There were 60 multibird cages (single deck, back waterer type) divided into 3 parallel lanes of 20 each.

B. Procedures for hen coop maintenance

Daily animal care was vital to the health, immune responses and productivity of the layers. Special care was particularly important in the often severe climatic conditions of the desert. Well-established daily maintenance procedures ensured good health for the hens, which translated into the high antibody titers and egg production necessary for this research project.

Eggs were collected daily, starting in mid-morning with the last collection at 2pm. The photoperiod settings and the collection times were established in an effort to minimize exposure of the eggs to warm afternoon temperatures. All eggs were labeled according to their cage number and date, and transported to the laboratory.

C. Immunizations

C.1. Antigens:

All batches of oocysts and antigen utilized for this studies were prepared in the same way as described in Chapter 2. To verify consistency between batches of antigen, samples of subsequent batches were compared by SDS-PAGE. Briefly, the *Cryptosporidium parvum* antigen was electrophoresed on 5-15% gradient polyacrylamide gels at 30 mAmps constant current, stained with Coomassie blue and band patterns from the different batches were compared.

Based on previous results which indicated that 25 μg/immunization of *C. parvum* antigen per hen were effective in inducing strong immune responses, sequential antigen reductions were evaluated to identify the minimal equivalent antigen concentration needed to hyperimmunize the hens.

C.2. Adjuvants:

Four adjuvant systems, emulsified with three different antigen concentrations plus a control were tested. Each adjuvant was mixed or emulsified with the antigen solutions as per the instructions from the manufacturer. The adjuvants tested were: Hunters Titermax (CytRx Corporation, Norcross, GA), Ribi poultry adjuvant LES (metabolizable lipid) + STM (mitogen) at a ratio of 1mg STM in 1.25 ml LES, (Ribi Immunochemical Research, MT) and Freund's complete (FCA) and incomplete adjuvant (Sigma Chemical Co, MO) for the primary and booster immunizations immunization.

A mixture of Freund's Incomplete adjuvant (FIA) in conjunction of heat-inactivated *Mycobacterium avium intracellulare* (MAI) 1mg/10ml was also tested. This material was kindly provided by Dr. Antonino Catanzaro, University of California San Diego Medical Center. Two different inactivated *M. avium* preparations were tested, a heat-inactivated preparation at three concentrations plus a control. One small group was established to test a preparation of *M. avium* inactivated by sonication.

C.3 Antigen-Adjuvant mixtures

The emulsions of Titermax were prepared using the pestle homogenizer method. Similar to Freund's, this adjuvant required the use of Luer lock, 10ml all-plastic syringes (AirTite Corporation, Virginia Beach, VA).

Ribi: To prepare the adjuvant-antigen mixture, the vial containing LES + STM was warmed to 37°C prior to use. To prepare the mixtures, the antigen had to be diluted to the desired concentrations considering the ratio of 3 volumes of antigen in PBS (3.75ml) to the 1 volume of LES + STM (1.25ml). The mixtures were vortexed until a milky emulsion was formed. Each prepared dose (0.5ml) contained antigen (25, 15, 7.5 or 0 μg) and 100 μg of STM.

Freund's adjuvant-immunogen emulsification: This procedure was used to prepare all preparations which included Freund's adjuvant, either complete or incomplete. The Freund's adjuvant was emulsified by using the two-syringe, double hub needle method (mixing/emulsifier needle). All plastic syringes with luer lock ends were used for this process (AirTite, VA). One syringe was loaded with the antigen solution, the other with an equal volume of Freund's adjuvant. The fluids were then transferred from one syringe to the other until a milky homogeneous emulsion was formed. The adjuvant antigen mixture was tested for completeness by observing if a drop placed in cold water could hold its form.

D. Hen immunizations study design:

A total of 17 groups were established with 6 hens included per group. The antigen concentrations tested were: 25, 15, 7.5 and 0 μg per hen per immunization (Table 3). All hens were immunized subcutaneously in the upper third of the dorsal cervical area and received a booster immunization in the same site 4 weeks later.

E. *In vitro* monitoring of immune responses

Blood samples from chickens were collected from the alar vein on the dates of the first and second immunizations and every two weeks for the next 14 weeks. Serum was separated as described in Chapter 2. Yolk samples were prepared from eggs collected on the same dates as the serum samples. Egg yolks were collected and separated from the egg white, rolled onto paper towels and the yolk contents poured into a 50ml sterile conical tube. The egg yolk was then diluted 1:1 with PBS, mixed using a vortex mixer and stored at –20°C until tested.

Serum samples were tested for specific antibody reactivity by ELISA as described in the general ELISA procedure of Apprendix 1. Briefly, sonicated *C. parvum* antigen was immobilized onto 96-well plates and the samples were allowed to react with the immobilized antigen. When antibodies were present, they bound to the antigen proportional to their concentration. A secondary antibody, labeled with an enzyme, was then allowed to bind to the primary antibodies present. A substrate solution for the enzyme plus a chromogen were added to the reaction. The intensity of the reaction was measured in an

ELISA Reader (Dynatech, MRX, VA) using a 450 nm wavelength optical filter. The resulting optical densities (OD) were directly proportional to the concentration of the *C. parvum* specific antibody present in the sample.

For the evaluation of serum samples, the extracted serum was diluted 1:1000 in PBS and used as primary antibody in ELISA. For generation of yolk data, all eggs per group were collected every Thursday and pooled to generate a representative data point. The pooled yolks were separated from whites, diluted 1:1 in PBS in 50 ml conical tubes and vortexed to homogeneity. Yolk samples were diluted 1:1000 in PBS and used as primary antibody in the ELISA testing. Results from the ELISA testing were tabulated in computer spreadsheets and analyzed. Statistical analysis was performed using two-tailed Student's t-test.

In addition to ELISA testing, the activity of the egg yolk antibodies was titrated by immune fluorescent assay (IFA) using excysted sporozoites. Ten-well slides were pre-coated with 1% poly-L-Lysine (Sigma Chemical Co. MO). Sporozoites purified as described in Chapter 2 were diluted to 50,000 sporozoites/ml and 10 ul of this preparation were applied per well. Slides were allowed to air dry and stored at -20°C until used. Two-fold serial dilutions from egg yolk preparations starting at 1:200 were made and applied at 10ul/well. Affinity purified goat anti chicken IgG FITC (KPL, MD) diluted 1:500 were used to detect the bound chicken antibodies. Slides were read in a epifluorescense microscope using 450-490 nm dichroic mirror excite filters.

C. parvum antigen recognition by egg yolk antibody samples was evaluated by Western Blot analysis (Appendix 2). Briefly, sonicated oocysts were boiled in SDS-PAGE reducing sample buffer, electrophoresed in 10-15% gradients polyacrylamide gels and the separated *C. parvum* fractions were electro-transferred onto nitrocellulose membranes. Blotted membrane strips were probed with egg yolk antibodies and the reaction was visualized by the use of a goat anti-chicken IgG HRPO followed by TMB chromogen (KPL, MD).

III. Results

A. Immunization groups and immunizations

Adverse immunization and blood sample collection reactions were not observed during this study. Local inflammatory reactions were not detected by palpation at the injection sites in the groups receiving the Ribi and Titermax adjuvants, regardless of the antigen concentration received. Moderate swelling at the injection site was detected in almost all hens from the groups receiving either Freund's complete adjuvant or Freund's incomplete adjuvant plus *M. avium*. Egg production was not affected by immunizations or blood sampling. No hens died nor were culled from the flock during the study period.

B. Sampling and evaluation of the immune potency of the samples

Seven blood samples were collected from all study hens for serum evaluations. A total of 13 egg collections per groups were made for ELISA evaluations. This lack of eggs for about two weeks was due to the young age and sexual inmaturity of the hens. Two data points for the ELISA yolk evaluation were not available. One was on July 10 for the Ribi 7.5 μg group, because that group started laying eggs 2 days later than the others. The other data point missing was for the Freund's 15 μg group, on date July 14, due to eggs accidentally damaged during transport.

B.1. *In vitro* activities: serum samples.

FIA+MAI groups: ELISA activities from these groups showed a marked increase in the anti-*C. parvum* activities from hens in the Groups receiving 25, 15 and 7.5 μg of *C. parvum* antigen plus 10 μg of heat inactivated *M. avium*. The group receiving 25 μg peaked at 4 weeks post prime immunization (OD=1.108) and maintained steady values for the next 6 weeks, decreasing to 0.725 by the end of the study. There was a moderate and transitory elevation in the antibody response six weeks after the booster immunization showing OD values of 1.055. The groups receiving 15 and 7.5 μg of antigen showed a rapid increase in antibody values by 2 weeks post prime immunzation (OD values 0.978 and 1.03 respectively) and showed peak OD values 6 weeks after the booster immunization (1.626 and 1.583 respectively). The hens receiving 25 μg of *C. parvum* antigen in combination with 10 μg of sonicated inactivated *M. avium* had the highest ELISA values 2 weeks after the booster immunization, with a gradual decrease in OD values over time (Figure 7).

The statistical analysis showed significant differences (all p values < 0.001) between the groups receiving *C. parvum* antigen and the control group. Significant differences were not detected between the hens receiving 25 μg antigen plus heat-inactivated *M. avium*, either heat-inactivated or sonicated, when compared to the hens in the groups receiving 15 and 7.5 μg of *Cryptosporidium* antigen (p values >0.133). Significant differences were not detected between the groups receiving 15 and 7.5 μg (p>0.8) (Table 4).

Titermax groups: ELISA activities from these groups showed a moderate increase in the anti-*C. parvum* activities from hens in the Groups receiving 15 and 7.5 μg of *C. parvum*

antigen. The group receiving 15 μg peaked at 4 weeks post prime immunization (OD=0.883) while the group receiving 7.5 μg peaked with an OD value of 1.0 at 2 weeks post prime immunization. In these groups, the ELISA values decreased over the next 8 weeks to OD values of 0.5 and 0.528 respectively, despite the booster immunization. The group immunized with 25 μg had a poor response, with OD peak values of 0.5133 four weeks after the prime immunization and decreasing to 0.476 at 8 weeks after the booster (Figure 8).

The statistical analysis shows marginally significant differences (p values < 0.05) between the groups receiving 15 and 7.5 μg of *C. parvum* antigen and the control group. Similar significance values were obtained when these two groups were compared to the 25 μg group (p values of 0.035 for the 15 μg group and p=0.023 for the 7.5 group). Significant differences were not detected between the hens receiving 25 μg antigen and the control group (p>0.7) and between the two moderately responding groups receiving 15 and 7.5 μg of antigen (p>0.4) (Table 4).

Freund's groups: ELISA activities from these groups showed a marked increase in the anti-*C. parvum* activities from hens in the Groups receiving 25, 15 and 7.5 μg of *C. parvum* antigen. Two weeks after the prime immunization these three groups had ELISA OD values of 1.065, 1.26 and 1.32 respectively. All three groups had similar antibody response curves for the duration of the study. The three groups peaked 4 weeks after the booster immunization with a slight decrease towards the end of the study. The 25 μg group

peaked with an OD value of 1.618, the 15 μg group had OD values of 1.522 and the 7.5 μg group had OD values of 1.815 (Figure 9).

The statistical analysis showed significant differences (p values < 0.001 for all groups) between the groups receiving *C. parvum* antigen and the control group. Significant differences were not detected between the hens receiving 25 μg antigen and 15 or 7.5 μg of *Cryptosporidium* antigen (p values >0.722). Significant differences were not detected between the groups receiving 15 and 7.5 μg (p>0.72) (Table 4).

Ribi groups: ELISA activities from the three groups receiving antigen plus Ribi adjuvant showed a moderate increase in anti-*C. parvum* activities. Hens in the group immunized with 25 μg of antigen, had a mild increase in OD values (OD= 0.387) by week 2 post prime immunization. Subsequent OD values were never greater than 0.45, with peak ODs six weeks after the last immunization (OD=0.482). The other two groups had very mild responses. Groups immunized with 15 μg peaked at 6 weeks after the second immunization with OD activities of 0.705. The hens immunized with 7.5 μg had a similar behavior in OD responses, with peak values (OD=0.663) six weeks after the booster immunization. In general, all groups had an increase in their OD values after the second immunization, but the persistence of this increased reactivity was almost undetectable. Eight weeks after the second immunization (booster) the OD values had decreased to levels similar to those detected at baseline (Figure 10).

The statistical analysis showed marginally significant differences (p =0.037) between the groups receiving 25 μg and the control (adjuvant alone). There were significant

differences between the group immunized with 15 μg of *C. parvum* antigen and the control group and no significant differences between the groups immunized with 7.5 and 0 μg of antigen (p=0.053). Even though the group immunized with 15 μg had better OD values, significant differences were not detected between the response of this group and the group immunized with 25 μg (p=0.085) or 7.5 μg (p=0.373). Additionally, no significant differences were detected between the hens receiving 7.5 μg and 25 μg of antigen (p>0.57) (Table 4).

B.2. *In vitro* activities: yolk samples.

Yolk samples were also quantitatively analyzed for their anti-*Cryptosporidium* activities. All groups had patterns similar to their OD values found in serum.

FIA+MAI groups: ELISA OD values from three groups showed marked increments in OD activities: two groups receiving heat-inactivated MAI plus antigen (15 and 7.5 μg) and the group immunized with sonicated MAI plus 25 μg antigen. Moderate erratic increments were also observed in the group receiving heat-inactivated MAI plus 25 μg antigen. The hens immunized with 25 μg antigen plus sonicated MAI had peak values of 1.22 two weeks after the booster; hens which received 15 μg plus heat-inactivated MAI peaked on the same week, with OD values of 1.86. The third group with significant increments was immunized with 7.5 μg of the immunogen and heat inactivated MAI, with peak OD values of 1.69 for two consecutive weeks following the booster immunization (Figure 11).

The Student's t-test analysis for these groups indicated statistically significant differences between all groups receiving *Cryptosporidium* antigen and the negative control ($p<0.01$). There also were significant differences when the group immunized with heat-inactivated MAI plus 25 μg of antigen (MAI-H-25) was compared with the remaining groups ($p<0.002$). This analysis determined that data from MAI-H-25 was significantly lower when compared to the other three groups. Analysis of the three groups with significant OD increments indicated there were non significant differences in the antibody responses between these groups ($p>0.19$ (Table 5)).

Titermax groups: The groups of hens immunized using Titermax adjuvant had mild immune responses, reaching their highest points on weeks 1 and 2 after the booster immunization. The peak OD values were 0.54 for the hens immunized with 25 μg, 0.47 for the group receiving 15 μg of antigen and 0.59 for the group immunized with the 7.5 μg of *C. parvum* antigen (Figure 12).

Statistically significant differences were seen between the hens immunized with 25 and 7.5 μg of immunogen, where the group receiving the lower dose (7.5 μg) of antigen had OD values that were significantly greater than those of the group receiving 25 μg. Analysis between all other groups immunized with Titermax, reflected differences with no statistical significance, where $p> 0.12$ (Table 5).

Freund's groups: In these study groups all three sets of hens receiving *C. parvum* antigen had statistically significantly greater ELISA OD values than the control group. The three groups had peak OD values of 1.56 for the Freund's + 25 μg group, 1.57 for the

Freund's + 15 μg group and 1.61 for the Freund's + 7.5 μg group at 2 and 3 weeks after the booster immunization. Those values decreased gradually over time, with optical densities greater than 0.6 by the end of the study (Figure 13).

The Students t-test analysis of data from this group indicates statistically significant differences between all groups receiving *Cryptosporidium* antigen and the negative control ($p<0.0001$). No significant differences were established between the three groups ($p>0.61$).

Ribi groups: All samples from the groups immunized with Ribi as an adjuvant exhibited mild to poor anti-*C. parvum* activities. There were marginal increases in the OD values (Figure 14).

The statistical analysis showed no signifcant differences between any of the groups immunized with amounts of *C. parvum* antigen and the control group ($p>0.195$). The only significant difference, event though marginal was between the ODs of the groups immunized with 25 and 7.5 μg, ($p=0.03$). No other significant differences were detected (Table 5).

C. Statistical analysis between groups:

Analysis of the data showed that the group immunized with heat inactivated MAI + 25 μg antigen had significantly lower responses than all other responder groups immunized with Freund's adjuvant ($p<0.001$).

No statistically significant differences were detected between the sonicated MAI plus 25 μg antigen and any of the three groups immunized with Freund's + 25 μg ($p=0.09$),

Freund's + 15 μg (p=0.26) and Freund's + 7.5 μg (p=0.11). Similar values were obtained when statistical testing was performed between group MAI-H-15 and the three Freund's groups (25, 15 and 7.5 μg), where the p values were greater than 0.8, and when the data from the group MAI-H-7.5 was compared to the three Freund's groups, p>0.74.

The groups immunized using Ribi and Titermax adjuvants had statistically significantly lower ELISA activities than the Freund's responders, the sonicated MAI+25 μg and the heat inactivated MAI + 15 and 7.5 μg, where the significance values of p were <0.007.

A total of 6 groups, Freund's 25 μg, Freund's 15 μg, Freund's 7.5 μg, MAI-H 15 μg, MAI-H 7.5 μg and sonicated MAI 25 μg, had elevations in antibody responses. The statistical significance between the groups demonstrated no differences between any of them (p>0.7).

D. SDS-PAGE, Western Blot and IFA

Three batches of antigen were compared by SDS-PAGE , with no detectable differences. Samples of pooled yolk were also tested by IFA and analyzed by Western Blot analysis. The IFA titers for samples of pooled yolks against excysted sporozoites were >10,240. The Western Blot analysis showed that egg yolk antibodies reacted with bands of 184.1, 155.2, 130.8, 88.5, 79.6, 68.7, 64.4, 59.8, 51.7, 44.6, 36.1, 32.8, 30.2, 23.6, 19.3, 16.3 and 14.7 KDa (Photo 1).

IV. Discussion

The production of anti-*C. parvum* hyper-immune yolks had different outcomes depending on the adjuvant and concentration of antigen used. Data generated from serum and yolk samples were complementary when statistically analyzed. Data and statistical analysis indicate that the use of Ribi or Titermax would not generate optimal responses for hyperimmunization. Unexpectedly, there was almost no response to immunizations in hens where Ribi was used as an adjuvant. Based on the information from the manufacturer, and on the previous experience with this kind of adjuvant in the production of bovine colostrum, a significantly enhanced antibody production was expected. Based on the data gathered in thisstudy, Ribi was deemed unsuitable as an adjuvant for poultry for the generation of anti-*C. parvum* HEY. Overall, Ribi does not provide the quantitative evidence to support its use as an adjuvant of choice in chickens, though the use of this adjuvant system may prove effective for other antigens.

Titermax is an adjuvant that has been successfully used in rodents. The overall responses obtained with Titermax showed a moderate enhancement of anti-*C. parvum* antibody production. The statistical analysis determined that those increments in antibody activities were significantly lower than the responses obtained when using Freund's adjuvants. Titermax may require further testing to elucidate its role in poultry applications, though for the purposes of this study it was not considered as the best candidate for the next stage of testing.

Freund's adjuvant was tested in two modalities, the standard protocol and a modified protocol. The standard protocol follows the manufacturers instructions, which consists of prime immunizations using Freund's complete adjuvant, with boosters of antigen emulsified in Freund's incomplete adjuvant. In the modified protocol, the objective was to determine if heat inactivated *M. avium* could also be used in conjunction with Freund's adjuvant. These modifications were made to evaluate if in prime immunizations, *M. avium* could replace *M. tuberculosis* as an adjuvant and to determine and if MAI affected the chicken immune responses to *C. parvum* antigens. The data supported the role of MAI as an adjuvant for the production of anti-*C. parvum* HEY.

Data analysis from both groups immunized with Freund's adjuvant indicates significantly enhanced antibody responses in the three groups immunized with FCA followed by FIA at the three *C. parvum* concentrations of 25, 15 and 7.5 μg/hen. Similar data was obtained from the 4 groups where *M. avium* was substituted for *M. tuberculosis* in the prime immunizations. When differences in the antibody activities between all these responder groups were compared, one of the seven groups was significantly lower than the others (MAI-H-25 μg). The other six groups had no statistically significant differences, with p values greater than 0.7, indicating strong similarities in the antibody activities evaluated. One group, however, had data sets with slightly greater values than the others. This antigen concentration FCA + 7.5 μg/hen was considered for further evaluations. Further testing of this antigen concentration using Freund's adjuvant was performed outside the scope of this research work. Antigen concentrations of 7.5, 5, 2.5

and 1 μg/hen were emulsified in Freund's adjuvants and tested following procedures similar to those described in this section. Those studies demonstrated that 7.5 μg/hen were was significantly better than any of the lower concentrations.

The conclusions from this study are that Freund's adjuvants, used by the standard protocol or by substituting *M. tuberculosis* with *M. avium*, generate greater anti-*C. parvum* antibody activities both in egg yolks and in serum. The antigen concentrations of 25, 15 and 7.5 μg/hen, when emulsified in Freund's adjuvant, engendered similar hyperimmunization levels in hens. The lowest dose level (7.5 μg/hen) was therefore used in follow up studies.

