MOLECULAR EPIDEMIOLOGY OF CRYPTOSPORIDIOSIS AND CHARACTERIZATION OF THE HUMAN IMMUNE RESPONSE TO *CRYPTOSPORIDIUM SPP.* IN SOUTHERN INDIA

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CERTIFICATE

This is to certify that the thesis entitled **"MOLECULAR EPIDEMIOLOGY OF CRYPTOSPORIDIOSIS AND CHARACTERIZATION OF THE HUMAN IMMUNE RESPONSES TO CRYPTOSPORIDIUM SPP IN SOUTH INDIA"** is based on the results of the work carried out by Dr. A. Sitara Swarna Rao during the period of study under my supervision and guidance. This work or thesis has not formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

The candidate has independently reviewed the literature, standardized and carried out the various techniques towards completion of the thesis. She has written up the thesis herself and has done the necessary statistical analysis and presentations independently.

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- **LIST OF ABBREVIATIONS**

bp - base pair
µ - micron
µl - micro litre
AIDS - acquired immunodeficiency syndrome
ART- antiretroviral therapy
BMT - bone marrow transplant
BSA - bovine serum albumin
CD - cluster of differentiation
CDC- Centers for Disease Control
CMC- Christian Medical College
COWP – Cryptosporidium oocyst wall protein
Cp – Cryptosporidium parvum
DHFR – Dihydrofolate reductase
DNA- Deoxyribonucleic acid
EDTA –Ethylenediamine tetra acetic acid
ELISA –Enzyme linked immunosorbent assay
EST – expressed sequence tag
ETEC – Enterotoxigenic E. coli
GIS – geographic information systems
gp – glycoprotein
GPI –Glycosylphosphatidylinositol
HAART – Highly active antiretroviral therapy
HAZ – Height for age Z score
HIV – human immunodeficiency virus
IFN – interferon
Ig - Immunoglobulin
IL – interleukin
IPTG Isopropyl β-D-1-thiogalactopyranoside
IQR Interquartile range
ITS – internal transcribed spacer
kDa Kilodalton
Mabs Monoclonal antibodies
Mb- mega base pair
Mg - milligram
MPN- most probable number
NFkB – Nuclear Factor k B
NNRTI – non nucleoside reverse transcriptase inhibitor
NTP Nucleotide triphosphate

N-WASP – Neural Wiskott–Aldrich syndrome protein
OD – Optical density
OI – Opportunistic infection
PBS - Phosphate buffered saline
PCR - Polymerase chain reaction
PV – Parasitophorous vacuole
RFLP – Restriction fragment length polymorphism
rRNA - ribosomal RNA
SD – Standard deviation
TCR – T - cell receptor
TNF- tumor necrosis factor
TRAP - Thrombospondin-related adhesive protein
WAZ- Weight for age Z score
WHO -World Health Organization
Xgal - 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

- **CHAPTER 1**

INTRODUCTION

Cryptosporidium spp. are apicomplexan parasites that are an important cause of diarrhea, both in children in developing countries, where it is endemic and in immunocompromised individuals where infection may result in protracted and life threatening diarrhea in HIV infected patients. In the west, cryptosporidiosis is causally associated with sporadic infections as well as waterborne outbreaks. In cryptosporidial infection, the immune status of the host plays an important role in determining disease. Immunocompetent persons develop a self-limited diarrheal illness but severe and persistent infections occur in subjects with compromised immune function. Cryptosporidiosis in humans is mainly attributable to 2 species - *Cryptosporidium hominis* and *Cryptosporidium parvum*.

Transmission occurs by multiple routes including direct person-to-person spread, ingestion of contaminated food or water or contact with infected animals. The spherical thick walled, environmentally hardy oocysts, shed in the fecal material of the infected host, are immediately infectious, unlike other coccidian parasites, and have a low infectious dose (42). The potential to cause explosive and massive waterborne outbreaks has also been well documented in developed countries. As there is a distinct potential for intentional contamination of water supplies, the CDC has included this parasite as a Category B pathogen for biodefense.