Table 3. Hen immunization groups

Groups	Adjuvant-Antigen preparation	Antigen concentration (μg/hen)
1	FIA+MAI (0.5mg/ml) (HEAT)	25
2	FIA+MAI (0.5mg/ml) (SONICATED)	25
3	FIA+MAI (0.5mg/ml) (HEAT)	15
4	FIA+MAI (0.5mg/ml) (HEAT)	7.5
5	FIA+MAI (0.5mg/ml) (HEAT)	0
6	Titermax	25
7	Titermax	15
8	Titermax	7.5
9	Titermax	0
10	Ribi	25
11	Ribi	15
12	Ribi	7.5
13	Ribi	0
14	FCA/FIA	25
15	FCA/FIA	15
16	FCA/FIA	7.5
17	FCA/FIA	0

Table 4. ELISA serum activities

SERUM OD CHICKENS						T-TEST	p value
	MAI-H 25	MAI-H 15	MAI-H 7.5	MAI-H 0	MAI-S 25	MAI-H	
6-23	0.288	0.367	0.292	0.263	0.203	25vs15	0.133
7-10	0.875	0.979	1.030	0.342	0.855	25vs7.5	0.189
7-24	1.108	0.991	0.997	0.325	1.648	25vs0	0.000
8-7	1.013	1.469	1.322	0.315	1.708	15vs7.5	0.848
8-21	0.953	1.470	1.467	0.277	1.270	15v0	0.000
9-4	1.055	1.626	1.583	0.273	1.300	7.5vs0	0.000
9-18	0.728	1.314	1.212	0.260	1.120	25vsMAI-S	0.203
						15vsMAI-S	0.950
						7.5vsMAI-S	0.911
	TITERMAX					0vsMAI-S	0.001
	25	15	7.5	0		TITERMAX	
6-23	0.270	0.323	0.213	0.363		25vs15	0.035
7-10	0.505	0.787	1.000	0.545		25vs7.5	0.023
7-24	0.513	0.883	0.930	0.558		25vs0	0.714
8-7	0.337	0.545	0.768	0.405		15vs7.5	0.472
8-21	0.415	0.627	0.753	0.473		15v0	0.049
9-4	0.470	0.620	0.725	0.417		7.5vs0	0.029
9-18	0.477	0.500	0.528	0.348			
	FCA 25	FCA 15	FCA 7.5	FCA 0		FCA	
6-23	0.305	0.355	0.262	0.358		25vs15	0.987
7-10	1.065	1.260	1.320	0.248		25vs7.5	0.722
7-24	1.095	1.113	1.390	0.375		25vs0	0.000
8-7	1.573	1.532	1.510	0.240		15vs7.5	0.722
8-21	1.618	1.522	1.815	0.327		15v0	0.000
9-4	1.507	1.458	1.592	0.273		7.5vs0	0.000
9-18	1.115	1.065	1.047	0.188			

Table 4. ELISA serum activities (cont.)

	Ribi 25	Ribi 15	Ribi 7.5	Ribi 0	Ribi	
6-23	0.370	0.345	0.312	0.375	25vs15	0.085
7-10	0.387	0.640	0.620	0.278	25vs7.5	0.574
7-24	0.448	0.517	0.525	0.335	25vs0	0.037
8-7	0.388	0.495	0.282	0.278	15vs7.5	0.373
8-21	0.440	0.492	0.323	0.287	15v0	0.002
9-4	0.482	0.705	0.663	0.135	7.5vs0	0.053
9-18	0.188	0.338	0.275	0.262		

TITERMAX	p value	Ribi	p value	MAI-H	p value
15vsFCA25	0.010	15vs FCA 25	0.003	25vsFCA25	0.137
15vsFCA15	0.006	15vs FCA 15	0.001	25vsFCA15	0.109
15vsFCA7.5	0.007	15vsFCA7.5	0.002	25vsFCA7.5	0.082
7.5vsFCA25	0.033	7.5vsFCA25	0.001	15vsFCA25	0.971
7.5vsFCA15	0.023	7.5vsFCA15	0.001	15vsFCA15	0.956
7.5vsFCA7.5	0.021	7.5vsFCA7.5	0.001	15vsFCA7.5	0.690
15vsMAI-H-25	0.076	15vsMAI-H-25	0.011	7.5vsFCA25	0.824
15vsMAI-H-15	0.008	15vsMAI-H-15	0.002	7.5vsFCA15	0.802
15vsMAI-H-7.5	0.012	15vsMAI-H-7.5	0.003	7.5vsFCA7.5	0.567
7.5vsMAI-H-25	0.303	7.5vsMAI-H-25	0.005	MAI-S-25	
7.5vsMAI-H-15	0.030	7.5vsMAI-H-15	0.001	vsFCA25	0.924
7.5vsMAI-H-7.5	0.044	7.5vsMAI-H-7.5	0.002	vsFCA15	0.909
15vsMAI-S-7.5	0.021	15vsMAI-S-7.5	0.007	vsFCA7.5	0.670
7.5vsMAI-S-25	0.059	7.5vsMAI-S-25	0.004		

Table 5. ELISA yolk activities

date	maH25	maH15	maH7.5	maH0	maS25	MAI-H	p value
10-Jul	0.06	0.54	1.01	0.09	1.110	25vs15	0.000
17-Jul	0.82	0.97	1.29	0.09	1.200	25vs7.5	0.000
24-Jul	0.07	0.88	1.27	0.05	0.960	25vs0	0.009
31-Jul	0.39	1.4	1.69	0.17	1.200	15vs7.5	0.873
7-Aug	0.18	1.86	1.69	0.15	1.220	15v0	0.000
14-Aug	0.8	1.46	1.51	0.16	0.910	7.5vs0	0.000
21-Aug	0.89	1.41	1.38	0.12	1.180	25vsMAI-S	0.002
28-Aug	0.09	1.19	1.27	0.14	0.590	15vsMAI-S	0.196
4-Sep	0.11	1.13	0.71	0.11	1.090	7.5vsMAI-S	0.170
11-Sep	0.05	0.68	0.66	0.08	0.071	Svs0	0.000
18-Sep	0.58	1	0.45	0.14	0.820		
25-Sep	0.78	0.75	0.66	0.07	0.520		
		Titermax					
date	25	15	7.5	0		Titermax	
10-Jul	0.39	0.69		0.18		25vs15	0.122
17-Jul	0.16	0.24	0.53	0.28		25vs7.5	0.015
24-Jul	0.15	0.22	0.41	0.46		25vs0	0.268
31-Jul	0.54	0.39	0.59	0.46		15vs7.5	0.296
7-Aug	0.021	0.45	0.36	0.54		15v0	0.608
14-Aug	0.25	0.37	0.6	0.44		7.5vs0	0.122
21-Aug	0.26	0.33	0.49	0.31			
28-Aug	0.39	0.47	0.2	0.2			
4-Sep	0.16	0.31	0.59	0.27			
11-Sep	0.11	0.22	0.23	0.19			
18-Sep	0.24	0.12	0.2	0.06			
25-Sep	0.07	0.15	0.23	0.18			
date	FCA25	FCA15	FCA7.5	FCA0		FCA	
10-Jul	1.08	0.9	0.25	0.11		25vs15	0.664
17-Jul	1.11	1.23	0.84	0.08		25vs7.5	0.875
24-Jul	1.15		1.19	0.11		25vs0	0.000
31-Jul	1.37	1.01	1.53	0.13		15vs7.5	0.615
7-Aug	1.56	1.42	1.61	0.11		15v0	0.000
14-Aug	1.36	1.57	1.59	0.17		7.5vs0	0.000

Table 5. ELISA yolk activities (cont.)

date	FCA25	FCA15	FCA7.5	FCA0		FCA	
21-Aug	1.35	1.52	1.22	0.1			
28-Aug	1.17	0.85	1.27	0.06			
4-Sep	1.22	1.33	1.42	0.22			
11-Sep	0.7	0.87	1.08	0.14			
18-Sep	0.71	0.56	0.96	0.02			
25-Sep	0.85	0.6	0.93	0.05			
date	Ribi25	Ribi15	Ribi7.5	Ribi 0		Ribi	p value
10-Jul	0.23	0.2	0.12	0.09		25vs15	0.916
17-Jul	0.16		0.1	0.1		25vs7.5	0.033
24-Jul	0.21	0.14	0.11	0.07		25vs0	0.088
31-Jul	0.32	0.28	0.19	0.09		15vs7.5	0.038
7-Aug	0.28	0.21	0.14	0.1		15v0	0.102
14-Aug	0.2	0.22	0.21	0.07		7.5vs0	0.569
21-Aug	0.12	0.12	0.12	0.15			
28-Aug	0.14	0.16	0.07	0.14			
4-Sep	0.26	0.22	0.15	0.23			
11-Sep	0.1	0.28	0.11	0.15			
18-Sep	0.07	0.06	0.05	0.14			
25-Sep	0.08	0.06	0.04	0.23			
TITERMAX		p value	Ribi		p value	MAI-H	
15vsFCA25		0.000	15vsFCA25		0.000	25vsFCA25	0.000
15vsFCA15		0.000	15vsFCA15		0.000	25vsFCA15	0.000
15vsFCA7.5		0.000	15vsFCA7.5		0.000	25vsFCA7.5	0.000
7.5vsFCA25		0.000	7.5vsFCA25		0.000	15vsFCA25	0.825
7.5vsFCA15		0.000	7.5vsFCA15		0.000	15vsFCA15	0.859
7.5vsFCA7.5		0.000	7.5vsFCA7.5		0.000	15vsFCA7.5	0.882
15vsMAI-H-25		0.523	15vsMAI-H-25		0.049	7.5vsFCA25	0.982
15vsMAI-H-15		0.000	15vsMAI-H-15		0.000	7.5vsFCA15	0.745
15vsMAI-H-7.5		0.000	15vsMAI-H-7.5		0.000	7.5vsFCA7.5	0.882
7.5vsMAI-H-25		0.993	7.5vsMAI-H-25		0.010	MAI-S-25	
7.5vsMAI-H-15		0.000	7.5vsMAI-H-15		0.000	vsFCA25	0.087
7.5vsMAI-H-7.5		0.000	7.5vsMAI-H-7.5		0.000	vsFCA15	0.258
15vsMAI-S-7.5		0.000	15vsMAI-S-7.5		0.000	vsFCA7.5	0.110
7.5vsMAI-S-25		0.000	7.5vsMAI-S-25		0.000		

Figure 7: ELISA serum activities M.avium+FIA

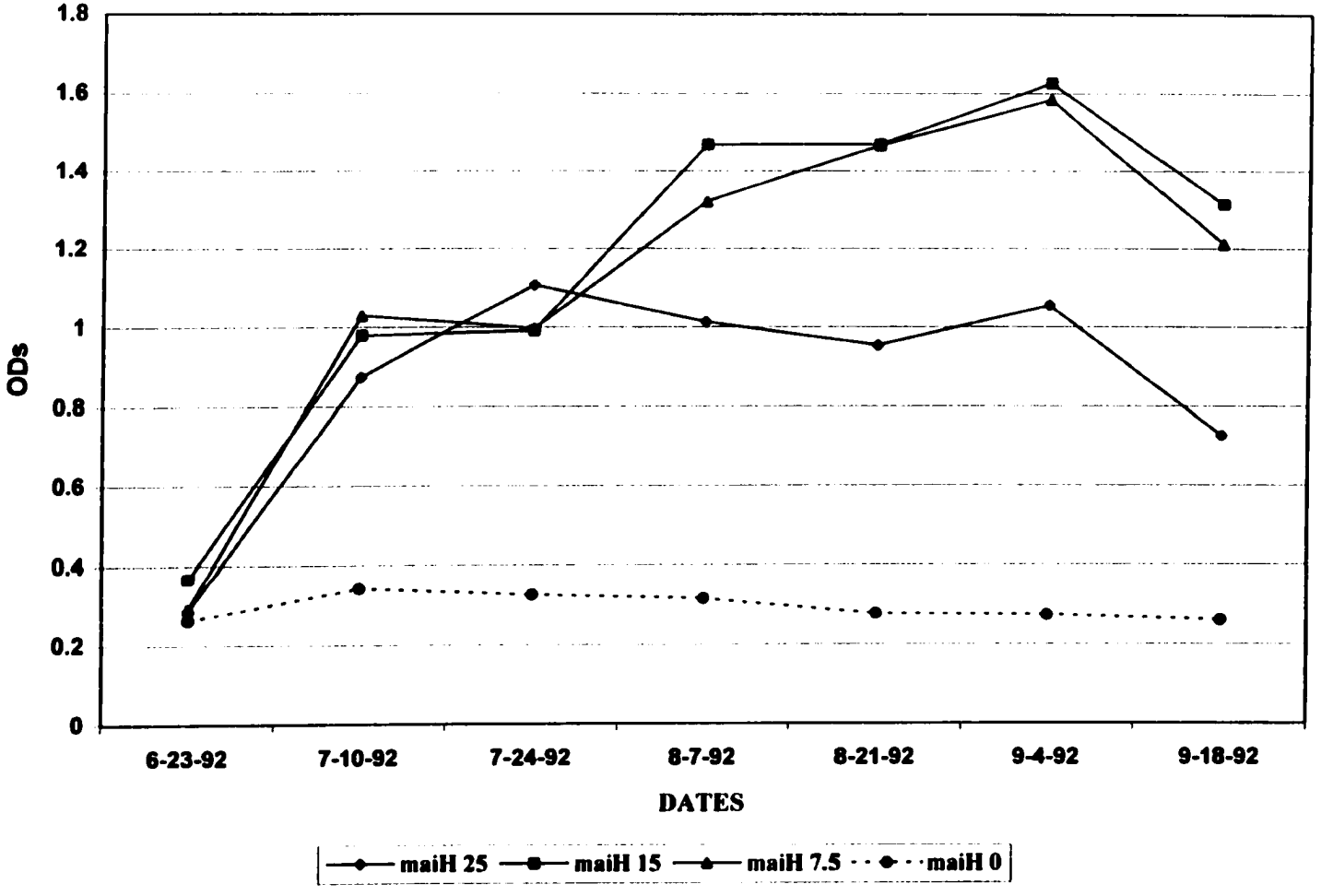


Figure 8: ELISA serum activities Titermax

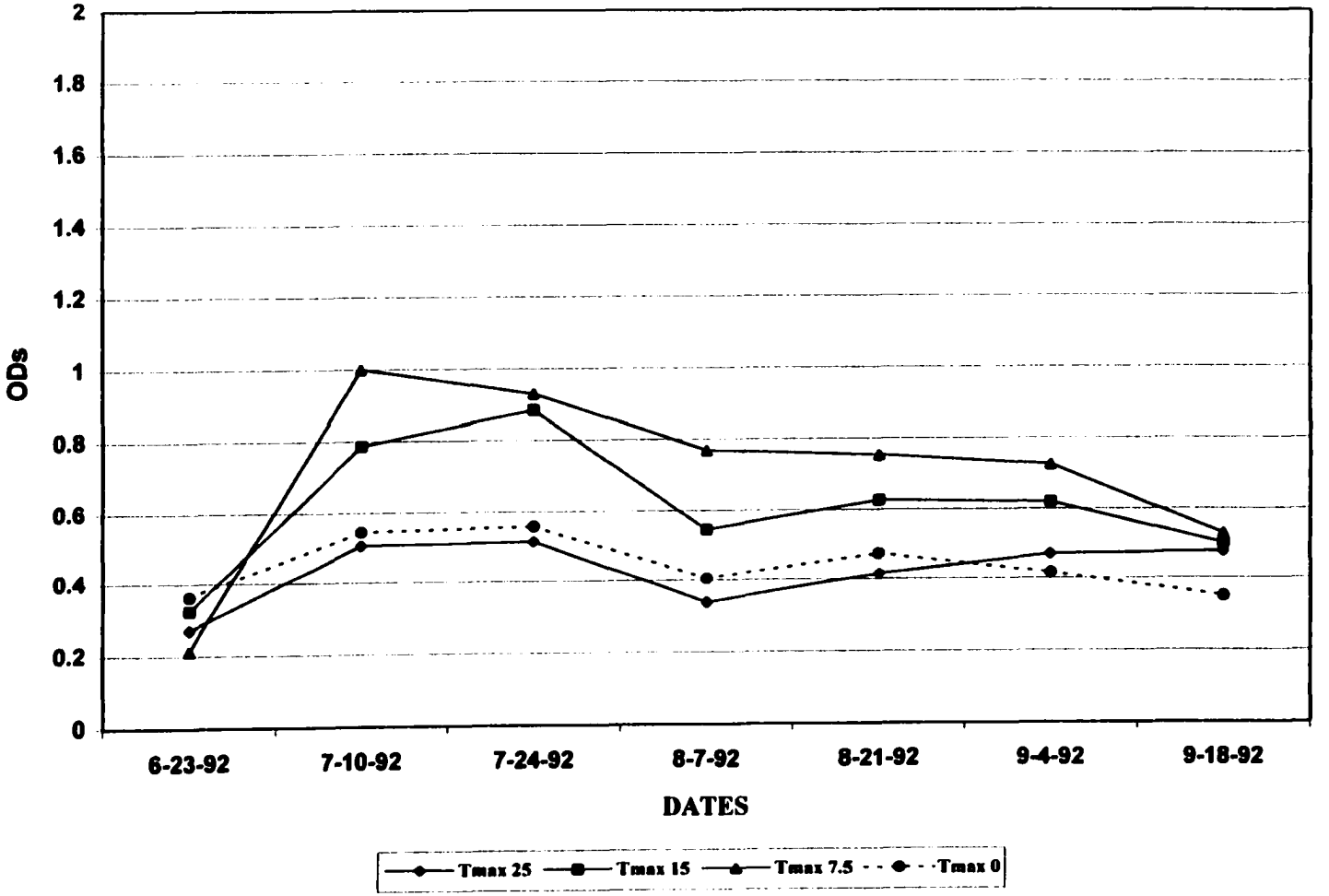


Figure 9: ELISA serum activities Freund's

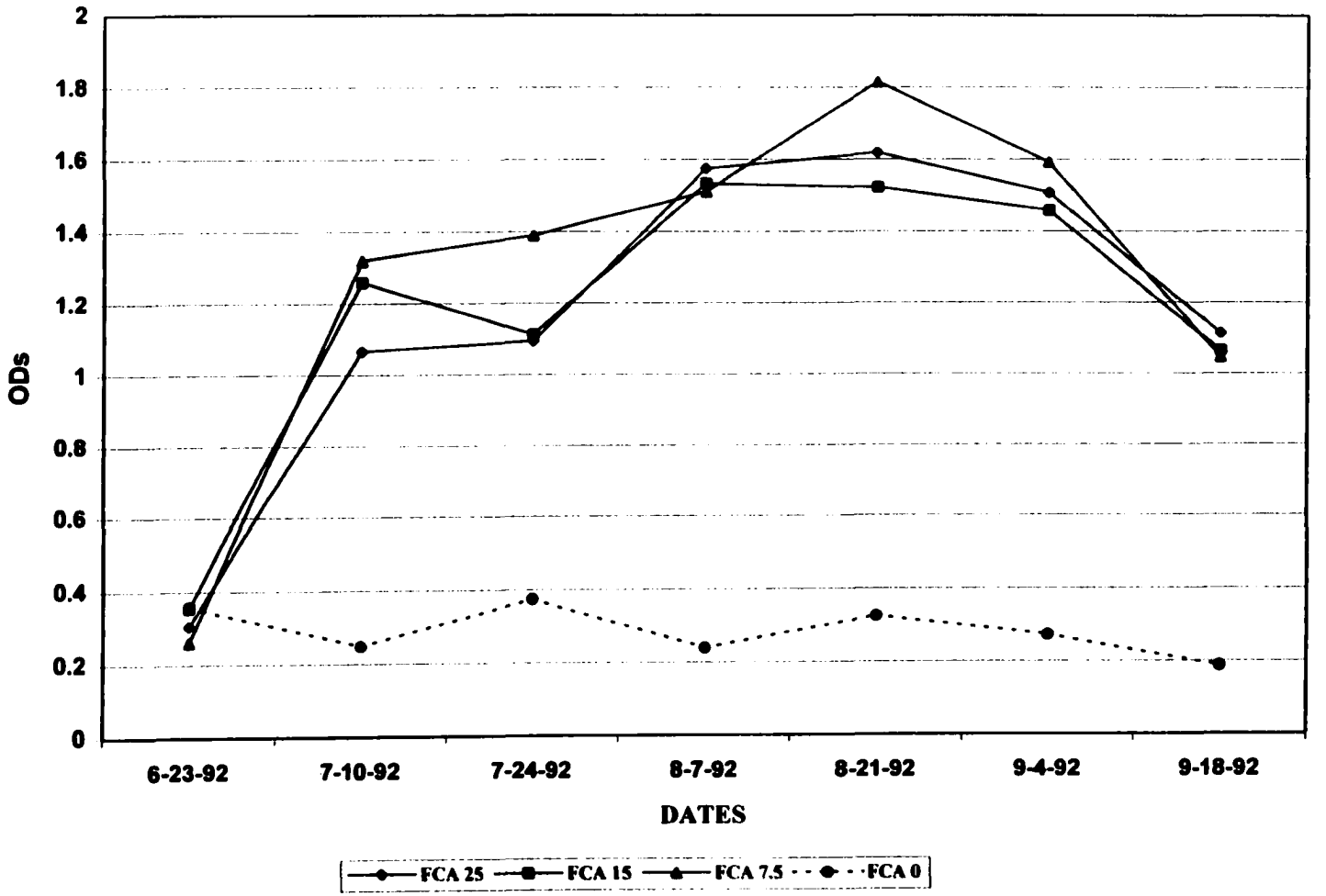


Figure 10: ELISA serum activities Ribi

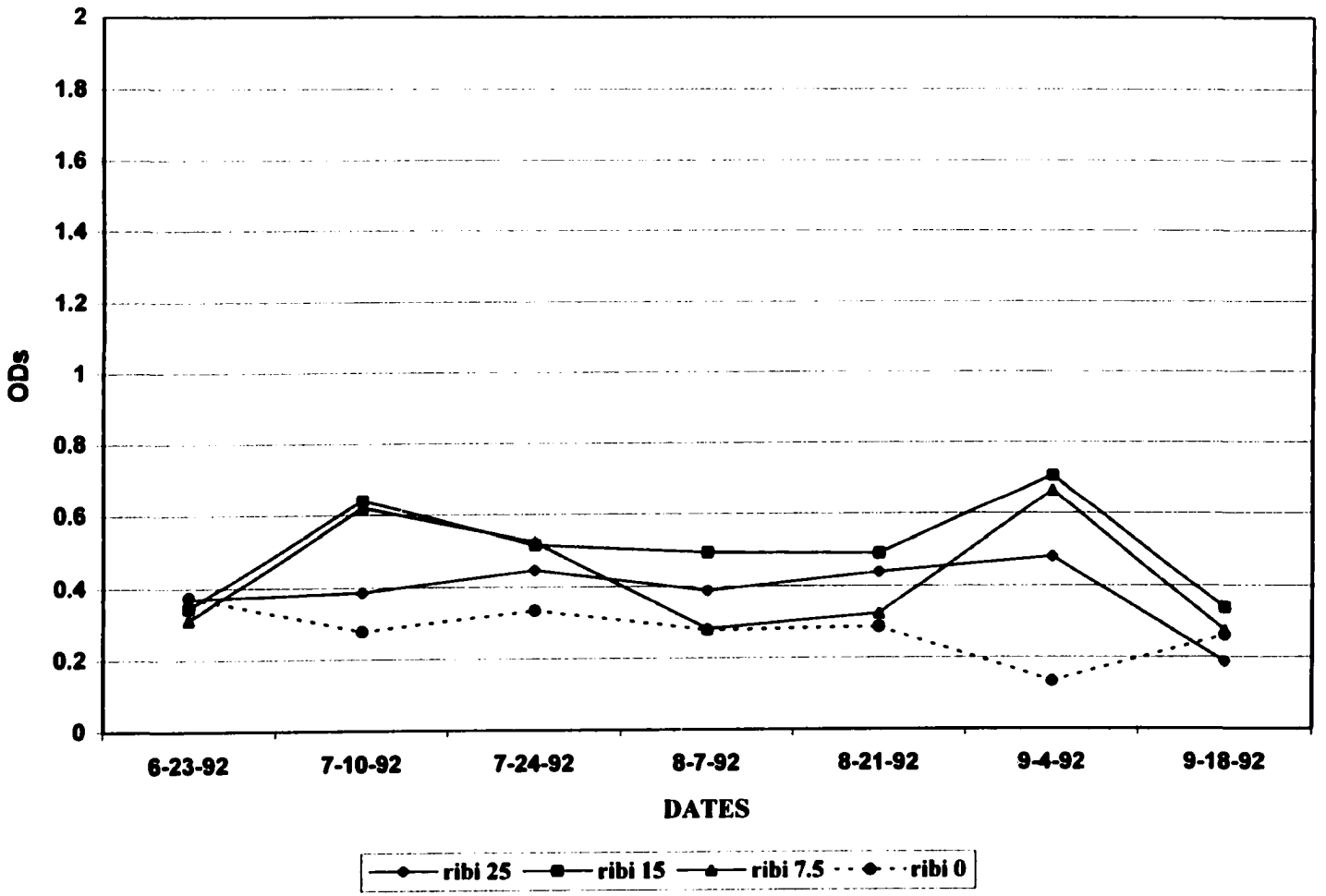


Figure 11: ELISA yolk activities M.avium+FIA

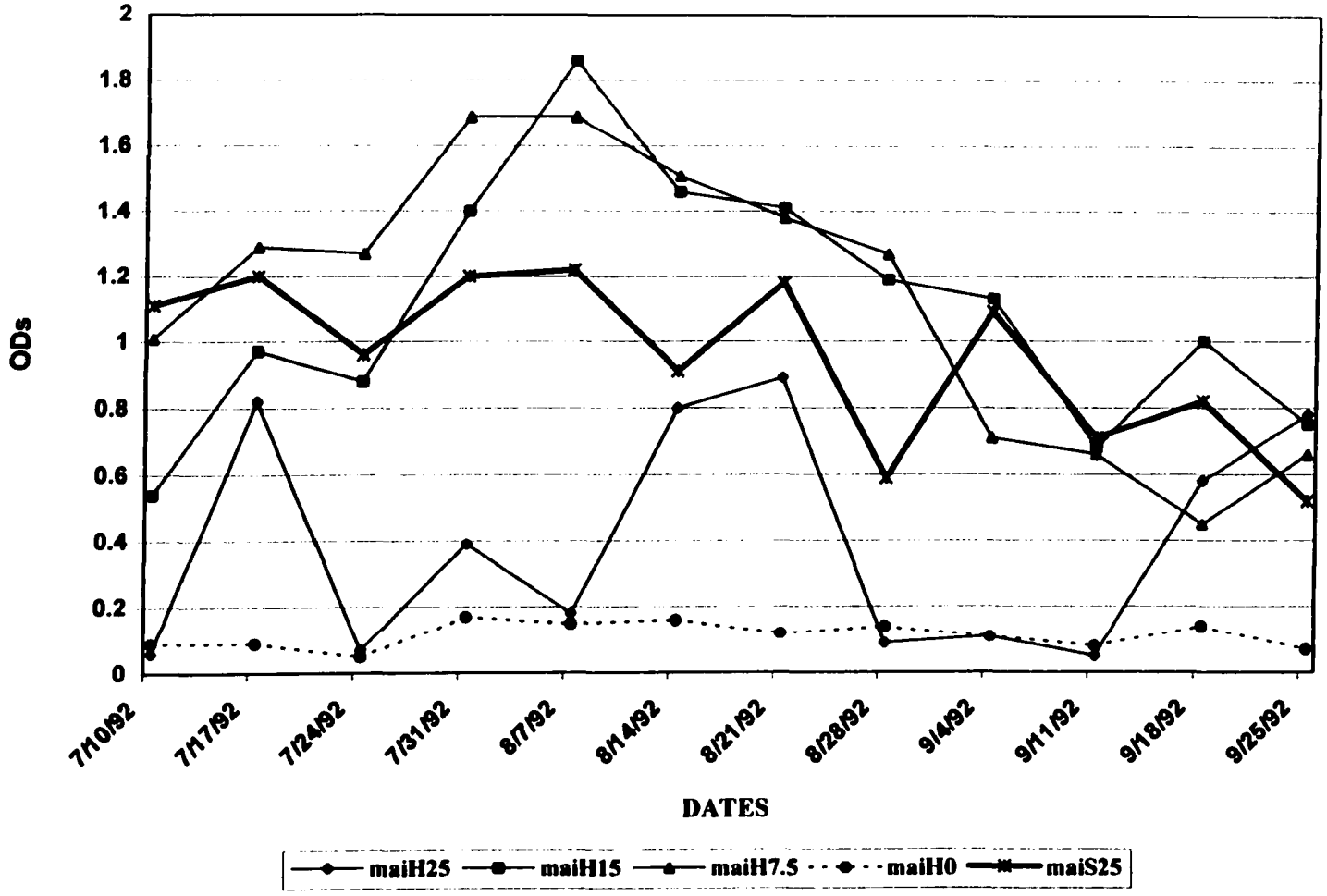


Figure 12: ELISA yolk activities Titermax

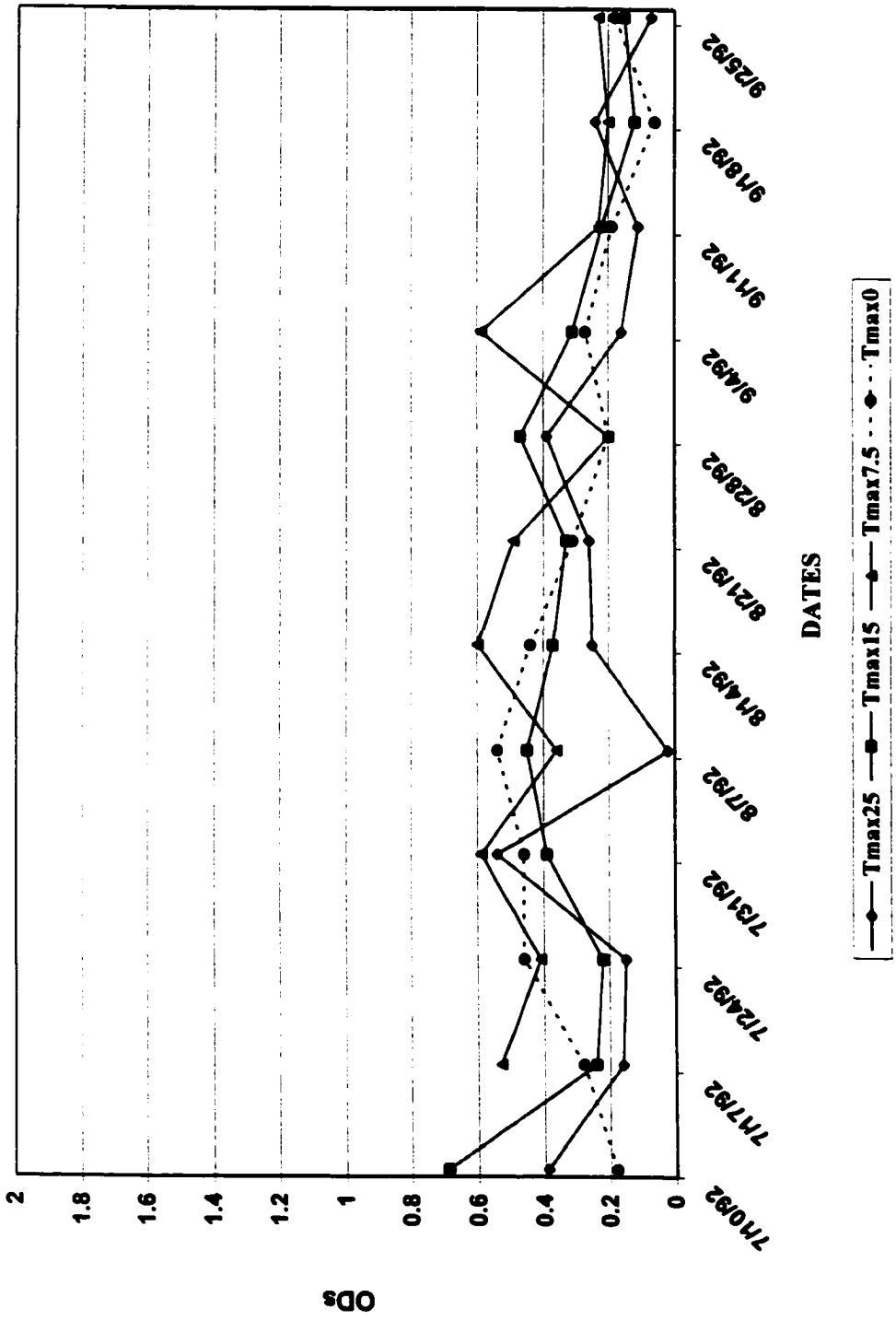


Figure 13: ELISA yolk activities Freund's

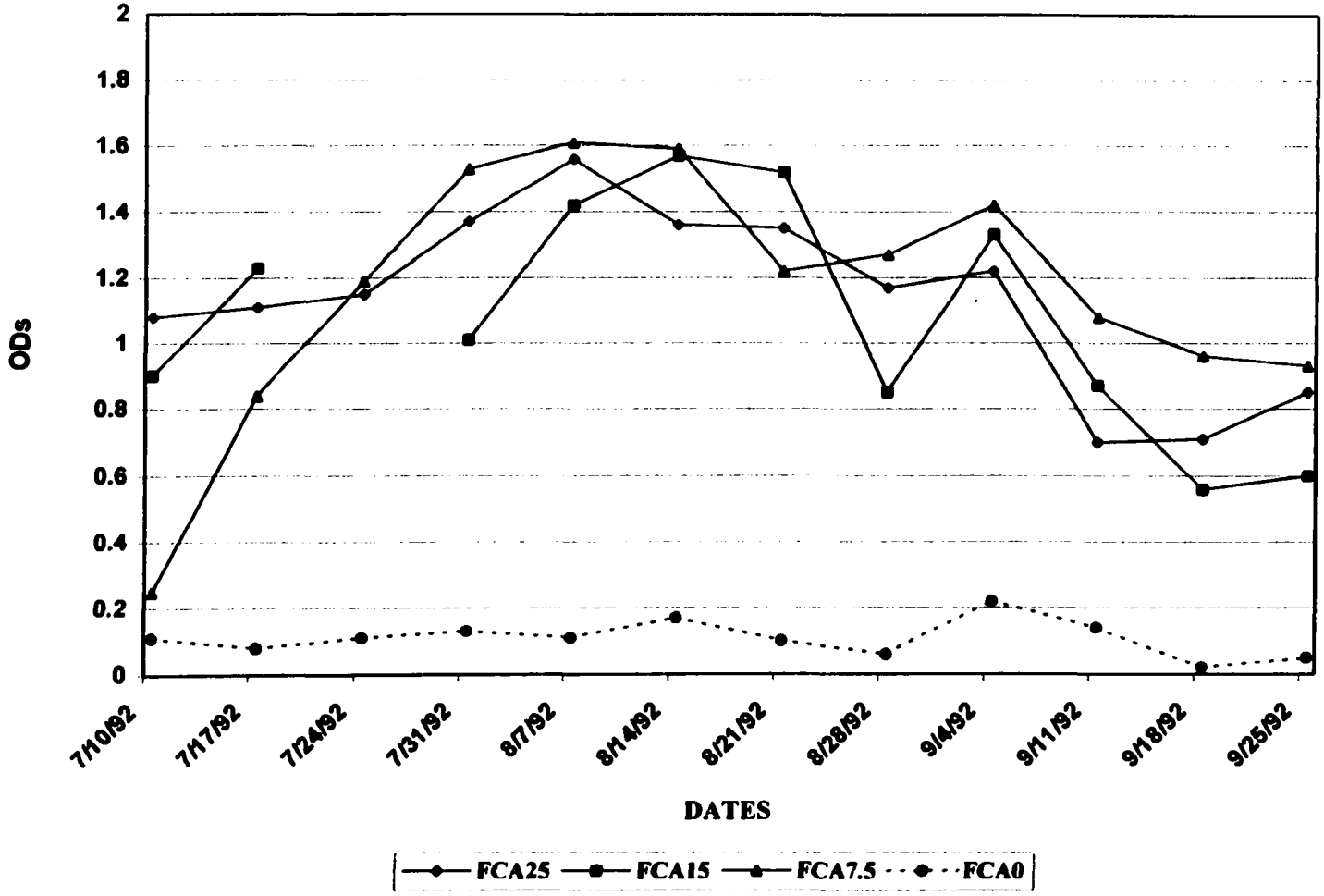
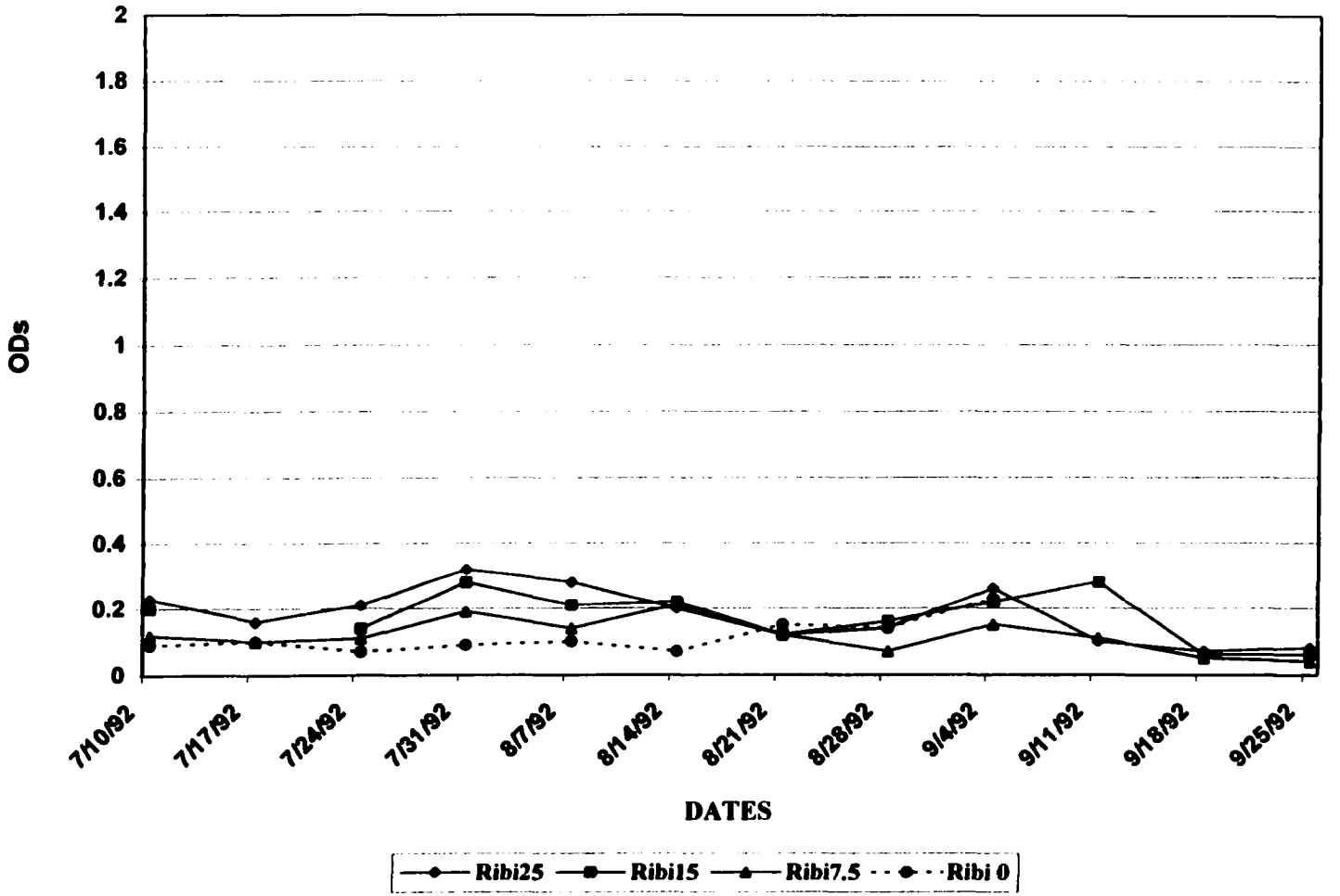


Figure 14: ELISA yolk activities Ribi



CHAPTER 4: *IN VITRO* ANALYSIS OF HUMAN BREAST MILK SAMPLES FOR ANTI-*Cryptosporidium parvum* ACTIVITIES

I. Introduction

Clinical cryptosporidial disease associated with infection is most prevalent in the immunocompromised and in the young, especially of less developed countries. Epidemiological data from urban and rural settings of countries in development indicate that up to 20% of their pediatric populations suffer bouts of diarrhea and dehydration due to infection with *C. parvum* (Ungar et al, 1988). Results of some studies suggested that protection from infection with *Cryptosporidium* may be afforded through breast feeding since infection was rarely detected in those children exclusively breast fed (Ziyane et al, 1999; Perera et al, 1999). Because of the published reports of immune treatment of cryptosporidiosis employing hyperimmune bovine colostrum and hyperimmune egg yolks, defining the role of breast milk immunity defined by anti *C. parvum* IgA antibodies will be important in determining strategies towards controlling infections caused by *Cryptosporidium* in humans and other mammals.

II Materials and Methods

A. Breast milk samples

The breast milk samples tested in this part of the study were obtained from a prospective epidemiological study conducted in San Juan de Mirafores, a shanty town in the periurban area of Lima, Peru. This population was selected for the epidemiological study because of the known presence of *Cryptosporidium* in the area and because on-going efforts from the non-government organization, PRISMA. Mothers and newborn children were sorted into cohort groups based on the mothers' breast milk antibody response to *Cryptosporidium* antigen as determined by a solid phase enzyme-linked immune assay (ELISA). The breast milk samples were collected weekly for 9 months, either in the field or at the San Juan de Miraflores medical station with the assistance and supervision of PRISMA personnel.

The distribution of breast milk samples from mothers and their newborn children into groups was based on OD values obtained from specific breast milk samples collected 3 weeks prior to the child's *Cryptosporidium* episode, during the episode (diarrhea and parasite detection in feces), and 5 weeks following the episode.

B. Breast milk analysis by ELISA

Milk samples obtained during the course of this epidemiological study were clarified. Two volumes of 0.05 M EDTA per volume of breast milk sample were added and centrifuged at 6000 RPM for 10 min in a micro-centrifuge (Baxter-VWR, PA). The clear fluid between the fat layer and small cell pellet was removed with a Pasteur pipette and stored at -20°C until tested.

The ELISA procedure was performed by coating microtiter plates (Nunc Immuno, Intermountain Scientific, UT) with 1 μg of solubilized *C. parvum* antigen per well. Breast milk samples were diluted 1/500 and added to the plates and incubated for 1 hour. Peroxidase labeled goat anti-human IgA (KPL, MD) was used in conjunction with TMB peroxidase substrate (KPL, MD) for color development. Color development was read at 450nm using a Dynatech MRX ELISA reader.

Based on the ELISA values from their milk samples, mothers were grouped as producing milk with: a) high antibody titer, b) intermediate antibody titer, and c) low antibody titer. The ELISA cut off optical density (OD) value between negative (low) and positive (intermediate) samples was 0.230. This value was established by using known negative breast milks and taking the mean ELISA value (mean OD= 0.034) plus two standard deviations (0.196). A similar method also was used to establish the cut off values between high and intermediate breast milk samples (value = 0.8). In this case, the mean of known highly positive samples (mean OD = 0.982) minus two standard deviations (0.182) was considered the upper cut off value for the intermediate group.

C. Western Blot analysis of breast milk samples

In chapters 2 and 3, the focus of the analysis was ELISA testing. Western Blot was used as qualitative tool. In the generation and testing of both HBC and HEY, the source animals for the test material were hyperimmunized with antigens of similar qualitative characteristics and at controlled times. Also, the immunogen concentrations utilized were associated with homogeneous immune responses and aimed to reduce individual variation between individuals.

The samples collected from this epidemiolgical study were evaluated with additional considerations. The Western Blot (WB) analysis, provided information on the specific and different antigens recognized by the mothers enrolled and its association with the evolution of *C. parvum* clinical disease. This study also provided data on antibody reactivity associated with the dynamics of *C. parvum* infections in children. The samples tested by WB were breast milk samples already clarified for ELISA testing purposes and diluted 1/25 in PBS. The overall WB procedure is similar to the ones performed for HBC or HEY (Appendix 2). The modifications were made after checker-board analysis for the concentration of the test antibody solution and the use of a HRPO labeled goat anti-human IgA secondary antibody.