Approximately one in five deaths in children aged less than 5 years in India is attributable to diarrhea and *Cryptosporidium spp*. is a leading cause of infectious diarrhea in children in India. Infection in early childhood has been associated with subsequent impairment in growth, physical fitness and cognitive function in children in developing countries (25, 86). In these countries, malnourished children may be at greater risk of developing cryptosporidiosis, and in addition, those who do so are at risk of further nutritional compromise during the course of infection. This protozoan parasite accounts for around 12% of diarrhea in children below? 5 years in developing countries with a peak incidence in children less than 2 years of age. A number of studies in India have reported *Cryptosporidium spp*. in diarrheal stool samples of children by microscopy with positivity rates ranging from 1.1% to 18.9%. There have however, been very few studies examining the molecular epidemiology of cryptosporidiosis in children and none examining immunity to cryptosporidiosis in Indian children.

Cryptosporidiosis is also a substantial threat to HIV-infected individuals and affects up to 50% of patients with AIDS in the developing world, (64). Patients can have chronic watery diarrhea that can last for more than 2 months, resulting in severe dehydration, weight loss, malnutrition, extended hospitalizations, and mortality. AIDS patients with cryptosporidiosis also have a significantly shorter duration of survival from the time of diagnosis (138). Cryptosporidiosis remains a major risk to immunocompromised because of the lack of effective specific therapy. Antiretroviral therapy greatly influences the outcome of cryptosporidiosis by immune reconstitution and increase in CD_4 counts

resulting in a sustained therapeutic effect on follow-up. However, despite the use of HAART, HIV-infected patients still present with coccidian diarrhea, possibly due to non-compliance with medications, viral drug resistance to the drugs or decreased drug bioavailability. Reports from the mid 1990s on the prevalence of symptomatic cryptosporidiosis in HIV infected adults from different parts of India, range from as low as 0.7% to 81%. Some of these studies that documented mean CD_4 counts in patients, showed that that symptomatic cases had CD_4 counts < 200 cell/mm^3 reinforcing the importance of CD_4 T cells in mediating resistance to this pathogen.

Due to its sequestered location within the host cell, ability to set up an auto-infective cycle in the host, innate antimicrobial resistance and ability to affect several host species, this parasite has a large survival advantage over other enteric pathogens. Till date, there is no effective treatment or vaccine for cryptosporidiosis. Nitazoxanide was licensed for use in immunocompetent patients, but a recent meta-analysis found it to be ineffective in HIV-infected individuals (3). The urgent need for development of tools for early detection, treatment and prevention of cryptosporidiosis especially in developing countries is highlighted by its inclusion in the 'Neglected Diseases Initiative' in September 2004 (203). This study attempts to present the much needed information on epidemiology, transmission and immune response to *Cryptosporidium* in 2 vulnerable populations in India.

- **CHAPTER 2**

AIMS AND OBJECTIVES

The aim of this study was to document cryptosporidial infections and immune response in children under the age of 5 years and HIV infected adults – the two populations most vulnerable to infection in India. Children in a longitudinal birth cohort study were included to study the commonly circulating species and subgenotypes in the community, transmission patterns, effect of multiple infections and longitudinal antibody responses. Hospitalized children were included to study severity of diarrhea and quantitate oocyst burdens and contrast species and subgenotypes obtained with that in the community. HIV infected adults recruited from the infectious diseases clinic in the hospital were included to study infecting species and subgenotypes, immune response and oocyst burdens.

The specific aims were:

1. **To study the molecular epidemiology of Cryptosporidiosis**

   a) To study the molecular epidemiology of cryptosporidial diarrhea in a birth cohort of children in the community

   b) To identify the effects of multiple symptomatic and asymptomatic cryptosporidial infections in early childhood

   c) To characterize cryptosporidial isolates in hospitalized children with diarrhea and quantitate oocyst burden with real time PCR

   d) To characterize cryptosporidial isolates associated with symptomatic and asymptomatic infections in HIV infected adults in the hospital and to determine oocyst burden using real time PCR

2. To study the immune response to cryptosporidial infections

a) To determine maternal and longitudinal serum antibody levels to recombinant cryptosporidial antigens in children in the birth cohort

b) To evaluate subtype-specific antibody responses in children before and after cryptosporidial diarrhea

c) To determine serum antibody responses to the immunodominant cryptosporidial antigen in HIV infected adults with and without diarrhea

- **CHAPTER 3**

REVIEW OF LITERATURE

Historical background – The parasite and its life cycle was first described in seminal studies in mice by a medical parasitologist, Ernest Edward Tyzzer, at Harvard University in an observation in 1907, and subsequent publications in 1910 and 1912 (231). He named this genus "*Cryptosporidium*" or "hidden sporocysts" due to the absence of sporocysts, which are seen in most other coccidian parasites. This protozoan then remained largely unrecognized as a human pathogen until the first cases reports in humans emerged in the late 1970s with the advent of the AIDS epidemic. Although the first human case report in 1976 was a child with a self limiting enterocolitis that lived on a farm (167), a review of the first 7 reported cases in 1980 showed that this was a disease of the immunocompromised (231).

The potential to cause waterborne outbreaks, due to the ability of the oocyst to survive in the environment, was documented in Milwaukee where the largest outbreak of water borne disease ever took place in 1993, affecting an estimated 403,000 persons (135). This and the inclusion of the parasite as a Category B pathogen for biodefense as a water safety threat by the Centers for Disease Control and Prevention, Atlanta (CDC) (http://www.bt.cdc.gov/agent/agentlist-category.asp) due to the potential for intentional contamination of water supplies, has resulted in a new interest in research on this pathogen.

Since the 1980s, the life cycle and invasive process of *Cryptosporidium* in the intestinal epithelial cell has been studied (139), the taxonomy and host specificity revised (247), the number of chromosomes established as 8, expressed sequence tag (EST) data made available (222) and *in vitro* culture attempted with partial success (15). In 2004, the 9 Mb genome sequence of *C. parvum* and *C. hominis* was released (2). This has resulted in a clearer understanding of the biology and pathogenesis of this protozoan parasite. However, in spite of these advances, till date there is no effective therapy for cryptosporidiosis in HIV and no vaccine for prevention of disease in susceptible populations.

Taxonomy and Classification – *Cryptosporidium spp*. belongs to the phylum *Apicomplexa*, group *Alveolata*. They are characterized by the presence of an apical complex containing apical rings, a conoid, micronemes, rhoptries, and dense granules which play an important role in host cell invasion. Although long believed to be related to the other coccidian parasites like *Sarcocystis* and *Eimeria* with whom this parasite shares an ecological niche, there is now phylogenetic evidence to show that *Cryptosporidium* is more closely related to another group of apicomplexan protists called gregarines (23). This new classification may also help explain the several differences between *Cryptosporidium* and other intestinal coccidians including its extracytoplasmic location in the host cell and absence of sporocyst as well as the unique attachment organelle that is also used to define this genus (247). Based on sequencing analysis,

Cryptosporidium was also found to lack the apicoplast and mitochondrial genome usually found in other apicomplexan parasites.

Early taxonomical classifications of species in this genus were based on oocyst morphology and size, infectivity and host species resulting in several erroneously named species. Recent advances in molecular characterization based on the *18S rRNA* gene sequence have now helped validate the presence of at least 16 species (31) and an additional ~ 40 isolates referred to as genotypes. Of importance is the emergence of the species '*C. hominis*' to denote what was previously described as *C. parvum* (human genotype) thereby clearly differentiating the zoonotic species *C. parvum* (previously known as bovine genotype) from isolates that were found in humans. This was based on multi-locus analysis and infectivity studies (150) although the 2 species share an average nucleotide similarity of 96.7%. These 2 species are the most common cause of disease in humans although infections with *C. felis, C. canis, C. muris, C. suis* and *C. meleagridis* have also been reported.