C.1. Antigen Preparation for WB:

Cryptosporidium antigen was heated for 15 minutes at 65-70°C in the presence of 0.1% SDS, 0.025% (wt/vol) bromophenol blue, 1% glycerol, 0.0025M Tris-HCl (pH 8.0), and diluted with 6% glycerol in distilled water to a concentration of 1.6 µg/ µl. Ten percent acrylamide gels were cast, and 5 µg/well *C. parvum* antigen was electrophoresed at 200 volts and 4°C until the running front reached the end of the gel. Pre-stained molecular weight (MW) standards (Bethesda Research Laboratories, MD) were used.

C.2 Immunoblotting

Electrophoretic transfer of protein onto nitrocellulose was carried out at 1 Ampere for 1 hour. The nitrocellulose strips were blocked overnight in 1% skimmed milk in 0.025M PBS, pH 7.2. The strips were then exposed to human breast milk samples (diluted 1/25) and incubated 1 hour at room temperature. After extensive washing in 0.4% Tween 20 in PBS (TPBS), peroxidase-conjugated goat antibody to human IgA (KPL, MD) diluted 1:100 was incubated with the strips for 1 hour at room temperature. The strips were washed and visualized using TMB (KPL, MD) substrate. (Appendix 2).

D. Data analysis

Data were statistically analyzed. One way analysis of variance (ANOVA) was used for ELISA values. Chi square was used to analyze proportional data and relative risk values (RR) were used to associate reactivities with infection.

III Results

A. ELISA

During a 15-month period (September 1990-December 1991) 211 children were surveyed for the incidence of *Cryptosporidium parvum* and their relationship with anti-*Cryptosporidium* antibody levels in breast milk. Sixty three of these children (63/211, 29.86 %) had at least one *Cryptosporidium* infection. A total of 90 episodes of *Cryptosporidium* infection were detected, with 88 episodes having a clear end (parasite excretion ceased).

Breast milk samples (6,475) from 211 mothers enrolled in the study were analyzed by ELISA. The distribution of mothers into high, intermediate, and low breast milk anti-*Cryptosporidium* antibody titers is presented in Table 6.

C. parvum infections were recorded in 63/211 children, for a prevalence rate of 30% and a mean parasite excretion of 2 weeks (Table 7).

When the incidence was stratified by OD levels, the number of episodes per child per week of surveillance showed no significant differences between children whose mothers belonged to either of the three different groups (Table 8).

Nineteen (29%) of the 63 children affected by *C. parvum* had mothers in the high antibody titer group, 30 (40%) had mothers in the medium titer group, and 19 (38%) had mothers in the low titer group. There were no significant differences in the prevalence or duration of infection among children of the different groups (Table 9).

A mathematical but non statistically significant difference was observed when the OD values were analyzed in conjunction with age of first infection. Differences with a significance level of p=0.06 were detected when the children receiving high titer breast milk were compared to the intermediate or low anti-*C. parvum* ELISA activities (Table 10).

B. Western Blot analysis: Band pattern recognition

Samples representing 6 mothers were characterized by WB. All these samples had high OD values (1.7 - 1.0) as per ELISA testing. Samples from 4 mothers represented 2-3 months of surveillance, samples from 6 mothers represented 4-6 months and samples from an additional 6 mothers represented 7 to 9 months of surveillance. A characteristic pattern of 13 protein bands was persistently recognized over time. These bands were identified as B1-B13. (Table 11).

Two bands may be of importance, the 20.5 and 24.5 KDa bands. The 20.5 KDa band may correspond to a 20 KDa band previously described in the literature, and the band of 24.5 KDa may be the band reported by others as the P23 antigen (Photo 3).

Sets of breast milk samples from 119 mothers were analyzed by WB, with a minimum of 4 milk samples per mother. Ninety one of these mothers (91/119) had children who did not present with cryptosporidiosis during the surveillance period. Every mother had one sample collected at the beginning and at the end of the surveillance period and least twice in between. Overall, 45 % (54/119) of mothers reacted with the 24.5 KDa band and 22% (26/119) reacted with the 20.5 KDa band.

In the breast milk samples from mothers of *Cryptosporidium*-infected children, 32% (9/28) recognized the 24.5 KDa band and 21% (6/28) recognized the 20.5 KDa band. Fifty percent (45/91) of samples from mothers whose children did not get infected with *C. parvum*, reacted with the 24.5 KDa band. Although the proportion of band recognition was higher in mothers of children who never got the infection (55 %), the difference was not significant (Chi square, $p>0.05$). Additionally, the 24.5 KDa band recognized by milk samples from mothers of children who got the infection was of mild intensity (Table 12).

Relative risks associated with the presence/absence of antibodies recognizing specific *C. parvum* antigens gave no indication of protection or susceptibility to the children receiving those milks. When the ELISA data were segregated into High ($OD>0.8$) and all other OD values, the relative risk was 1.406. Further analysis of the relative risks based on ELISA values between High and Low OD ($OD<0.196$) gave a relative risk value of 1.356 (Table 13).

C. Cryptosporidiosis in mothers

Only one mother was identified as positive with *C. parvum* infection by examination of acid fast stained fecal samples but had no clinical symptomatology. Western Blot analysis was performed on a serum sample collected 1 month prior to the positive *C. parvum* test. It demonstrated recognition of several bands, with a clear band recognizing a 24.5 KDa antigen.

IV Discussion

A previous report provided preliminary information on these studies (Sterling et al, 1991). This report evaluated the role of maternal breast milk with respect to infection rates, mean duration of infection, and age to first cryptosporidial infection in peri-urban Peruvian children from birth until termination of breast feeding or after a total of 9 months.

In this chapter, the data confirmed that increased anti-*C. parvum* ODs in the breast milk samples were not correlated with reduced incidence of the disease. The data do not support the notion that there is protection from *Cryptosporidium* infection afforded to children whose mothers have demonstrable breast milk antibodies against the parasite. The relative risk values for High ELISA reactivity and incidence of *Cryptosporidiosis* were greater than 1.0. From these values it could be inferred that high OD values were likely indicators of recent exposure or clinical disease in the child, or indicators of increased risk of exposure.

The Western Blot analysis of the breast milk (BM) samples provided evidence of specific antigen recognition by the samples. Several of the antigens identified by breast milk had MW similar to antigens previously described in the literature or antigens recognized by sera of convalescent patients.

A total of 13 bands were clearly identified by the BM samples. From those 13 bands, bands of 204, 79, 44, 30, 24.5, 20 and 15 KDa might be correlated with other bands associated with convalescence of cryptosporidiosis or neutralization of the parasite.

Due to multiple reports of sporozoite neutralization with antibodies directed against the 23 KDa protein antigen and the 20 KDa antigen, particular interest was paid to these two bands. One of the bands recognized by the breast milk samples was identified as B11, with an estimated MW of 24.5 KDa. This band might be the same as the reported P23 antigen. B12 has the same molecular weight as a reported 23 KDa neutralizing antigen. The statistical analysis showed no significant differences between reactivity of breast milk samples against B11, indicating that breast milk samples with antibodies against B11 were neither an indicator of exposure in the children nor an indicator of protection (Chi square>0.05).

The epidemiological evaluation of these values provided similar information. The determination of relative risks showed that the presence of antibodies against B11 had an inverse relationship with the occurrence of disease, RR=0.484. The inverse analysis gave a RR=2.065, indicating a marginal association between B11 and protection.

The results in this chapter indicate that there is little difference seen in protection against *Cryptosporidium* infection afforded children whose mothers have demonstrable breast milk antibodies versus those having little or no titer activity against the whole parasite or reported neutralizing antigens, such as B11 or B12. The data reinforce the notion that the passive transfer of naturally produced milk products having specific antibody activity against whole or specific *Cryptosporidium* antigens may not affect the outcome of infection. There are reports from calves and humans receiving colostrum from naturally exposed and non-immunized cows where the treated subjects did not appear to

be offered protection (Saxon and Weinstein, 1987; Current and Bick, 1989). This notion was again reinforced by the fact that most newborn calves which receive colostrum products from non-immunized cows are susceptible to cryptosporidiosis.

Further testing of all these products in defined and controlled animal studies are described in the next chapter and the information may offer additional information on the potential of passive immune transfer of antibodies in controlling infections by this parasite.

Table 6: Classification of 211 mothers according with OD values into three groups :

Group	Number	%
Low O.D.	65	31
Medium O.D.	107	51
High O.D.	39	18
Total :	211	

Table 7. Incidence of *Cryptosporidium* :

Number of episodes	Number of children		Mean excretion of *Cryptosporidium* in weeks (*)
0	148	(70.14 %)	----
1	42	(19.90 %)	2.01
2	17	(8.06 %)	2.16
3	4	(1.90 %)	1.45
Total	211	(100.0 %)	1.99

ANOVA : p=0.3626

(*)Mean age of first infection : 6.76 months of age.

Table 8. Incidence of *Cryptosporidium* according with level of antibodies in mother's milk.

	Number of mothers	Incidence of *cryptosporidium* (episodes/child/week)
Low O.D.	65	0.0071
Medium O.D.	107	0.0072
High O.D.	39	0.0109
Total :	211	
ANOVA : p>0.05		

Table 9. Proportion of children with *Cryptosporidium* according with level of antibodies in mother's milk

	Number of mothers	Number of children with *Cryptosporidium*	
Low O.D.	65	19	29 %
Medium O.D.	107	30	28 %
High O.D.	39	14	36 %
Total :	211	63	30 %

Chi Square : 0.86 p=0.6503

Table 10. Age of first infection with *Cryptosporidium*

	Number of mothers	Age of first infection with *Cryptosporidium* (months)
Low O.D.	19	6.6364
Medium O.D.	30	5.6107
High O.D.	14	3.5044
Total :	63	5.4520

ANOVA : F ratio : 2.86, p=0.06

Table 11. WB Band denomination and pattern recognition of *C. parvum* antigens by breast milk samples from an endemic area

Band	KDa	M.W. Standards	
B1	204	204.9	
B2	190	103.9	
B3	144	67.4	
B4	120	43	41.8
B5	85		28.2
B6	79	25.5	
B7	50	14.5	15.1
B8	44		
B9	30		
B10	28.5		
B11	24.5		
B12	20.5		

Table 12: *Cryptosporidium* antigen bands recognized by WB:
Breast milk samples from mothers of
infected and non infected children.

Child with *Cryptosporidium* infection

	Total		Yes		No	
	N	%	N	%	N	%
# mothers	119	100	28	24	91	64

Band recognition per group

	Total		Infected		Non infected	
	N	%	N	%	N	%
B1	119	100	28	100	91	100
B2	119	100	28	100	91	100
B3	99	83	20	71	78	87
B4	99	83	20	71	78	87
B5	61	51	12	43	49	54
B6	61	51	12	43	49	54
B7	35	29	9	32	26	29
B8	35	29	9	32	26	29
B9	27	23	4	14	23	26
B10	33	28	4	14	29	32
B11	54	45	9	32	45	50
B12	26	22	6	21	20	22

Table 13: Relative risks and reactivity of breast milk

High OD and infection in children

High OD	infection	no infection	total	
with	14	25	39	
without	49	123	172	
TOTAL	63	148	211	Relative risk: 1.406

High vs. Low OD and infection in children

OD	infection	no infection	total	
High	14	25	39	
Low	19	46	65	
TOTAL	33	71	104	Relative risk: 1.356

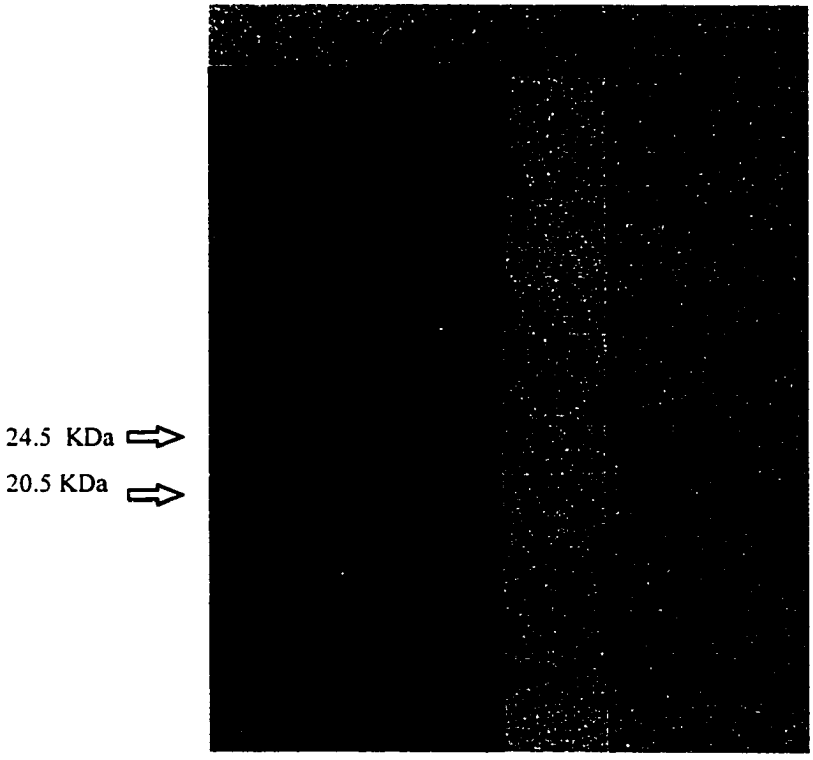
Band 12 (MW = 20KDa)

band	infection	no infection	total	
with	6	20	26	
without	22	71	93	
TOTAL	28	91	119	Relative risk: 1.014

Band 11 (MW=24.5 KDa)

band	infection	no infection	total	
with	9	45	54	
without	19	46	65	
TOTAL	28	91	119	Relative risk: 0.484

Photo 3. Western blot of 3 pools of high OD breast milk samples



CHAPTER 5: *IN VIVO* EVALUATION OF ANTI *C. parvum* HYPERIMMUNE BOVINE COLOSTRUM, HYPERIMMUNE CHICKEN EGG YOLKS AND HUMAN BREAST MILK SAMPLES

I. Introduction

One of the intriguing facts of *Cryptosporidium* is its particular location within the cell, where the parasitophorous vacuole is located intracellularly, but extra-cytoplasmatically. The parasite, once established in this peculiar localization, connects with the cytoplasm via a feeder organelle. It has been proposed that an ABC protein possibly located within this structure, plays an important role in the selective transport of substances into the parasite habitat (Perkins et al, 1999). This structure may be the reason why antimicrobial drugs commonly used to treat other coccidial infections are ineffective in controlling cryptosporidiosis (Griffiths et al, 1998).

Since the intracellular stages are not affected by traditional therapies and cannot be targeted by the immune system, most immune therapies have focused on the extracellular stages: sporozoites and merozoites. These therapies are mainly antibody based and are known as passive immune transfer. The antibodies target surface antigens of the zoite stages and either neutralize the parasite or interfere with its attachment to enterocytes, interfering and affecting the parasitism of healthy intestinal cells. As part of this research work, hyperimmune bovine colostrum, hyperimmune egg yolks and human breast milk samples were tested *in vivo* in neonatal BALB/c mice to determine the efficacy of these preparations.

II Materials and methods for *in vivo* evaluation

A. Neonatal BALB/c mouse model

Hyperimmune bovine colostrum, hyperimmune hen egg yolk product and human breast milk, 220 AU/mouse per treatment were tested for *in vivo* efficacy in the neonatal mouse model. Doses administered were adjusted to match antibody activities between the different compounds, dilution factors and volumes were set to 100 µl. Term pregnant BALB/c mouse mothers were received at the University of Arizona and within 24 hours of delivery (day-1) all mice (adults and neonates) were randomly re-assigned at 5-7 neonates to each mother. This procedure was performed to reduce biological differences between litters. Treatment groups were established to compare the efficacy of the three polyclonal preparations. The non-therapeutically treated control groups received filter sterilized saline or phosphate-buffered saline (PBS) and preparations from animals immunized with control antigen-adjuvant mixtures. Controls for the breast milk groups received low dose OD samples. At day 6, all neonatal mice were orally infected with 1 x 10^5 *C. parvum* oocysts by gastric gavage using polyethylene tubing (1.09 mm OD., Intramedic, Beckton Dickinson, MI) and 27-gauge needles. Mice were treated on days 8 to 12, receiving 100 µL of the respective solutions assigned to each group. Treatments were performed every morning between 8-9 am and afternoon between 4-5pm. On day 13, experimental animals were euthanized, the terminal ileum collected, placed in 10% formalin and ultimately processed for histopathology.

B. Parasite enumeration:

The H&E stained histological sections were examined at 400x magnifications under light microscopy. *C. parvum* densities per villus were determined by counting the number of developing parasite forms along the villus surfaces of at least 25 longitudinal villi, counting only villi with visible intact architecture and from crypt to crypt. Cross sections or other sections were not considered.

C. Strategy used for testing hyperimmune preparations

For the hyperimmune polyclonal preparations, base studies were initially performed to determine the *in vivo* potency and efficacy of the preparations. In these base studies, different concentrations of materials from each of the different hyperimmune polyclonal preparations were evaluated in the neonatal mouse model. Samples of HBC considered to have high and low antibody (adjuvant group) activities were tested. Samples of HEY considered to have high (FCA plus 7.5 μg *C. parvum* antigen), low (Titermax plus 7.5 μg antigen) and no antibody activities (FCA+MAI alone immunized group) were tested. Information form these evaluations was used for the subsequent *in vivo* studies comparing the three different polyclonal preparations (comparison studies).

D. Data analysis and selection of hyperimmune polyclonal preparation.

Within each group of the two hyperimune polyclonal antibodies, the preparations with better anti-*Cryptosporidium* efficacy were selected based on the reduction of

parasitism along the intestinal surface as determined by the counts of parasites per villus. Results from the *in vivo* testing were statistically analyzed by one way analysis of variance.

E. Standardization of the potency of the materials tested

Samples were matched for potency by ELISA determination of activity units. The activity units represent the potency of the compounds to be evaluated and was determined for samples of HBC, HEY and human breast milk. The HEY samples were hyperimmune egg yolks diluted 1:1 with sterile PBS. Breast milk samples with high ELISA reactivity were selected and pooled to prepare the desired test materials providing 220AU/100 µl. The HBC samples were processed by Mead Johnson, the sponsor of the HBC studies. For the preliminary testing, a solution of 5% w/v HBC was received from the sponsor. The final testing, where multiple preparations were tested under identical conditions, were performed using a 10% w/v preparation sent from Mead Johnson. As in the other preparations, concentrations were adjusted to the 220AU/ml criterion.

E.1. Activity unit determination :

Definition of One Activity Unit (AU): One AU is defined as the amount of anti-cryptosporidial activity present in a sample dilution that yields a minimal positive OD by ELISA. A minimal positive OD is defined as the mean of the ODs obtained for a subset of negative samples plus 2 standard deviations. Optical densities from 30 negative yolk

samples yielded a mean of 0.097 and a standard deviation of 0.045, therefore, mean plus 2 standard deviations (0.090) equals 0.187. The dilution of a sample that gives an OD of 0.187 contains 1 AU of activity; therefore, the undiluted sample contains the number of AU reciprocal of the dilution. These values were 0.050 as the average with a standard deviation of 0.055 for HBC (1AU= 0.160) and a mean of 0.034 with a standard deviation of 0.098 for the breast milk preparations (1AU=0.230).

Freshly produced anti-*C. parvum* polyclonal preparations were tested for its anti-cryptosporidium ELISA activity and adjusted to contain the same number of AU per dilution in at least two different ELISA runs performed on different days. The material had to have similar AU activities (5% variation allowed) in two ELISA evaluations. Once these requirements were fulfilled, this new material was used as the anti-*C. parvum* polyclonal preparation standard and stored in aliquots at -20°C.

E.2. Calculating AU for Other Samples of anti *C. parvum* polyclonal preparations

In order to estimate the number of AU in other samples of anti-*C. parvum* polyclonal preparation, a mathematical predictive model was established. Plotting the AU of anti-*C. parvum* polyclonal preparation against the ODs obtained from ELISA yielded a non-linear model, but taking the log of the OD versus the log of the AU produced a straight line from which other values could be estimated using linear regression analysis.

Anti-*C. parvum* polyclonal standards were thawed and standard dilutions of

1:500, 1:1000, 1:2000, 1:4000, and 1:8000 were made in ELISA dilution buffer. The standard was run in triplicate. Samples for AU determination were re-suspended and diluted to 1:1000 in ELISA dilution buffer and tested in triplicate at 1:4000 and 1:8000 dilutions.

Anti-*C. parvum* polyclonal standard and samples were applied to ELISA plates and ELISA was completed according to the procedure described in Appendix 1. Average ODs of the anti *C. parvum* polyclonal preparation standard obtained from the test were transformed into log OD and plotted against log AU to obtain a line ($r = 0.95$ or better). The log average OD from each sample was then used to calculate AU by linear regression analysis.

The resulting values represented the number of AU present in 1 ml of the liquid anti-*C. parvum* polyclonal preparation to be tested. Upon AU determination for each material to be tested, the different anti-cryptosporidial preparations were matched by potency, adjusting the potency to 220 AU per 100 µl dose.

III. Results

A. Base *in vivo* studies testing of HBC

A total of 120 neonatal mice were randomized into 20 cages (1 mother and 6 neonates per cage) and divided into 10 treatment groups (Table 14). The MPV in the groups receiving low HBC at all dilutions showed no statistically significant differences between any of the groups when compared to each other ($p=0.91$) and to the saline control ($p>0.27$) (Table 15, Figure 15). The results from the high activity groups showed statistically significant differences between the groups treated with 1:1 high HBC and the control ($p<0.0001$) or the respective serial dilutions ($p<0.005$). No significant differences were detected between the diluted HBC and the control group (Table 15, Figure 16).