Life cycle, Excystation and Invasion – Several factors have made cryptosporidial research extremely difficult, including the inability to maintain this organism in culture instead requiring passage in larger animals like calves or pigs and the species that affects humans, C. *hominis* is even more difficult to obtain. Therefore *C. parvum* has been used for most research even though *C. hominis* is responsible for most of the human infections.

The life cycle of *Cryptosporidium spp*. is completed within the small intestine and colon of the host, with the developing stages associated with the luminal surface of the intestinal epithelial cells, where it remains intracellular but extracytoplasmic (95). Infection in a new host occurs following the ingestion of as few as 10 oocysts (ID_{50} 132 oocysts), as evidenced in healthy adult volunteer studies (42). A clear dose response relationship in *C. hominis* was also found with diarrhea in 40% of cases when infected with 10 oocysts increasing to 75% with 500 oocysts.

The oocyst wall is a trilaminar structure and is interrupted at one pole by a single seam or suture that extends one-third to one-half the way around the periphery. Sporozoites exit the oocyst via this suture during excystation. The outer wall is thought to be a glycoprotein while the central layer of the wall is thought to be a glycolipid/lipoprotein responsible for the acid-fast staining property of the oocyst. Thin-walled oocysts differ in that the wall does not contain a thick inner layer. The release of 4 motile sporozoites from each oocyst is triggered by temperature, exposure to acidic pH followed by bile salts and trypsin along with proteases released by the parasite (215). Prior to invasion, the adherence of oocyst wall and sporozoites to the intestinal mucous layer is thought to be mediated by surface Gal/GalNAc lectins (215). Using a process of helical or gliding motility, based on the parasites intracellular actin-myosin like mechanism called the actin-aldolase-TRAP complex, the sporozoites move over the surface of cells and deposit trails of proteins that later mediate invasion (38). These include the TRAP C1 proteins and heavily *O*-glycosylated mucins and mucin like proteins like circumsporozoite surface

Table 3.1: Cryptosporidial Proteins Associated with Host – Parasite Interactions

Protein	Size and type	Location and Function
CSL	1300 kDa glycoprotein	Seen in the apical region of sporozoites and released in trails, binds to host cells via 85 kDa receptor
gp900	~ 900 kDa mucin like glycoprotein	Seen on the surface of sporozoites and shed in trails – associated with adherence to mucin and gliding motility
gp40/15	40 and 15 kDa (Cp17) mucin like glycoproteins	gp40 seen on apical region and associated with adherence gp15 over the entire surface, shed in trails, gliding motility
Cp47	47kDa glycoprotein	Apical region, adherence
P23	23/27 kDa glycoprotein	Seen over entire surface, shed in trails, associated with adherence and gliding motility
Cpa 135	135 kDa glycoprotein	Apical region, Co-localizes with gp900
P30	31 kDa protein	Gal/GalNAc lectin, adherence to intestinal mucin layer and epithelial cell epitopes
Cp2	82 kDa, glycoprotein	Associated with PV membrane, sexual development
TRAP C1	76 kDa protein	Apical region, Adhesion and gliding motility
CPS 500	Glycolipid	Gliding motility
Cp12		Oocyst and sporozoites surface adherence
CpMuc 1-7	Family of mucins, 28-55 kDa	Apcial region, adherence

ligand (CSL), gp900, gp40/15, p23 and Cpa 135 (38, 215). Other proteins described to be involved in host cell adhesion include Cp47, Cp12 and Cp2 (38, 215) (Table 3.1).