B. Base *in vivo* studies testing HEY

A total of 24 neonatal mice were randomized into 6 cages (1 mother and 4 neonates per cage) and divided into 3 treatment groups: high, low and negative. Based on the previous experience with HBC, no serial dilutions were tested.

The histological examination of the slides demonstrated that all the study mice became infected. The results from the mice treated with high activity HEY showed statistically significant differences when compared to both the low and control groups ($p<0.0016$). Individual statistical significance values were $p=0.0014$ when compared to

the low HEY and p=0.0025 when compared to the control group. No significant differences were observed between the low and control groups. (Table 16, Figure 17).

C. Comparison studies: *In vivo* evaluation of the three polyclonal preparations

These studies were conducted to determine if polyclonal antibodies from hyperimmunized cows (HBC), hyperimmunized hens or breast milk samples from naturally exposed humans had better effects in treating *Cryptosporidium* infections. The samples of HBC and HEY were standardized to 220AU/100 µl.

Compared to the hyperimmune products, the breast milk samples had lower activity units and selected samples from the group with high OD values had to be pooled for this study. Chicken yolk polyclonal antibodies against MAI, non reactive colostrum samples from cows immunized with adjuvant alone and pooled breast milk samples from the low group were used as controls for each of the polyclonal preparations. An additional group received saline and was the control for *C. parvum* infectivity and study procedures. Overall, the groups treated with the breast milk samples and the groups receiving bovine colostrum did not show significant reductions in the MPV when compared to the saline control. Significant reductions in the MPV were detected in the groups receiving the HEY polyclonal preparations. The group treated with HEY anti-*C. parvum* was significantly better (p=0.009) than the MAI antibody control, though this value reflects some kind of background activity present in the anti-MAI antibody preparations (Table 17, Figure 18).

IV. Discussion

The objective of the work presented in this chapter was to elucidate the *in vivo* efficacy of three different polyclonal preparations and their potential role as therapeutic agents in treating *C. parvum* disease. Information in the previous chapters described *in vitro* characteristics of these compounds. Two products were generated through hyperimmunization of study animals and the antibody rich products were tested for quantitative increments in the recognition of *C. parvum* antigens. In the case of the breast milk samples, these samples were obtained from an epidemiological study aimed to study the role of breast feeding in protecting children from cryptosporidial diarrhea. The samples were further tested by ELISA to determine quantitative antibody reactivities against *C. parvum* antigens. These samples were also analyzed via Western Blot to determine the recognition of specific *C. parvum* antigens.

Breast milk samples were tested due to reports that associate breast feeding with protection or decreased incidence of diarrheal diseases. Several studies have reported reduced incidence of viral diarrheas when breast feeding is started early and continued until the child is at least 6 months of age (Naficy et al, 1999; Mulder-Sibanda et al, 1999; Clemens et al, 1999). The breast milk samples evaluated in these studies did not show therapeutic efficacy. There are several explanations on the protective role of breast feeding. One is the decreased or lack of exposure to pathogens of those exclusively breast fed when compared to those receiving solid food within the first 4 months of life (Ziyane et al 1999). In the developing world, another effect that is very evident is the improved

nutritional status of the breast-fed infants when compared to non breast-fed children. This difference is more evident during the first 6 months of life, but gradually disappears as children are weaned and switched to solid foods. Another explanation is the passive transfer of antibodies from the mother to the newborn. In humans, passive transfer via colostrum is not as critical as it is in other mammalian species. It has been reported that infants who were given colostrum and breast milk had fewer diarrheal and respiratory attacks (Perera et al, 1999.) These reports may suggest more than just the nutritional advantages of the mothers milk and the prevention of exposure to pathogens in children exclusively breast fed.

As part of the evaluation performed in this dissertation, breast milk samples were further tested via ELISA and specific *C. parvum* antigen recognition was performed and analyzed by Western Blot. Pooled samples with relatively high antibody activities were then tested in the neonatal mouse model. Even though for the epidemiological evaluation the samples were characterized as having high reactivity when compared to the negative controls, the IFA titers of those samples were in the range of 1/128, which were significantly lower than the obtained from HBC (>1/32,000) or HEY (>1/10,240). The data analysis of the *in vivo* evaluation demonstrated that breast milk samples, regardless of antibody reactivities agains whole *C. parvum* antigen or the specific antigen recognition reported in WB analysis, had no significance. These results confirmed the initial findings already reported (Sterling et al, 1991), both at the WB level and at the *in vivo* level, indicating the lack of efficacy of maternal sIgA antibodies in conferring

protection against cryptosporidiosis. Interestingly, some of the specific antigens recognized by these breast milk samples correspond to antigens reported to be neutralization sensitive epitopes, such as GP15, P23, p15/60, or a protein of 40KDa (Enriquez and Riggs. 1998; Sagodira et al, 1999b; Perryman et al, 1999; Riggs et al. 1997; Tilley and Upton, 1997). These discrepancies may be explained by more than one fact. First, all the reported epitopes have been first identified and or characterized by monoclonal antibodies or by polyclonal antobodies immunized with purified antigenic fractions. Antibodies present in breast milks might have reacted poorly or with a different epitope within the same antigenic fraction. Based on data obtained from hyperimmunization studies, it seems that either one or more of the factors mentioned above could explain the lack of protection from the breast milk samples.

The ELISA and IFA testing showed strong antibody responses to immunizations for both HBC and HEY. The Western Blot analysis demonstrated that both products recognized multiple antigenic fractions of *C. parvum*. HBC recognized 23 bands and HEY recognized 18 bands. Both products recognized bands with MW similar to antigens previously described and associated with the neutralization of *C. parvum*: HBC reacted with bands of 15, 23.8, 44.8 and 61 KDa, while the banding pattern for HEY included protein fractions of 14.7, 19.3, 23.6 and 44.6 KDa. Based on these results, it was expected that both HEY and HBC would have parasite neutralizing capabilities and would aid in reducing the parasite burden or eradicating the disease.

The *in vivo* data of the base or preliminary evaluation of HBC did not meet therapeutic expectations. From all dilutions tested, only the undiluted colostrum had demonstrable efficacy against *C. parvum* infections (Table 15), with less than 40% reduction in the parasite burden.

The preliminary evaluations of the HEY showed significant reductions in the infection as quantitated by the parasite densities along the villus border (about 76% reductions). There were non-significant parasite reductions observed in the animals treated with the control material, i.e. yolks hyperimmune for MAI.

In the comparison study evaluating the efficacy of the three polyclonal preparations, the group receiving HEY preparation had statistically significant parasite reductions when compared to the controls or the other groups. An unexpected result was the reduced efficacy of the new batch of HBC received, which presented no significant differences when compared to the colostrum or the saline controls. Data from the mice treated with breast milk samples with either high or low ELISA OD values had no significant differences either between them or when compared to the saline control. These results from breast milk were not totally unexpected but were necessary to have a direct confirmation of the *in vitro* data discussed at the beginning of this section.

The data from colostrum was unexpected and controversial because there were differences in the outcomes of the *in vivo* testing when a different batch of material was tested. The 5% HBC preparations initially tested conferred benefits to the mouse and those results were consistent with *in vitro* and *in vivo* studies conducted with the same

HBC preparations (Riggs et al, 1994). Data from the 5% HBC was considered for the selection process, however no ANOVA statistical analysis was possible between studies conducted at different times. The HEY treated mice had an 82.3% reduction in parasite burden when compared to the saline treated mice, while the 5%HBC induced a statistically significant reduction of parasitism, but only of 28.7%. Differences from other studies were the length and frequency of administration. When HBC was administered to SCID mice for 10 consecutive days, the therapeutic effects were more evident (Rigss et al, 1994). Other studies evaluated the use of HBC in humans, administered through nasogastric intubation for 48 consecutive hours, reported resolution of clinical disease and cessation of parasite excretion (Ungar et al, 1990b).

Prophylactic studies where HBC was administered 30 minutes prior and after infection of human volunteers, and three times daily for the next 5 days showed no differences in oocyst excretion when compared to the placebo group (Okhuysen et al, 1998). Treated subjects exhibited a non significant trend to less diarrhea. This report seems to correlate with our observations that when HBC was administered less frequently or for shorter periods or both, the antiparasitic effect was less pronounced.

Considering that both HEY and HBC were preparations similar in concept, with the main difference being the fluid in which the antibodies were collected and the antibody isotype, and that the data analysis demonstrated that the HEY was significantly better than the other two polyclonal preparations, HEY was pursued for further testing as a therapy for enteric *C. parvum* infections.

Table 14. Distribution of Treatment groups per cage

Low activities HBC	High activities HBC
Saline control	Saline control
1/1 low	1/1 high HBC
1/5 low	1/5 high HBC
1/25 low	1/25 high HBC
1/125 low	1/125 high HBC

Table 15. Means of parasite per villus (MPV) mice treated with HBC at serial dilutions

	Treatments				
	Low HBC (serial dilutions)				
	Control	1:1	1:5	1:25	1:125
MPV	32.66	30.5	29.1	28.3	25.9
Std. dev.	4.78	18.8	8.5	8.8	15.2
P value		0.70	0.22	0.60	0.16
	High HBC (serial dilutions)				
	Control	1:1	1:5	1:25	1:125
MPV	26.04	15.9	27.4	27.2	30.8
Standard dev.	9.83	9.54	5.2	7.8	14.7
P value		0.000	.67	0.76	0.36

Table 16. Means of parasite per villus (MPV) mice treated with HEY

	Treatments		
	Control	High HEY	Low HEY
MPV	17.4	4.13	14.3
Standard dev.	16.4	6.20	9.88
P value		0.001	0.550

Table 17. Means of parasite per villus (MPV) mice treated with HBC 10%, HEY and human breast milk polyclonal preparations

	Treatments						
	Control	HBC 10	HBC(-)	HEY	MAI	BM(H)	BM(L)
MPV	26.7	22.3	25.8	4.6	18.5	31.1	27.4
Std. dev.	8.4	6.9	15.3	2.2	11.6	15.9	12.0
P value		NS		NS	p<0.001	NS	NS

NS: not significant when compared to the control

Statistical significance				1 way ANOVA		
	HBC 10	HBC(-)	BM L	BM H	HEY	MAI
Saline	0.139	0.838	0.875	0.375	0.000	0.069
HBC 10		0.474	0.264	0.113	0.000	0.392
HBC(-)			0.829	0.501	0.002	0.319
BM L				0.654	0.000	0.221
BM H					0.001	0.009
HEY						0.009

Figure 15: Base studies with low HBC
MPV per study group

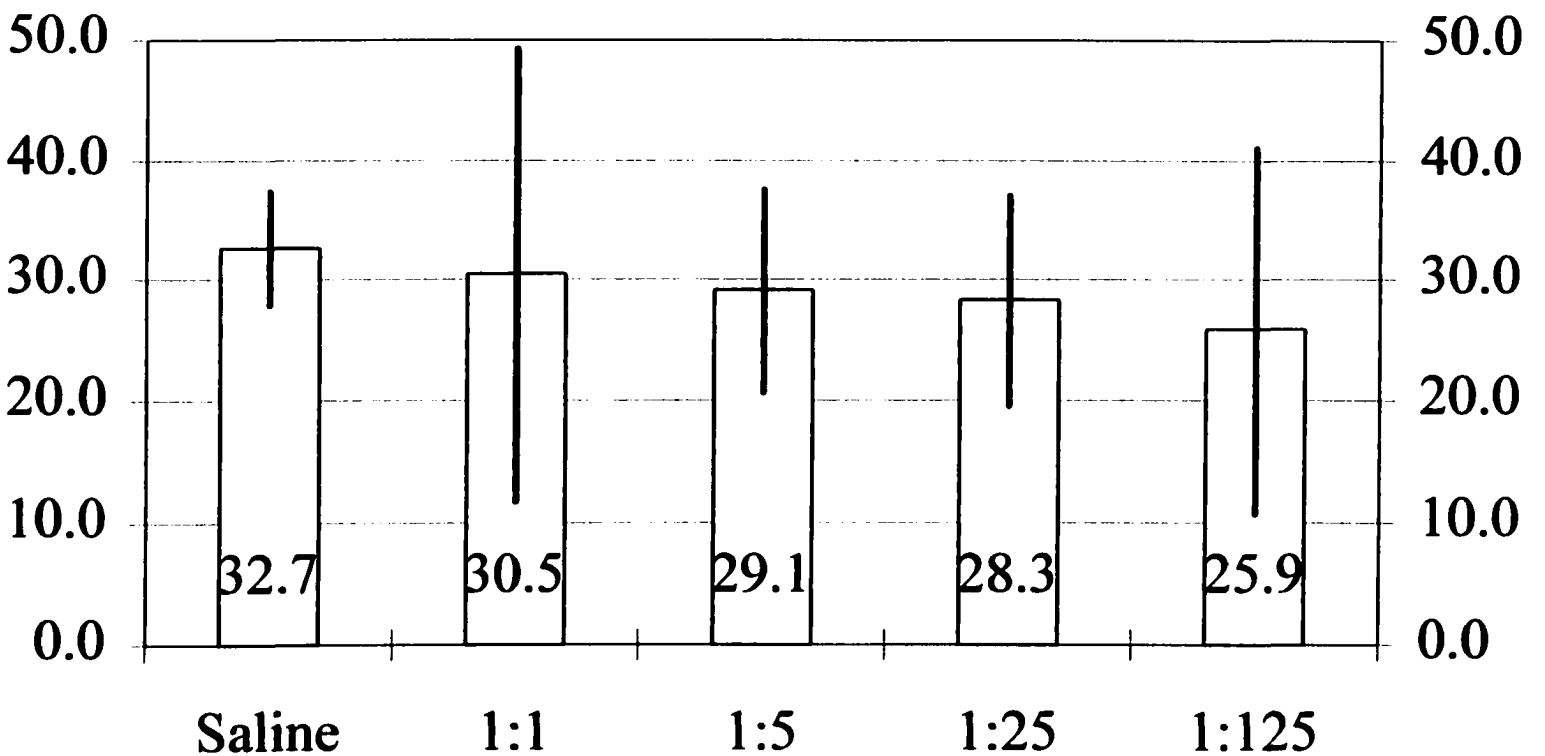


Figure 16: Base studies with high HBC
MPV per study group

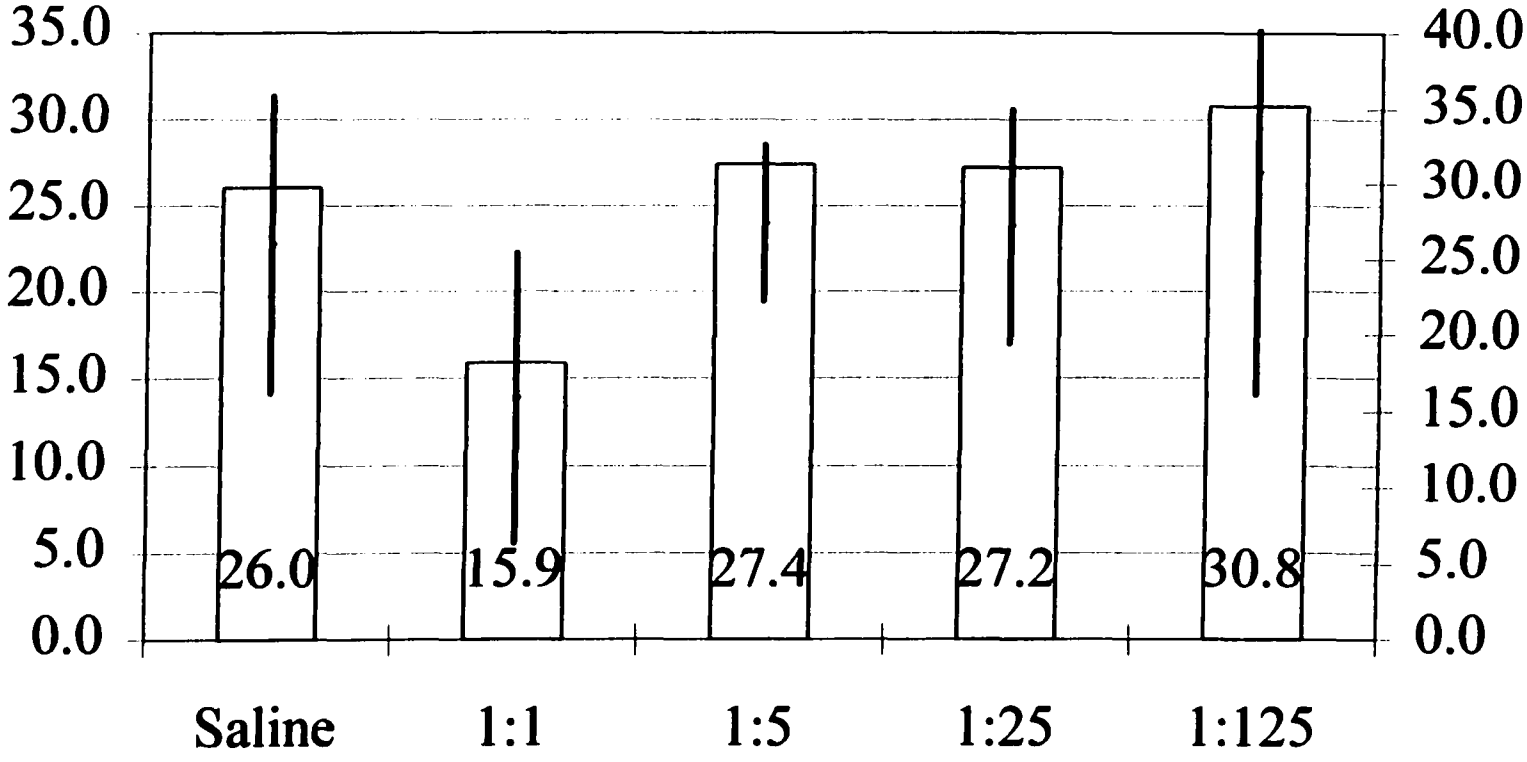


Figure 17: Base studies with HEY
MPV per study group

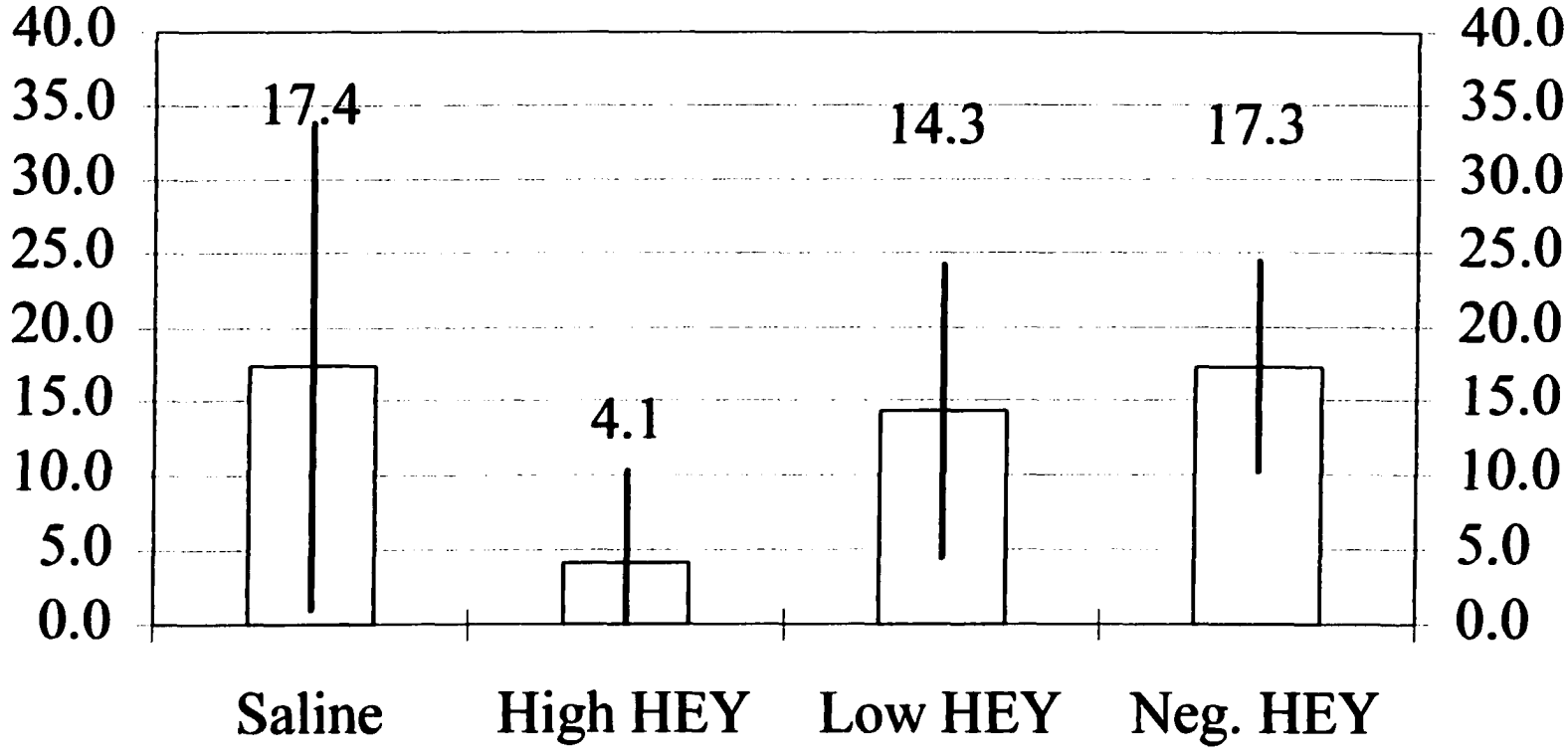
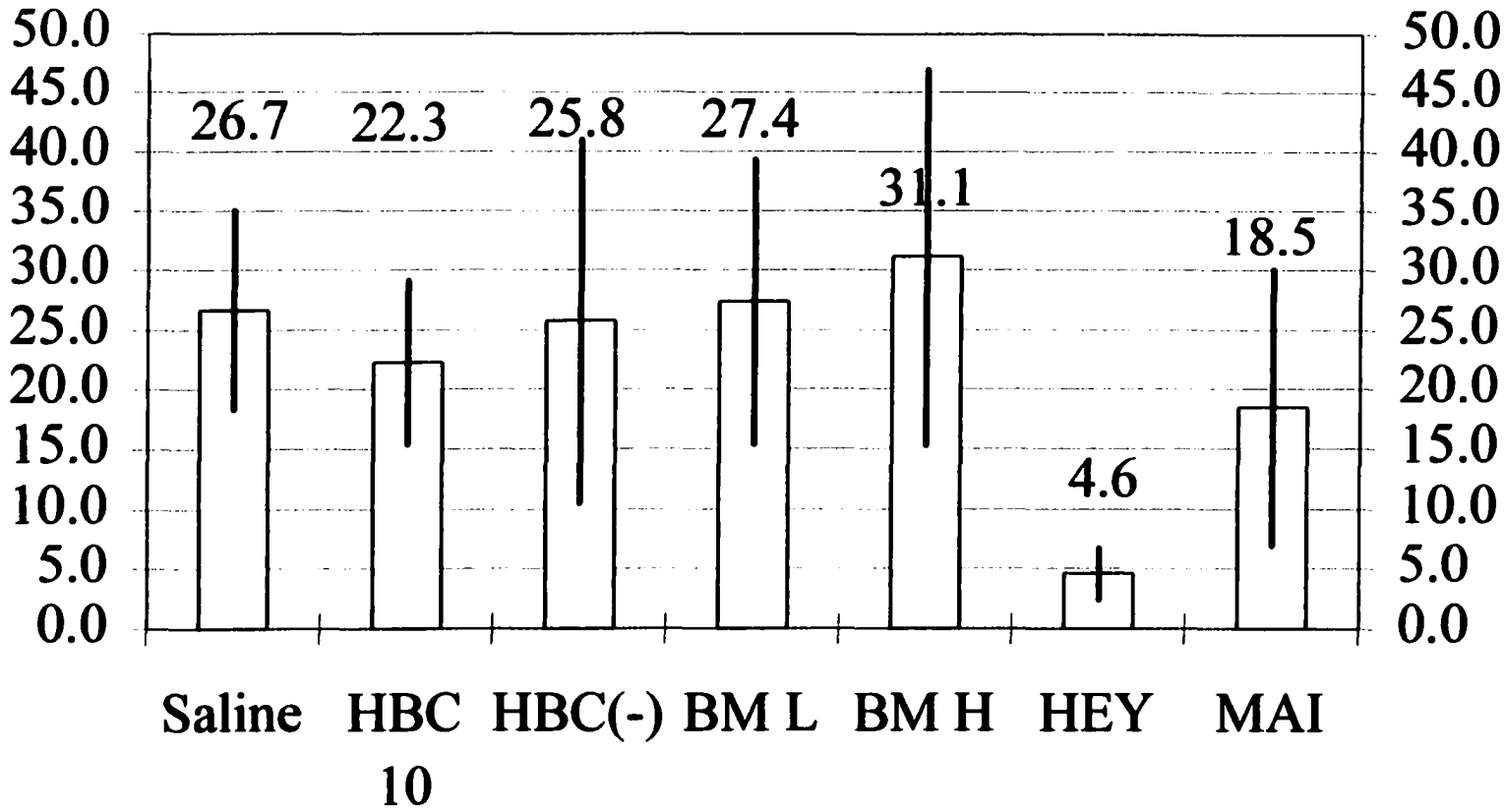


Figure 18: MPV of comparison studies



CHAPTER 6: DATA ANALYSIS OF THE EVALUATION OF A HYPERIMMUNE CHICKEN EGG YOLK PRODUCT (HEP) IN AIDS PATIENTS

I. Introduction

The use of eggs from hyperimmunized hens is an alternative to the production of hyperimmune bovine colostrum suitable for passive transfer. Eggs laid by hens hyperimmunized with *C. parvum* oocyst antigens have high anti-*C. parvum* activity (similar to HBC) and have been shown to produce significant parasite reduction when administered to *C. parvum* infected neonatal mice (Cama and Sterling 1991).

The analyses of data generated in a Phase I/II clinical trial evaluation is presented in this chapter. Hyperimmune chicken egg yolks were tested for safety and efficacy in treating chronic cryptosporidiosis in AIDS patients. The test materials were prepared from yolks of eggs laid by hyperimmunized hens containing anti-*Cryptosporidium* antibodies. This Phase I/II trial (Study No. CP-94-01), evaluating the safety and preliminary efficacy of HEP in the treatment of cryptosporidiosis in AIDS patients, was performed under the Food and Drug Administration (FDA) number BB-IND 5426. The study was conducted at Cornell University Medical Centre in New York under the supervision of Dr. Rosemary Soave and Dr. Lawrence Davis. The polyclonal preparations were manufactured by the Sponsor of the study, IgX Corporation, and the required approvals were obtained by the same company. The results presented were the basis for continuing efforts aimed at developing a successful therapy.

II. Brief Description of the Regulatory Requirements

Any new potential product aimed to treat or prevent a human or animal condition shall follow the procedures described in the Code of the Federal Register (CFR) and interact with the FDA.

Prior to any evaluation, the sponsor must file a dossier with information relevant to all aspects of rationale, activity, safety and stability of the compound. This filing includes a clinical trial protocol, the signatures of all medical personnel involved in the study and the approval of the Institutional Review Board from the hospital or clinics where the evaluation will take place.

Once the protocol has been approved by the sponsor, the medical investigators and the respective Institutional Review Boards, the sponsor may proceed to submit to the FDA an application for an Investigational New Drug (IND). Once the FDA has reviewed the dossier, and no queries are pending, the sponsor may start a Phase I study. These studies usually include a small number of subjects. The main objective is to determine the safety of the new drug candidate. Any event affecting the health of the participants of the study, and that was not expected to occur within the evaluation, must be reported by the investigators to the Sponsor within 72 hours. Serious and fatal adverse events (such as hospitalizations or death) must be notified to the sponsor in writing within 24 hours and the sponsor must also report such events to the FDA within 72 hours. Any protocol modifications require prior approval from the IRB and the FDA.

Upon completion of the study, the data is audited for completeness, adherence to protocol and compliance with Good Clinical Practices (GCP). These requirements and procedures are similar for all study phases. Once there is assurance that the study was performed in compliance with the protocol and current regulations, a final report is submitted to the FDA for their evaluation. This report is the basis for a meeting with the sponsor prior to the conduct of the next phase of studies.

Phase II studies are usually expanded Phase I studies, and the primary outcomes are safety and preliminary efficacy. Phase III studies are conducted after all requirements from a Phase II studies are fullfilled. All Phase III clinical trials are double blinded, placebo controlled and multicenter studies. The main outcomes in a Phase III study are all related to efficacy and safety. In this chapter we will analyze data from a Phase I/II study, conducted to evaluate the safety and preliminary efficacy of a hyper-immune egg product (HEP) for the treatment of cryptosporidial diarrhea in patients with AIDS.

III. Methods

A. Hyperimmune hen egg yolk product

The test material was prepared from yolks of eggs laid by hyperimmunized hens contained anti-*Cryptosporidium* antibodies. It was a frozen (-20°C) solution composed of hyperimmune egg yolk (50%), purified water (45%), and sucrose (5%). All doses of HEP were irradiated (Vindicator Inc. Florida) at 1.5 – 2.5 kiloGrey (kGy) and stored at -20°C until shipment for patient use. The HEP was formulated to provide 412,000 Activity Units (AU)/unit about 70 to 100 mL. Individual doses were provided frozen in bags.

B. Description of Phase I/II Clinical Trial

The objective of a Phase I/II clinical trial evaluation was to determine the safety of the HEP as well as to determine efficacy, dosage, tolerability and toxicity profiles..

C. Study design:

Open label, ascending dose study of two dose levels of HEP in patients with AIDS and cryptosporidiosis. HEP was administered 5 times daily for 3 or 6 weeks at two dose levels: 412,000 AU/dose and 824,000 AU/dose. Concomitant to the HEP, patients took an H_2 blocker to increase the gastric pH and minimize antibody degradation. Patients had to go to the clinic once a week while in the study and had to go back for follow up visits at 2 and 4 weeks post termination of their participation in the study.

D. Study Population:

The target population were HIV positive patients suffering from chronic diarrhea due to *C. parvum*. Patients were 18 years or older and meeting the CDC criteria for AIDS with documented $CD4^+$ counts below 180/mm. All had positive HIV tests by ELISA and Western Blot and a documented history of *C. parvum* chronic diarrhea. All prospective patients provided stool samples for the detection of other diarrhea causing enteropathogens, such as *Salmonella, Shigella, Giardia, C. difficile, E. histolytica.* Positive results to any of these pathogens resulted in exclusion from the study.

Once there was confirmation of *C. parvum* in stools or colon biopsy and confirmation of the absence of other diarrhea causing conditions, the patients were enrolled into the study. Treatment phase: patients received the HEP orally, 5 times daily while awake, for a 3 week period. Patients were to come back at weekly intervals for clinical examination and sample collection. Patients who completed at least three weeks of therapy were considered evaluable patients.

After the conclusion of the patient treatment phase, data from the patients was audited, tabulated and reviewed for accuracy with the clinical investigators. Data from the physician's case report forms and patients diaries was used to determine: the patient's compliance with the study protocol, safety, tolerability, toxicity and dosage of the HEP.

Adverse events (AE) were those unexpected events that affected the health condition of the patient and had to be reported to the clinical investigator. The safety analysis was based on the information from clinical laboratory exams and AE.

E. Efficacy and response

The efficacy endpoints for evaluating this study were classified as primary and secondary. Primary efficacy endpoints were the daily frequency of bowel movements, stool consistency and *Cryptosporidium* scores in stool.

The following values were assigned for quantitating *Cryptosporidium* in stool samples: 0 when no organisms were detected per 22 mm^2 coverslip area, 1 when 2-5 organisms were observed per 22 mm^2 coverslip, 2 when one oocyst was detected per 5-10 400x high power fields, 3 when up to 1 organism per 2-3 400x high power fields were observed and 4 when there were more than 2 organisms per every high power field.

The clinical response was based on the frequency of bowel movements. Patients were considered clinical responders if diarrhea resolved or had an average of no more than 3 bowel movements per day (BMD). Partial responders were those patients with reductions greater than 50% but still had more than 3 BMD. Non responders had less than 50% reductions in their average BMD.

Parasitologic responses were based on the parasite quantitation. Eradication was when at least two consecutive weekly stool examinations were negative for *Cryptosporidium*; substantial reductions were when the decreases in oocyst quantities

were at least two grades, and non-responders were defined as those with no substantial reductions or increases in the parasite grade.

F. Study medication: Study Drug, Administration, and Dosage

Patients took HEP, 412,000 AU (one unit) or 824,000 AU (two units), orally every 4 hours while awake (*e.g.*, from 6 am to 10 p.m.) five times daily, for a total of 2.06 $\times$ 10^6 AU/day or 4.12 $\times$ 10^6 AU/day. Famotidine 20 mg (Pepcid) was provided in manufacturer blister packs to be taken orally twice a day.

IV. Results

A. Study population:

A total of 42 patients were screened for this study. Only 24 patients met all inclusion criteria and were enrolled in the study; the first 15 patients at the low-dose level and the next nine patients at the high dose level. All patients met the CDC criteria for AIDS and were diagnosed with cryptosporidiosis. Patients had an average of 7.5 stools/day, and mean duration of cryptosporidiosis was 8.6 months. Mean $CD4^+$ count was 32.3/mm^3. Sixteen of the 24 patients completed at least 2 weeks of treatment; eight patients discontinued within the first week. Mean compliance during the 6 week study was 95% at the low dose level and 60% at the high dose level (Tables 18 and 19).

B. Efficacy

Efficacy endpoints included daily frequency of bowel movements, stool consistency, and stool oocyst quantitation. The evaluable patient population comprised 16 patients who were treated for at least 2 weeks; 11 at the low dose and five at the high dose. Although patients were assigned to two dose levels (the high dose two-fold higher than the low-dose), due to poor compliance at the high dose two clear dose levels did not exist and a comparison of the two doses was not performed. The mean number of bags of HEP consumed during the 6 week study was 33.4 at the low dose and 41.9 at the high dose (Table 20).

Among the 11 evaluable patients receiving low dose HEP, six patients (54.5%) demonstrated a decrease in the daily frequency of bowel movements relative to baseline after 3 weeks of treatment; at 6 weeks, six of eight patients (75%) who were still in the study demonstrated a decrease. Daily frequency of liquid stools improved in eight of the 11 evaluable low dose patients (73%) after 3 weeks relative to baseline; and after 6 weeks, in six of the eight (75%) patients who were still in the study. Among patients receiving the high dose, the frequency of daily bowel movements relative to baseline decreased in three of four evaluable patients (75%) after 3 weeks; after 6 weeks of treatment, two of five evaluable high dose patients (40%) demonstrated a decrease. The daily frequency of liquid stools after 3 weeks on the high dose decreased in three of four (75%) patients relative to baseline; only one of five (20%) high dose patients still demonstrated a decrease after 6 weeks (Figure 19).

Four of the 16 evaluable patients (25%) were classified as clinical responders by the clinical investigators. Clinical response occurred after at least 6 weeks of treatment. Parasitologic response was evaluated by comparing oocyst score on the last day of treatment to baseline. Eradication occurred in one patient after 1 week on low dose HEP; his oocyst grade decreased from 1 to 0, and remained 0 for 5 weeks. Two patients experienced substantial reductions at the end of treatment; their oocyst grades decreased from 3 to 1 and from 4 to 1, respectively. One of 3 patients terminating their participation at 3 weeks, had a one grade reduction in the oocyst grade. From 13 patients whose end of treatment was by week 6, only 4 had reduced oocyst grades. Four patients continued on

compassionate extension beyond 6 weeks and 2 had reductions in the parasite quantitations. The stool consistency evaluated as the daily frequency of liquid bowel movements decreased on 9 of 16 patients (Figure 20, Table 21).

C. Safety

Sixteen AE occurred in 14 patients following treatment with HEP; nine occurred during treatment and seven, all unrelated to treatment, occurred following completion of treatment or premature discontinuation. Three of the 16 AEs were considered to be severe in intensity. All three resulted in hospitalizations; only one, acute pancreatitis, was considered to be possibly related to treatment. None of the other 15 AE were considered to be treatment-related.

Several patients were unable to tolerate HEP, especially in the high dose group. They reported satiety, inability to continue ingesting egg yolks, fullness and bloating. The clinical investigators felt strongly that in this patient population (*i.e.*, patients with advanced AIDS and anorexia) there was poor tolerance of all food, including HEP, and that these symptoms did not represent AE to treatment.

No deaths were reported during the study. Seven deaths however, occurred following termination of treatment (4.5 to 18 weeks after the last dose). None of the deaths were considered to be treatment related and all were a result of progressive end-stage AIDS. There were no laboratory abnormalities that lead to a reduction in dosage and no patient was discontinued from the study because of an adverse laboratory event.

V. Discussion

Data to date suggest that HEP, up to doses of 4.12 x 10^6 AU/day, was safe for administration to patients with AIDS. Although the possibility that HEP may have contributed to exacerbating pancreatitis in one patient cannot be excluded, HEP was not felt to be causative by the clinical investigator. There were no other AE or laboratory abnormalities that were directly attributed to HEP therapy. Important information on HEP tolerability was obtained from this study. Satiety, bloating and fullness were the reported causes of intolerance. Three of 10 subjects discontinued HEP therapy prematurely due to problems with satiety; all three patients were receiving up high dose HEP. Satiety was not experienced in subjects receiving low dose HEP. Also, a dosing regimen of five times per day was considered maximal.

Analysis of study CP-94-01 suggested that HEP had a favorable effect on cryptosporidial diarrhea and a less pronounced effect on oocyst eradication. Sixteen subjects completed at least 3 weeks of therapy and were therefore considered evaluable. Of those, four (25%) met the study definition for a clinical and/or parasitological response. Improvement was very similar in subjects receiving either 3 weeks or 6 weeks of therapy: bowel movement frequency was reduced in 60% of subjects completing 3 weeks of therapy and in 67% of subjects completing 6 weeks of therapy.

The discontinuation of the patient affected by pancreatitis and lower compliance of patients enrolled at the high dose level were closely analyzed with the clinical investigators. It was concluded that the reported satiety and fullness and bloating could be

attributed to the high lipid content of HEP. In any future clinical evaluations, a low lipid presentation of HEP was strongly recommended for administration to patients.

Based on the response observed in the patients, the dose and duration of treatment was adjusted. No adjustments were made to the low dose of 5 bags per day at 412,000 AU each. Compliance was 79.6% in the first week and ≥ 95% in subsequent weeks. Low compliance in the first week was directly related to the patients who discontinued after taking very few doses. Compliance with treatment in the 11 evaluable patients was ≥ 95% throughout the study. All evaluable patients in the low dose group reported no problems ingesting HEP. All five evaluable patients enrolled in the high dose group required adjustments to dose levels of HEP.

High dose patients were intended to receive twice as much HEP as low dose patients (10 units per day at 412,000 AU each), but compliance in this therapy group was low. On average, patients enrolled in the high dose group received an additional 20 to 40% (7 to 14 units) of HEP instead of the intended double dose. Overall, it was estimated that the maximal tolerated dose of the HEP as formulated, was 6-7 bags (412,000 AU each) per day, equivalent to 2.88×10^6 AU per day.

Only one patient met both the clinical and parasitological criteria for response. This patient was detected negative for parasites by week 2 of the compassionate extension, but thereafter only substantial reductions and no eradication were detected. Despite clinical improvements in the number of bowel movements, no defined trends in parasite eradication were detected. The stool oocyst grade as performed in this study was

a semi-quantitative measurement of the presence of *Cryptosporidium* in stools. Since measurement of the total volume of stools per day was not allowed in this study, the oocyst stool grade reflected the parasite densities in the stool samples. Improved methodology for the quantification of *Cryptosporidium* in stools should be implemented in future studies.

All patients were routinely monitored for vital signs and laboratory work was performed at screening and at weeks 1, 3 and 6. Information was gathered from hematological, biochemical and lipid profiles. No significant abnormalities in clinical or laboratory values attributable to the administration of HEP were detected.

One adverse event that could have been directly attributable to the administration of HEP was the patient with acute pancreatitis on the first day of treatment. Although the possibility that HEP may have contributed to exacerbating his pancreatitis cannot be excluded, investigators attributed the event to intolerance to lipid ingestion rather than to antibody ingestion. In the case of one patient who received HEP for 36 weeks (compassionate extension), no AE or laboratory abnormalities attributable to the administration of HEP was detected during the length of his participation.

Satiety was the main symptom reported as a tolerability problem. Three of the 4 discontinuations from the high dose group were associated with satiety. The fourth patient who discontinued from the high dose group reported intolerance to HEP. Overall, it was estimated that the maximum tolerated dose of HEP was 2.88×10^6 AU/day (equivalent to 7 bags per day), when administered 5 times daily.

The clinical trial evaluation in end-stage AIDS patients was particularly challenging. The patients evaluated in this study were very ill, as reflected in their CD4+ counts (average of 32), and seven of them died within a few weeks after their participation in the study. The characteristics of this patient population created a multifactorial complex of variables that were considered in the protocol. When designing the study a top consideration was the patients health and well being, as well as procedures that were deemed simple enough for the patients to perform on a reliable basis. Another challenging aspect was the compliance with the inclusion/exclusion criteria for the study, where only 24 of the 42 patients who wanted to participate in the study (about 57%) were actually enrolled into it. Additionally, one of the most important factors for a successful study in end-stage AIDS patients was the positive interaction between the clinical investigators and the patients. Without a direct participation of the physicians, the clinical evaluation in these patients would had been almost impossible to accomplish. Any future evaluation must consider the doctor-patient interaction as part of the criteria for selecting a study site.

In conclusion, HEP in combination with famotidine was safe for use in this patient population, however, the high lipid content limited the tolerability of doses greater than 2.88×10^6 AU/day. For the doses and duration of HEP used in this trial, an overall favorable effect on diarrhea was seen with a lesser effect on *C. parvum* eradication. Recommendations from the investigators also included changes in the formulation to increase the potency of the HEP with a minimum of 50% lipid reduction.