The attachment and invasion process is dependent on temperature, intracellular calcium, and the host cell cytoskeleton and is an extremely rapid process with invasion occurring in less than 7 minutes (48, 215), with release of proteins by the apical complex. Activation of cell signaling pathways that trigger cdc42, a key regulator of actin remodeling, induces host cell actin remodeling at the attachment site with accumulation of actin and related proteins like tropomyosin 5, N-WASP and Arp 2/3 (46, 48). This results in the formation of a parasitophorous vacuole (PV) (95), a unique sequestered extracytoplasmic niche. A feeder organelle on the parasitophorous vacuole membrane comprised of a tunnel-like structure is believed to function as a portal to allow nutrients to pass from the host cell to the parasite (215). Within the PV, the sporozoites develops into a transitional trophozoite stage which give rise to meronts, which replicate by an asexual cycle (merogony) to form merozoites that infect adjacent epithelial cells (within 3-5 days post infection) and by a sexual cycle resulting in the production of micro- and macro-gamonts (within a week of infection) which fuse to form zygotes. How microgametes detect cells containing the macrogamonts is not known, but once it does, the microgamete and macrogamont fuse (139). The resultant zygotes then mature to form sporulated thin-walled and thick-walled oocysts. The parasite also affects host cell apoptotic pathways causing down-regulation early in infection followed by moderate promotion at later stages (132), thereby ensuring survival in the host cell. Thin-walled

oocysts can excyst endogenously, resulting in autoinfection, which, along with repeated first-generation merogony, helps to explain persistent infections in AIDS patients. The spherical thick-walled, environmentally resistant oocysts (3–6 μm in diameter), shed in the fecal material of the infected host, are immediately infectious, unlike other coccidian parasites, and hence can be transmitted from person to person. The more common routes of transmission are by the fecal-oral route with ingestion of contaminated food or water or contact with infected animals.

Pathophysiology of cryptosporidial diarrhea - Cryptosporidial diarrhea has a pre-patent period ranging from 4 to 22 days for humans as shown in volunteer studies (60). The median duration of diarrhea in these studies was 6 days with oocyst shedding recorded up to 30 days after challenge. The alterations in the intestinal structure and physiology that lead to the pathogenesis of cryptosporidiosis include rapid loss of the microvillus border, shortening and fusion of the villi and lengthening of the crypts which occurs in a dose dependent manner, resulting in malabsorption due to loss of membrane-bound digestive enzymes, decreased absorption, reduced glucose-NaCl absorption and increased chloride anion secretion. (173, 230).

Cryptosporidium infection stimulates the production of proinflammatory cytokines, IFN-γ, IL-8 and TNF-α. While IFNγ is important for the resolution of symptoms, TNFα contributes to the pathogenesis of cryptosporidiosis by increasing production of

prostaglandins, neural peptides and reactive nitrogen intermediates (128). Neutrophils and mononuclear cells infiltrate the lamina propria. Substance P has also been found to be up regulated in cryptosporidiosis and could also cause elevation of pro-inflammatory cytokines (197). A marker of inflammation, fecal lactoferrin has been found to be elevated in diarrheal stool of children with cryptosporidial diarrhea (119). *Cryptosporidium* has also been found to cause disruption of the epithelial tight junction leading to a leaky and dysfunctional epithelium and alteration of solute transport (230). In a suckling rat model cryptosporidial infection was found to impair nutrient intake, growth and muscular protein synthesis and this persists beyond the duration of infection (227). Most infections present with a self-limited watery diarrhea although people with pre-existing antibodies are found to be less susceptible or may have asymptomatic infections. Associated symptoms like vomiting, malaise, loss of appetite and fever may also occur. The disease is usually self limited in the immunocompetent but can result in a prolonged, protracted infection in HIV infected adults.

Molecular typing of cryptosporidial isolates – PCR based identification of *Cryptosporidium* and differentiation of species based on polymorphisms has been carried out using several loci. These include β-tubulin, actin, TRAP-C1, TRAP-C2, ITS1, polythreonine repeat (Poly-T), dihydrofolate reductase (DHFR) and heat shock proteins (*Hsp70*) (105) (Table 3.2). Currently however, most workers carry out a nested PCR and RFLP at the small subunit 18S RNA gene (SSU rRNA) due to increased sensitivity associated with a high gene copy number and the ability to differentiate species (105).