Table 18. Patient enrollment by dose and categorization of treatment duration

	Patient Enrollment In Each Dose Group		Total
	Low Dose	High Dose	
Total no. patients enrolled	15	9	24
No. patients completed study:			
• 3 weeks	5	-	5
• 6 weeks	6	3	9
No. patients discontinued prematurely:			
• < 3 weeks	4	4	8
• > 3 weeks, but < 6 weeks	-	2	2

Table 19. Number of patients prematurely discontinued

Categorization	Dose level	Week of study						Total (%) of premature discontinuations
		1	2	3	4	5	6	
Adverse event	Low	1	--	--	--	--	--	1 (7 %)
	High	--	--	--	--	--	--	--
Poor tolerability	Low	--	--	--	--	--	--	--
	High	2	--	--	--	--	--	2 (22 %)
Intercurrent or concurrent illness	Low	1	--	--	--	--	--	1 (7 %)
	High	--	--	--	--	--	--	--
Treatment failure	Low	--	--	--	--	--	--	--
	High	2	--	--	1	1	--	4 (44 %)
Patient request/refusal	Low	2	--	--	--	--	--	2 (13 %)
	High	--	--	--	--	--	--	--
TOTAL	Low	4	--	--	--	--	--	4 (27 %)
	High	4	--	--	1	1	--	6 (67 %)

Table 20. Mean dose (Number of Bags of HEP) and percent compliance per week by dose level

Dose Level	Mean Number of Bags of HEP/Week (% Compliance)						
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Overall Mean
Low	N = 15 27.9 (79.7%)	N =11 34.9 (99.7%)	N = 11 34.4 (98.3%)	N = 6* 34.7 (99.1%)*	N = 6* 34.7 (99.1%)*	N = 6* 34 (97.1%)*	33.4 (95.4%)*
High	N = 9 38.1 (54.4%)	N = 5 50 (71.4%)	N = 4** 51.5 (73.6%)	N = 5 43.2 (61.7%)	N = 4 38.5 (55%)	N = 3 30.3 (43.3%)	41.9 (59.9%)

Table 21. Changes in primary efficacy parameters at end of treatment relative to baseline *versus* treatment duration

Duration of treatment	Total No. Patients	Number of Patients (% Patients)								
		Oocyst Grade			Frequency of bowel movements			Stool Consistency*		
		Decr.	No change	Incr.	Decr.	No change	Incr.	Decr.	No change	Incr.
2-3 weeks	3	1 (33%)	2 (67%)	0 (0%)	2 (67%)	0 (0%)	1 (33%)	2 (67%)	0 (0%)	1 (33%)
4-6 weeks	13	4 (31%)	6 (46%)	3 (23%)	8 (62%)	0 (0%)	5 (38%)	7 (54%)	0 (0%)	6 (46%)
>6 weeks**	4**	2 (50%)	2 (50%)	0 (0%)	3 (75%)	0 (0%)	1 (25%)	3 (75%)	0 (0%)	1 (25%)

*Evaluated as the daily frequency of liquid bowel movements.
**Compassionate extension beyond 6 weeks

Figure 19. Daily Frequency of BM
at 3 and 6 weeks

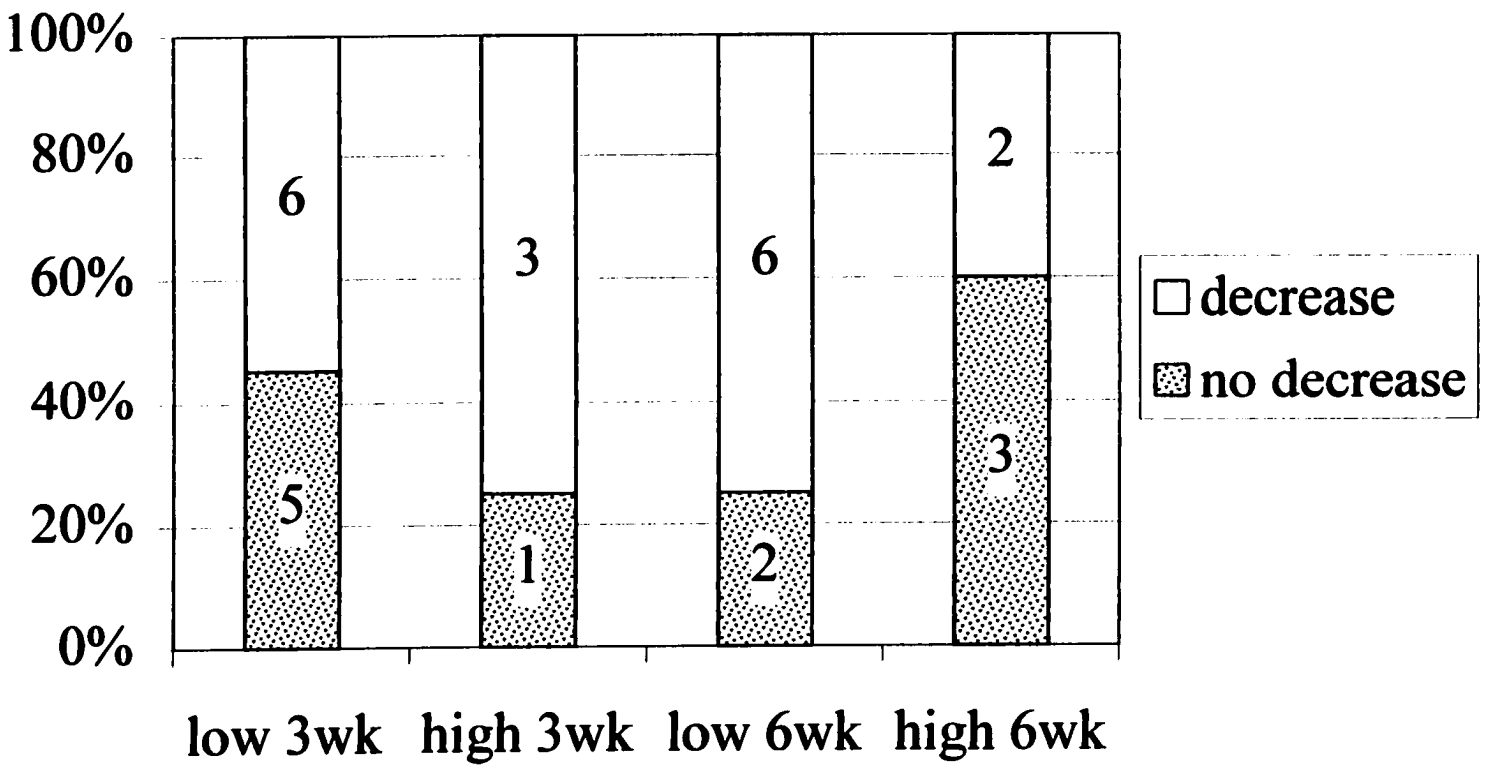
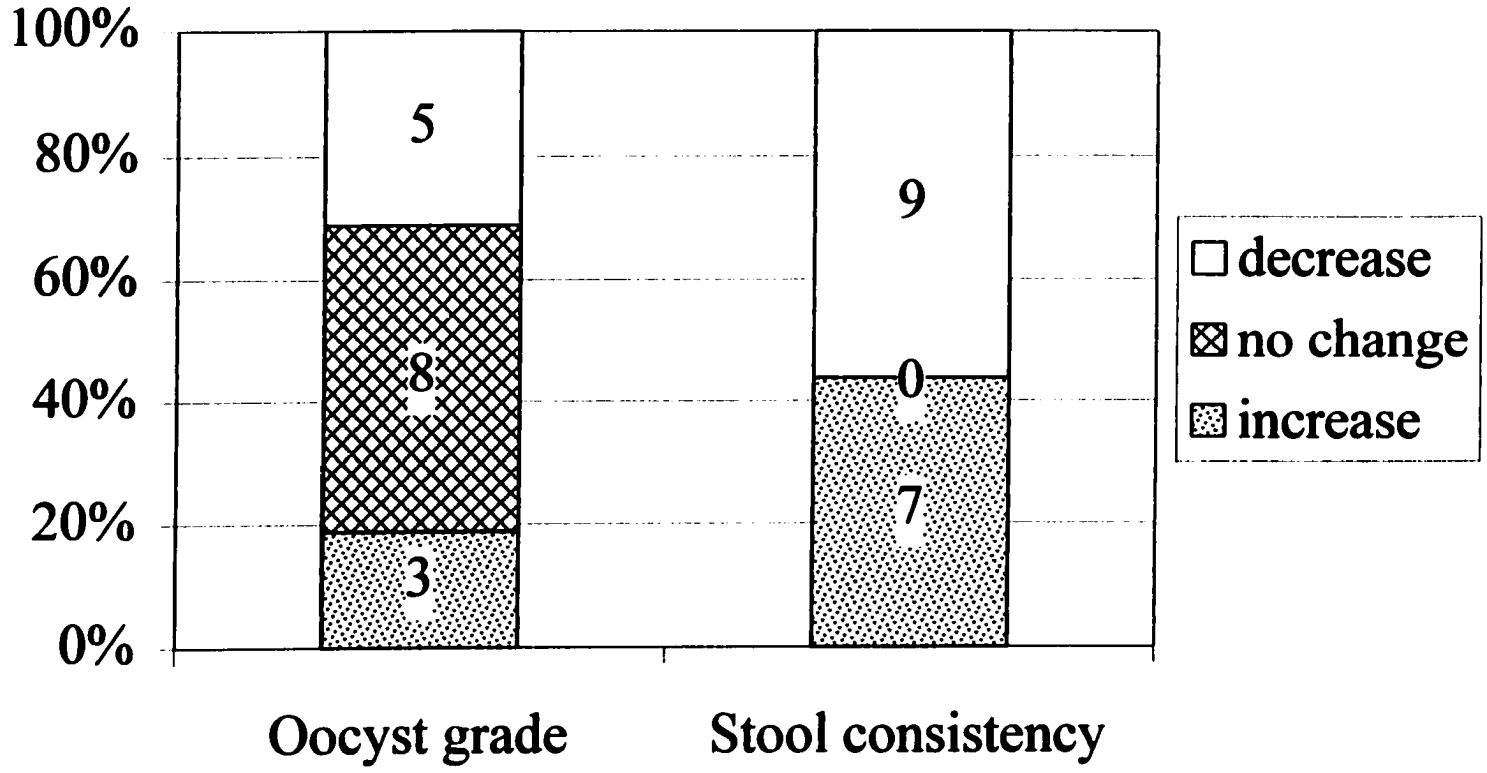


Figure 20. Oocyst grade and stool consistency by end of treatment



CHAPTER 7: IMPROVEMENTS IN THE HEY FORMULATIONS FOR TREATING ENTERIC CRYPTOSPORIDIOSIS.

I. Introduction:

The information gathered through the experiments described in the previous chapters reflects the efforts towards evaluating polyclonal antibody preparations as potential therapeutic agents for the treatment of enteric cryptosporidiosis, selecting the most efficacious preparations based on *in vivo* testing with subsequent testing in human clinical trials.

Some additional remarks regarding the use HBC and breast milk need to be mentioned. The use of bovine colostrum in *in vivo* mouse studies showed reductions in the parasite burden, however, the degree of infestation of the enteric surface was still significant. Other evaluations of bovine colostrum in treating calves (Fayer et al, 1989) described improvements in the condition of the treated animals, but no indications of therapeutic clearance of the parasite during the evaluation time. The prophylactic administration of HBC to human volunteers infected with *C. parvum* provided some beneficial effects, but did not show clear clinical evidence towards efficacy of the product (Okhuysen et al, 1998a) . These findings indicated that HBC raised against whole *C. parvum* antigens was not highly efficacious in controlling infections in immune competent humans and bovines, with benefits associated only with it's prophylactic administration.

The use of monospecific colostrum, targeting specific epitopes such as P23 and GP15, has renewed the focus on determining the potential of bovine colostral polyclonal therapies for the treatment of acute cryptosporidiosis (Perryman et al, 1999; Jenkins et al, 1999). In the case of chronic human disease, it would be of great value to the scientific community and the patients in need to have access to data from monoclonal antibodies and monospecific HBC in treating AIDS patients with cryptosporidiosis.

The conclusions from the human clinical trial described in Chapter 6 were that the HEY preparations provided benefits to the patients, but those benefits were limited by the formulation of the product. The formulation tested was a homogenized liquid preparation of egg yolk contents, with potency adjusted by AUs, sugar added as an anti-gelation agent and dispensed into stand-alone plastic pouches. The analysis of the data demonstrated safety, partial efficacy and that small volumes of the product were very well tolerated by the patients. However, the proposed dose escalation testing of the product reported led to patient tolerability problems. In order to achieve a higher dose, the only option with the formulation tested was to increase the total volume of egg yolk product administered. Patients were asked to take larger volumes, equivalent to twice as much as administered to the patients in the low dose arm of the study. As a result, most patients in the high dose arm of the study were unable to comply with the protocol, mainly due to satiety and bloating.

The analysis of the data gathered in the clinical trial demonstrated that the product was safe as observed in the data from the laboratory reports (blood chemistry, urianalysis,

lipid profiles, etc.) and that most patients in the high dose group reported satiety and bloating. Further analysis with the clinical investigators and the nutritionist led to the conclusion that the low dose administered was beneficial but that the efficacy of the product could have been enhanced by the administration of more antibodies, as initially planned for the high dose patients. In summary, the necessary changes to the product to be used in the subsequent clinical trials were to double the potency in a formulation with at least 50% lipid reduction when compared to the product already tested.

This technical request and challenge had another component. All work had to be done in compliance with the regulatory requirements for the clinical trial testing of compounds. There are numerous protocols for lipid reduction and antibody purification already described in the literature. A few are dedicated to the areas of egg yolk products (Bizhanov and Vyshniauskis, 2000), with lipid reductions achieved by the use of organic solvents (Hassl et al, 1987), lipid precipitation (Akita and Nakai, 1993) or the use of poly-ethylene-glycol (PEG) for antibody purification (Polson et al, 1990). The protocol of choice had to keep in mind several considerations including: preservation of efficacy of the antibodies, safety of the product and compliance with the current regulations.

Most antibody purification procedures do not significantly affect the antibodies, however, the method of choice had to maintain the antibody efficacy and demonstrate so in *in vivo* assays. The safety of the product was a top consideration. For HEY products, the safety can be demonstrated by assuring that the antibodies are generated in a safe source and that intermediate products, components and additives are safe and within

specific quality limits and in agreement with the regulatory agency. With these constraints, the ideal components or additives to be used in the lipid reduction process had to be safe chemicals with minimal or no toxicity profiles.

To meet the regulatory requirements that allow the continuation of clinical trial evaluations, the material had to be similar to the one already tested in the Phase I/II trial described in chapter 6, with only nutrients being reduced, such as lipid, protein or carbohydrates. The ideal method would consist of one or maybe two steps, and achieve both the lipid reduction and enhancement of antibody concentration. An additional consideration was that the method of choice had to be compatible with upscaling and manufacturing regulations (current Good Manufacturing Practices or cGMPs).

A review of the literature found multiple options for reducing egg yolk lipids. Solvent extraction methods are commonly used to remove lipid contents of organic preparations that are going to be tested *in vitro*, one being the use of chloroform, ethanol and sample in a ratio of 1:2:0.8 (Bligh et al, 1957). These methods were briefly tested, but antibody activity was diminished after the lipid solvent extraction. There were practical limitations for purifying antibodies from several eggs since procedures had to be performed in the fume hood. Also, the removal of the residual solvents was an additional process and difficult to accomplish. Solvent extraction methods did not meet the selection criteria because they did not preserve the antibody efficacy and the purified preparations were not safe due to the solvent residues.

The use of PEG at different concentrations followed by centrifugation at high speeds is a well described method for the purification of hen egg yolk antibodies (Polson et al, 1980; 1985; 1990). This method was initially used for purification of antibodies against other pathogens and the purity and efficacy of the resulting antibodies was very high (Cama, 1988). Under the selection criteria established PEG purification had several limitations. Safety of the product was affected due to the residual presence of PEG with the antibodies and to the toxic profile of this chemical. The partially purified antibodies would have required further purification to achieve the removal of PEG. These additional steps, plus the use of high-speed centrifugation (about 22,000 x g for 15 minutes each time, a minimal of 4 times per batch), would have created regulatory conflicts in the generation of the product. If no other processing alternative would be available, this option had to be considered.

Another method described in the literature is the precipitation of antibodies by saturated salt solutions followed by removal of the excess salts by dialysis. This method worked very well with chicken serum and the process yielded antibodies that are highly pure, with reduced salt contents and high efficacy. In the case of HEY, the lipid content interferes with the precipitation and the precipitated materials are a mixture of lipids and multiple proteins in a very viscous mixture. This process did not satisfy the requirements already determined and could be considered only after the yolks were rendered low lipid. A potential limitation for salt precipitated antibodies would be the removal of the residual salt contents, especially if the salt of choice would be ammonium sulphate. Currently

described protocols for dialysing salt precipitated antibodies require large volumes of water and several hours per step.

Xantham gums were also described in the literature to aid in the lipid precipitation from egg yolks (Hatta et al, 1990) . Observations in preliminary laboratory testing using carrageenan showed a very firm precipitated yellow fraction and a clear almost transparent supernatant fluid. Antibody and protein measurements revealed that antibodies were almost 100% in the pellet and the supernatant fluid had no detectable ELISA reactivity. Several repeat testings at varying concentrations of carrageenan consistently showed no separation between lipids and antibodies; when clear supernatant fluids were observed, the antibodies were trapped in the pellet; when less carrageenan was used, no separation occurred. Based on this observations, the xantham gums were no longer considered an option for lipid reduction.

The acid and water dilution methods were tested, with preliminary results showing that lipids could be precipitated and antibodies would remain in the supernatant fluid.

II. Materials and Methods

A. Hyperimmune hen egg yolk product

Eggs were obtained from *Salmonella*-free white Leghorn hens that had been hyperimmunized with *Cryptosporidium parvum* antigens. Briefly, sonicated *C. parvum* oocysts to a final concentration of 7.5ug/0.5ml were emulsified in Freund's complete adjuvant and injected subcutaneously into the hens. Booster immunizations were carried at 4 and 8 weeks post prime immunization replacing Freund's incomplete for Freund's complete adjuvant. Hens for control HEY were hyperimmunized with inactivated *C.difficile* cells kindly provided by Dr. Glenn Songer, at doses of 5μg/hen/immunization.

For initial laboratory experiments, the yolks from the eggs were manually separated and diluted about 1:1 with purified water. For larger volume experiments, the yolks were automatically separated using a MB-104 Seymour Egg Washer/Yolk Separator (Sanovo Seymour, KS) and also diluted 1:1 with distilled water. The pre-diluted yolk material was subsequently diluted and precipitated to evaluate the lipid reduction of the various test methods.

B. Hydrochloric Acid (HCl) and Caprylic Acid (CA) precipitation methods

The yolks were separated and diluted with purified water in the proportions of 1 part yolk to 9 parts water. The final dilution of yolk was made in water, the pH adjusted to 5.2 with 0.1M HCl or 0.5M CA and the preparations were stored overnight at room

temperature or 4°C. The following day the material was centrifuged at 3,000 rpm (1870 x g) for 15 minutes at room temperature, the supernatant fluid containing the IgY antibodies collected and the pelleted material discarded. The supernatant fluid was concentrated by either dehydration or tangential flow ultrafiltration (TFF).

C. Water precipitation methods

The yolks were diluted with distilled water either at room temperature (water-RT) or with water pre-chilled (cold water) to 4-6°C in a 1:10 proportion and stored overnight at 4°C. The following day the material was centrifuged at 3,000 rpm (1870 x g) for 15 minutes at room temperature, supernatant fluid containing the IgY antibodies was collected and the pelleted material discarded.

D. Control samples for lipid reduction

The pre-diluted material was centrifuged at 1870 x g for 15 min. Three different layers were differentiated: a thin yellowish clear top layer, an intermediate liquid phase which contained IgY, and a yellow pellet. The top layer was carefully removed and the second aqueous layer collected. All test materials from the different methods were stored at -20° C until used in the study.

E. Lyophilization

E.1. In-house lyophilizations

Initial lyophilizations were performed in-house using a Thermovac lyophilizer. Small volume samples, not exceeding 100 ml., were frozen as homogenous thin layers in 2L round bottom glass flasks. The flasks were attached to the lyophilizer, the vacuum set at 100mTorr and the samples lyophilized overnight. The next morning the vacuum was turned off and the dehydrated samples collected and stored at 4°C until tested.

E.2. Contract lyophilization

Samples from protocols that presented therapeutic potential were lyophilized in larger volumes (>10L) at a contract facility (BioServ Corporation, CA). For the generation of a lyophilization protocol, samples of 25ml of selected low lipid material were sent to Phase Technologies Inc. (Conshohocken, PA) for thermal analysis. The objective of these studies was to identify the key thermal properties of the formulation that would help design a detailed lyophilization protocol to be used at a contract facility (Appendix 3).

F. Tangential flow ultrafiltration concentration (TFF)

A Centrassette (Pall Filtron, MA) unit assembled with Omega membranes (Molecular weight cut-off 50,000 Daltons, Pall Filtron, MA) was used to concentrate the supernatants. Briefly, the solution was concentrated by continuos filtration and

recirculation through the Centrassette unit. Molecules larger than the cut off were retained (retentate) and molecules smaller than cut-off were filtered (filtrate). The retentate was recirculated through the unit until the desired concentration was achieved. To match the yields from concentration by dehydration, solutions had to be concentrated to 1/10 of the original volume (10X). All TFF concentrations were performed at 4°C.

G. Lipid measurement

Samples of materials were sent to ABC Research, Division of Food and Agricultural Chemistry (Gainsville, FL), for determination of moisture, fat, protein, ash and carbohydrate content, as the basis for the nutrient composition determinations. Methods used by ABC Research were analytical methods conforming with USDA regulations for food products.

H. Animal Studies

For the initial screening, samples from the different methods were resuspended to 30% wt/vol and orally administered to neonatal Balb/c mice for 5 consecutive days. The materials concentrated by TFF were the equivalent to the 30%wt/vol, in this case concentrated to 1/10 of the original volume. The final evaluations were performed by the administration of 600AU/100 ul. In order to be able to perform statistical analysis, 12 neonatal mice were included in each treatment group. The procedure and data analysis was described in detail in Chapter 5.

I. Tolerability

Because of the intended oral use of these preparations in humans, the flavor of the preparations needed to be tested. Partially purified HEY preparations obtained by methods showing *in vivo* efficacy were evaluated by two people using a scoring system from 1 (not palatable) to 10 (very palatable). The preparations were tested alone or in combination with sugar, artificial sweetener or chocolate flavor.

J. Strategy

In order to select a process that increases the potency of the preparation, with reduced lipid contents and prepared into a tolerable formulation (flavor), the following steps were followed: Primary screening of candidates based on retention of efficacy. The preparations from the different methods were initially tested for activity and efficacy in the mouse model. The methods which preserve the efficacy of the antibodies were then subjected to the next screening stage, which was based on its tolerance (palatability). The preparation with the highest efficacy and tolerability was further tested for efficacy and also used to produce several pilot run batches. Samples from these batches were used for detailed nutrient composition analysis.

III Results

A. Primary screening: Retention of efficacy

This screening was accomplished by the *in vivo* evaluation of the low lipid preparations obtained by the HCl, CA and water dilution and precipitation methods. Study 1 examined the efficacy of HCl or CA lipid reduction methods in the production of a low lipid HEY product for treating *Cryptosporidium parvum* infections in neonatal Balb/c mice. Preparations from antibodies generated by these methods were lyophilized in-house, resuspended to 30%wt/vol and tested for *in vivo* efficacy against *C. parvum* infections. One sample of HCl precipitated material was lyophilized at the contract facility. Three control groups were established, two receiving sterile water (diluent) and one containing non relevant chicken antibodies (anti-*Clostridium difficile* IgY resuspended at 30 % w/v). The diluent control groups had mean parasite-loads of 31.58 and 24.71 parasites per villus (MPV) while the negative control group receiving anti-*Clostridium difficile* IgY had counts of 31.72 MPV. HCl formulations lyophilized in-house and at the contract facility showed loads of 8.24 and 7.36 MPV respectively, with no significant differences ($p>0.05$). These values gave an indication that preparations lyophilized in-house or in the contracted facility were comparable. The group treated with the CA preparations had parasite-loads of 12.99 MPV. The statistical analysis showed significant parasite reductions in the groups receiving HCl or CA lipid reduced preparations ($p<0.01$, ANOVA) when compared to the controls. This data indicates that

HEY preparations with lipids reduced by the use of HCl or CA retained significant anti-crytposporidial efficacy (Table 22).

Study 2: The objective of this study was to examine the efficacy of HCl, CA and water lipid reduced materials which had been lyophilized and the efficacy of HCl and CA lipid reduced supernatant fluids concentrated by TFF.

Results from the diluent treated control group showed mean values of 33.14 parasites per villus. The groups receiving HCl, CA and water-RT formulations, resuspended at 30% w/v, provided parasite-loads of 5.64, 7.34 and 7.31 MPV, respectively. Tangential flow ultrafiltration (TFF) concentration of the CA formulation showed parasite loads of 17.77 parasites per villus while the groups receiving HCl supernatant fluids concentrated by tangential flow had loads of 27.21 parasites per villus (Table 22).

The precipitation of lipids by HCl, CA or water in the HEY formulation, followed by lyophilization and resuspension in water to 30% wt/vol. reduced parasite-loads significantly ($p<0.01$) when compared to that seen in the control group. The TFF concentration to 10% of the original volume of the liquid supernatants from the HCl and CA methods did not reduce parasite loads significantly when compared to the control groups, with values of 27.21 and 17.77 MPV ($p>0.05$). No significant differences between the data from the groups treated with HCl, CA or water preparations, concentrated by lyophilization, were observed.

B. Secondary screening: Tolerability

Considering the potential use of these materials in an oral indication, the acceptability (palatability) of the solutions in suspension alone or with the addition of 1% sucrose, 1% artificial sweetener or 3% chocolate flavoring compounds was evaluated by two individuals. A scoring system from 1-10 was established, with 1 being less acceptable and 10 very acceptable. All CA and HCl preparations scored 1, regardless of the additive used. The water preparations scored better than 7 when used in conjunction of any of the three additives, water preparations without additives scored 5.

C. Verification of efficacy

Based on the results from study 2 and the palatability testing, the focus of the following evaluations was the use of water purified antibodies. Additionally, the conditions for water precipitation included pre-cooling of water to 2-6°C prior to addition to the yolks. In Study 3, two different water preparations were tested: lyophilized and resuspended in diluent to 600AU/100ul and TFF supernatants concentrated to 600AU/100ul. Three control groups were established: diluent, HCl preparations matched to 600AU/100ul (positive control) and a lyophilized anti *C. difficile* water precipitated negative control. Mean parasite loads per villus observed in the terminal ileum were significantly reduced ($p<0.001$) in the water group (0.5 MPV) when compared to the diluent and lyophilised negative controls (27.3 and 22.6 MPV, respectively). Significant

differences were also observed when compared to the group receiving TFF concentrated antibodies or the HCl positive control ($p<0.01$) (Table 22).

Study 4: This study had the objective to evaluate the effects of a flavouring substance (sucrose) in the water preparations. The efficacy data showed similar results to those of study 3, where the mean parasite density per villus observed in the terminal ileum was significantly reduced ($p<0.001$) in the water preparation flavoured with sucrose (0.34 MPV) when compared to the diluent and lyophilized negative controls (32.03 and 18.09 MPV, respectively). These data indicated that the addition of sugar did not have a negative impact on the formulated materials and might have provided some benefit (Table 23).

D. Lipid reductions:

A nutrient composition analysis comparison was made between egg yolk preparations administered in the human clinical trials (OLD)and the new water lipid reduced preparations (NEW). The materials tested in the previous human evaluation had either 412,000 (low dose) or 824,000 (high) activity units (AU) per dose. To provide a patient a total of 824,000 AU per dose, the patient was required to drink 141.58 mls of the HEY. This value was the average from 30 manufactured batches. Considering the *in vivo* data reported in TABLE 1, the water preparations were selected and several batches following that protocol were generated and compared for nutrient composition. The average percentage and mass compositions of the previous HEY and the new low lipid preparations matched to 824,000 AU are presented in Table 24.

The average mass for 824,000 AU of the water precipitated and lyophilized formulations was 14.49 g; this value was the average from 69 batches, 200L each (average = 14.49 g, SD = 3.57). For comparison purposes this value was rounded to 15 g. The analysis of the nutrient composition values showed an almost 90% reduction in the mass of the dose and in the net fat contents per dose, and a 45% reduction in the grams of protein per dose (Table 24).

IV. Discussion:

The three lipid reduction methods tested were based on the principle of breaking the emulsion present in yolk and allowing the precipitation of the suspended lipids. The use of HCl and CA had the purpose of precipitating the suspended lipids by adjusting the pH to their reported isoelectric point (pI), resulting in lipid insolubility and subsequent precipitation. The water method induced the precipitation of lipids by breaking the emulsion and reducing the lipid solubility at refrigerated temperatures. The efficacy data showed that all methods retained active antibodies when concentrated by lyophilization, with similar *in vivo* results between the in-house and contracted lyophilization.

The main objective of the thermal analysis was to identify the critical temperatures associated with large scale lyophilization. It was determined that the complete freezing of the water preparations required temperatures <-40°C (mobility of water in the interstitial matrix reaches zero) and that during the first stage of lyophilization (primary drying) the temperature of the product should never be above –28°C. Warming of the product at a temperature greater than –28°C could increase the likelihood of collapse of the frozen matrix, trapping water molecules within the material to be dehydrated. When the temperatures are ramped towards 0°C, the water trapped within the collapsed cake could boil and damage the antibodies.

Studies 1, 2 and 3 (Table 22) tested the three different preparations in matched weight/vol concentrations, showing various degrees of antiparasitic efficacy in the *C. parvum* infected mice. The supernatant fluids concentrated by TFF were not as

efficacious. This low efficacy observed in the TFF materials could be attributed to the continuous passage of the preparations through the membranes, which eventually caused mechanical shearing of the proteins resulting in a significant loss of efficacy. Subsequent evaluations of TFF demonstrated that TFF concentrations up to 40% of the original volume (2.5X instead of the 10X tested) were the maximum limit that did not affect antibody efficacy.

Because the intended use of these preparations was oral administration, a basic palatability testing was performed using two laboratory volunteers. Both HCl and CA preparations were scored the lowest possible, since those preparations were not tolerable even when diluted 1/10. The water preparations were significantly better tolerated, with scores of 7 when sucrose was added to a concentration of 1%. The best scores were obtained when the preparation was mixed with chocolate (score = 9). Considering that the preparations had to be frozen for lyophilization processing, and sugar is an antigelating agent for egg yolks, sugar was chosen as the additive.

The water preparations resulted in a significantly reduced mass when compared to the original formulation tested in humans, with an average 89.4% reduction. In terms of lipid composition, the water preparations had at least 72.98% less lipid, and on average, 90.26% less lipid in a 824,000 AU dose. The water preparations plus sucrose to 1% were tested for *in vivo* anti-cryptosporidial activities. Previously tested HCl materials matched by potency were used as positive controls. The results indicated a significant

improvement in the efficacy of the HEY preparations when lipids were reduced by water precipitation.

These partially purified HEY antibodies enhanced the potential use of chicken antibodies. A more potent formulation with increased potency was generated, achieving significant reductions in *C. parvum* infections of neonatal Balb/c, with MPV values of less than 1. These results were not possible with the previous formulation tested in the clinical trial nor with the material previously reported (Cama and Sterling, 1991), where the efficacy of parasites reduction was to about 5 parasites per villus.

One of the major difficulties associated with egg yolk antibodies was the lipid rich environment in which they were produced. The reduction of lipid content allowed a significant volume reduction and facilitated tolerability for direct oral applications, also it would permit further purification of the antibodies by more refined techniques.

These results demonstrated a lower lipid preparations for formulating an enhanced product. Because HEY polyclonal antibodies are poly-specific, this concentrated lipid reduced preparation could be significantly improved by the simultaneous administration of specific epitope-binding antibodies. These antibodies could be monoclonal or mono-specific polyclonal antibodies. Additionally, the simultaneous use of these HEY antibodies and other antiparasitic compounds could provide a product aimed towards the cure instead of control of enteric cryptosporidiosis.

Table 22: Efficacy of low lipid preparations tested in the mouse model.

Study #	Materials Tested	MPV
1	Sterile diluent	Group 1 = 31.58
	Sterile diluent	Group 2 = 24.71
	Negative Control (anti-C. difficile IgY)	31.72
	HCl preparation	8.24[1]
	HCl preparation @	7.36[1]
	Caprylic acid	12.99
2	Sterile diluent	33.14
	HCl preparation	5.64[1]
	HCl by TFF	27.21
	CA preparation	7.34[1]
	CA by TFF	17.77
	Water RT	7.31[1]
3	Sterile diluent	27.3
	HCl 600 AU	7.3[1]
	Cold water 600 AU	0.5[1,2]
	Cold water 600 AU by TFF	13.6
	Negative controls (anti *C. difficile)*	22.6

[1] Statistical significant difference ($p<0.01$) when compared to control
[2] Statistical significant difference when compared to HCl and TFF preparations

Table 23: Efficacy of a flavored cold water low lipid preparation tested in the mouse infectivity model

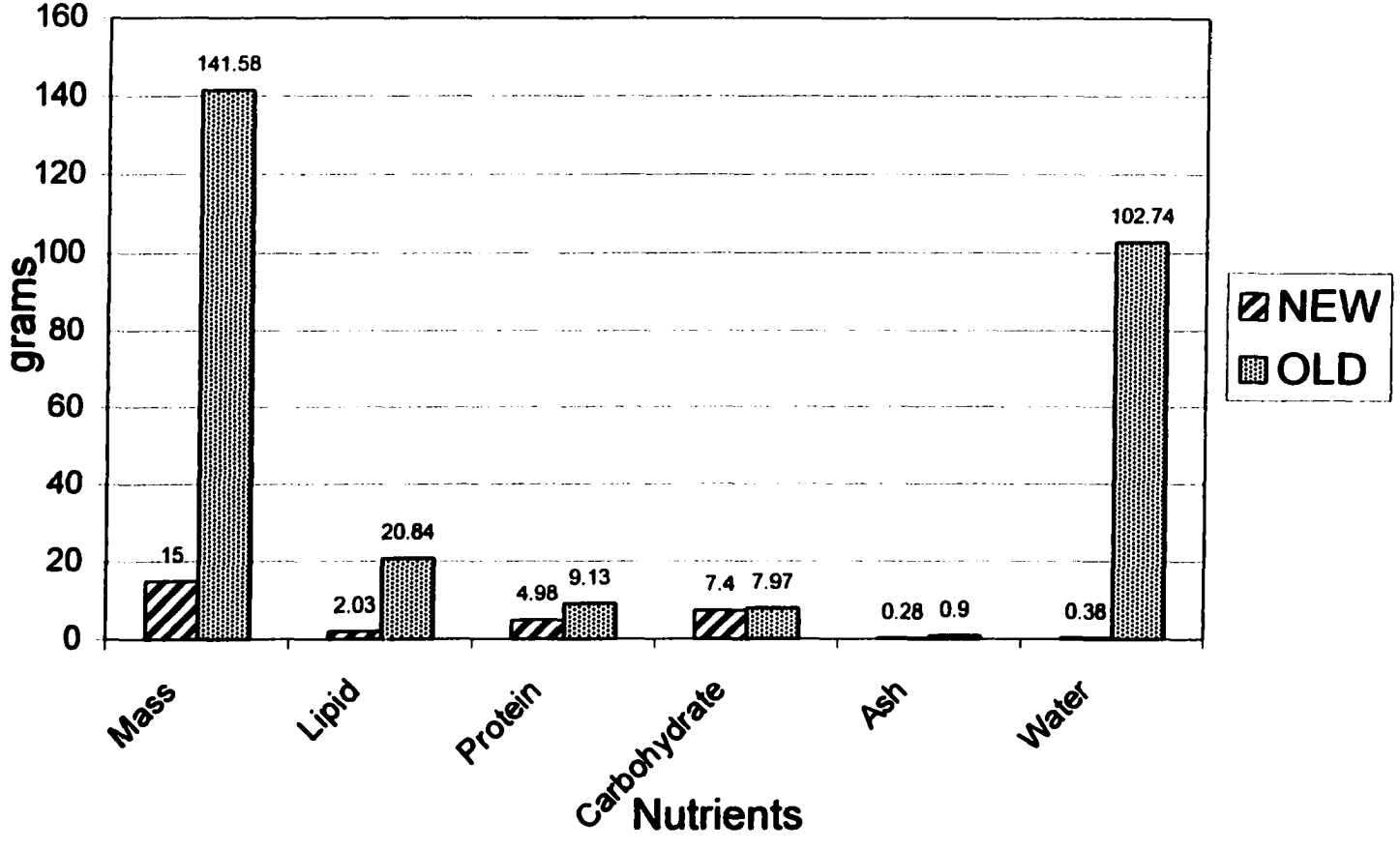
Materials Tested	MPV
Diluent Control	32.03
Cold water @	0.34[1]
Negative control	18.09

[1] Statistical significant difference ($p<0.01$) when compared to control
@ The cold water preparation +1% Sugar, flavor rating of 8 (1-10 scale).

Table 24: Nutrient composition of preparations tested in clinical trials and the new lipid reduce compositions.

	Protein	Lipid	H_2O	Ash	Carbohydrate
OLD formulation 141.58ml/dose	9.13g 6.45 %	20.84g 14.72%	102.74g 72.57%	0.90g 0.63%	7.97g 5.63
NEW formulation 15g/dose	4.98g 33.17%	2.03g 13.55%	2.51g 2.51%	0.28g 1.85%	7.4g 49.36%
Differences					
Mass	Protein	Lipid	H_2O	Ash	Carbohydrate
-126.58g -89.4%	-4.15g -45.5%	-18.81g -90.26%	N/A	-0.62g -68.8%	-0.57g -7.51%

Graph 22. OLD and NEW preparations
Nutrient Composition Analysis



APPENDIX A

ELISA Procedure

1. Dilute antigen (Ag) (frozen sonicated oocysts) to 0.2 μg/100 μL in carbonate buffer. Make 7.5 mL of carbonate buffer for each 96-well plate.
2. Coat columns 1,5,9 of 96-well plate with carbonate buffer only (minus Ag controls). Coat the rest of the wells with Ag in carbonate buffer (100 μL/well).
3. Incubate plates overnight at 4°C or for 1 hour at 37°C.
4. Wash plates 3 times with washing buffer. (Do not allow wells to dry out at any point).
5. Block all wells with casein buffer (200 μL/well). Incubate for 1 hour at 37°C or overnight at 4°C.
6. Wash plates 3 times with washing buffer.
7. Apply primary Ab (human breast milk, bovine serum or colostrum, and chicken serum or yolk diluted in PBS, 100 μL/well) in triplicate (column No.s 2-4, 6-8, or 10-12). Reserve the last row (H) for positive control, negative control and PBS only (in that order). This scheme allows for 21 samples per plate.
8. Incubate plates for 1 hour at 37°C.
9. Wash plates 3 times with washing buffer.
10. Apply secondary Ab 100 μL/well according to the primary antibody used [Peroxidase labelled goat anti-bovine IgG for bovine colostrum, goat anti-human IgA for human milk samples, or goat anti-chicken IgG/IgY for chicken and yolk samples] (Kirkegard and Perry Laboratories Inc., MD), diluted 1:1000 in washing buffer.
11. Incubate plates for 1 hour at 37°C.
12. Wash plates 5 times (to be sure to remove all non-specific secondary Ab) with wash buffer.
13. Mix equal portions of substrate (TMB) and H_2O_2 to a total volume of 10ml per plate and apply 100 μL/well. Allow reaction to proceed for about 3 minutes (positive reaction = blue). Stop reaction with 100 μL/well of 1M phosphoric acid (turns yellow).
14. Read the OD of each plate well using an ELISA reader at 450 nm. Data are sent directly to computer files using the Procomm program. Each 96-well plate is represented on a separate computer file. Files are then processed by a Lotus 123 macro program and results are printed out with the mean values for each sample tabulated.

APPENDIX B

Western Blot Procedure

Protein antigen samples are solubilized with SDS and run reduced on a 10% SDS-PAGE gel. The proteins are transferred to a nitrocellulose membrane using a semi-dry transfer apparatus.

1. Prepare reduced samples/controls and separate proteins using a SDS-PAGE gel. Use 10% SDS-PAGE gel for C. parvum antigen. Pre-stained MW standards (BRL) were used as a indication of a successful transfer.
2. Remove stacking gel and sides of running gel beyond sample wells with a razor blade. Notch bottom right-hand corner of gel for orientation. Note: Equilibration of running gel in Cathode Buffer is generally not required but may improve results if bands appear diffuse.
3. Prepare transfer membrane by soaking in transfer solution for 15 seconds and equilibrate membrane in Anode Buffer II for 5 minutes.
4. Wet two pieces of filter paper in Anode Buffer I and place on anode plate of blotter. Avoid trapping air between electrode and filter paper by laying filter paper on electrodes at an oblique angle.
5. Wet one piece of filter paper in Anode Buffer II and place on top of filter papers previously placed on electrode.
6. Remove transfer membrane from Anode Buffer II and place on top of filter paper stack.
7. Place gel on top of transfer membrane taking care not to trap air bubbles between gel and membrane.
8. Wet three pieces of filter paper in Cathode Buffer and place on top of gel. Use a clean plastic test tube to roll out air bubbles.
9. Place cathode plate of blotter on top of transfer stack (see Fig. 1).
10. Connect high voltage cords to power supply. Apply a constant current of 1.9 - 2.5 mA per cm2 of gel area for 30 - 60 minutes. Transfer time depends upon proteins being transferred.
11. After transfer is complete, turn off power supply and remove cathode plate of blotter. Remove transfer membrane and cut lower right corner of membrane to mark orientation of gel.
12. If immunostaining is to be performed immediately, rinse the membrane in distilled water and proceed to the immunostaining portion of protocol. If staining is to be performed at a later time, place membrane on a paper towel and allow to dry. Store membrane in a dry container at 4 °C.

Immunostaining Procedure -

1. After transfer, thoroughly dry the blot by incubating the blot at 37 °C for 30 minutes.
2. Dilute 1° antibody 1/250 in Diluent buffer.
3. Add 20 mL of diluted 1° antibody to membrane. Incubate on rocker/shaker at room temperature for 1 hour.
4. Pour off 1° antibody and wash membrane for 10 seconds with TTBS. Repeat wash.
5. Dilute 2° antibody to 1:8000 to 1:50,000 (or manufacturer's recommended dilution) in diluent buffer.
6. Add 20 mL of diluted 2° antibody to membrane. Incubate on rocker/shaker at room temperature for 30 minutes.
7. Pour off 2° antibody and wash membrane for 10 seconds with TPBS. Repeat wash.
8. Dilute peroxidase labeled 2° antibody (HRPO) diluted 1:250 in Diluent buffer.
9. Add 20 mL of HRPO reagent to membrane. Incubate on rocker/shaker at room temperature for 30 minutes.
10. Pour off HRPO reagent and wash membrane for 10 seconds with T-PBS. Repeat wash.
11. Prepare Substrate Development Solution by adding 1ml membrane enhancer to the TMB peroxidase membrane developing system. Do not allow membrane to dry while preparing Substrate Development Solution.
12. Add Substrate Development Solution to membrane. Incubate on rocker/shaker for 5-10 minutes or until the desired degree of staining has been reached.
13. Stop substrate development by pouring off development solution and rinsing membrane in deionized water. Allow membrane to air dry on a paper towel. Blots can be stored dry for long periods if protected from light.

APPENDIX C

Lyophilization Protocol

1. Trays are filled with 1.0 litres of the partially purified IgY from egg yolk
2. Place trays in lyophilizer so that there are no more than 4 pans on each shelf.
3. Lyophilization cycle

The pressure is maintained at 100 microns in the lyophilizer and the condenser temperature is maintained at -5°C throughout the drying process procedure.

Step 1: The egg yolk is soaked for 30 minutes and the temperature will then be ramped for 4 hours to -40°C.

Step 2: The temperature is maintained at -40°C for 6 hours and then ramped for 2 hours to -20°C.

Step 3: The temperature is maintained at -20°C for 36 hours and then ramped for 1 hour to -10°C.

Step 4: The temperature is maintained at -10°C for 1 hour and is then ramped for 1 hour to 0°C.

Step 5: The temperature is maintained at 0°C for 12 hours and is then ramped for 2 hours to 15°C.

Step 6: The temperature is maintained at 15°C for 8 hours whereupon the cycle is complete.

Step 7: Nitrogen purge (optional)

a. Increase the pressure to 2 Torr for about 2 minutes by using USP nitrogen.

b. Turn gas bleed off. Allow pressure to return to 100 mTorr.

c. Repeat 7 times.

4. Drying cycle is completed. The dried material is removed from the trays and placed in designated transport tanks to be returned to the manufacturing facility.

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