More heterogenous loci used for subgenotyping of cryptosporidial isolates in epidemiological and population genetics studies include micro satellite 1 and 2, mini satellites, ITS-2 and *gp60* or *Cpgp40/15*.

Extensive polymorphisms among *Cryptosporidium* isolates at the *Cpgp40/15* locus has also been used as a epidemiological tool for subgenotyping in several studies (130). This gene encodes a precursor glycoprotein which is proteolytically cleaved into two glycoproteins, gp40 and gp15 (Table 3.1) which are implicated in attachment and invasion (170). A striking feature of the *Cpgp40/15* gene (also referred to as *GP60*) is the high degree of polymorphism. Numerous single nucleotide and amino acid polymorphisms in the *Cpgp40/15* locus, define at least 8 allelic subtypes that can be reliably identified by PCR-RFLP. The identifying polymorphisms are predominantly clustered in the variant domain of the gp40 portion of the molecule, located directly C-terminal of the polyserine domain. Currently, *Cpgp40/15* subtype analysis is commonly used as a tool to investigate transmission patterns, waterborne outbreaks and clinical correlates of cryptosporidiosis. The common subgenotypes of *C. hominis* are Ia, Ib, Id, Ie and If and that of *C. parvum* are IIa, IId and the anthroponotic IIc (248). Additional subgenotypes in both *C. hominis* and *C. parvum* have been described using sequencing.

Table 3.2: Tools for Molecular Typing of Cryptosporidial Species

Primers		**PCR products (bp)**	**method**	**Author**	**year**
SSU rRNA	(Small subunit of nuclear ribosomal RNA)	1,750	PCR-RFLP	Xiao	1999
SSU rRNA		840	Nested PCR-RFLP	Jiang	2005
ITS1	(Internal transcribed spacer of nuclear ribosomal DNA)	513(hu)519(bov)	PCR	Morgan	1999
COWP	(Cryptosporidium oocyst wall protein)	550	PCR-RFLP	Spano	1997
TRAP-C1	(Thrombospondin related attachment protein gene)	1,200	PCR-RFLP	Spano	1998
TRAP-C2		369	PCR-RFLP	Peng	1997
Hsp70	(70 Kda Heat shock protein)	1950	Nested PCR-RFLP	Sulaiman	2000
Actin		1066	Nested PCR-RFLP	Sulaiman	2002

Molecular epidemiology – Among the 9 species of *Cryptosporidium* that cause human disease, *C. hominis* and *C. parvum* alone account for greater than 90% of cases. In developed countries, there are geographic differences between the 2 species with a slightly greater burden of *C. parvum* than *C. hominis* in the United Kingdom, New Zealand and some parts of Europe (248) but in North America and Australia, *C. hominis* is more common. This difference could be attributed to different sources of infection and route of transmission with *C. parvum* associated with farming and rural areas. Also, in these countries, the *Cpgp40/15* subgenotype of *C. parvum* is usually IIa or IId indicating a zoonotic transmission. In these countries, cryptosporidiosis is a sporadic disease that occasionally causes outbreaks due to contamination of drinking or recreational water.

In developing countries, the majority of infections are due to *C. hominis* not *C. parvum*. A higher prevalence of more exotic species like *C. felis, C. canis* and *C. meleagridis* are also seen. Cryptosporidiosis is endemic in developing countries and mainly affects susceptible populations like young children, malnourished and immunocompromised. Transmission of cryptosporidiosis is anthroponotic in these countries and is reflected in the fact that most *C. parvum* isolates belonged to the IIc subgenotype (129, 248). In this setting, transmission is both endemic involving person to person routes, contamination of food and from animals as well as epidemic involving the water supply. Seasonality has also been demonstrated in several developing countries and usually is associated with rainfall.

The importance of differentiating between species using molecular tools is highlighted by several recent findings. These include a report of longer periods of oocyst shedding in *C. hominis* compared to *C. parvum*-infected patients in Peru (245), significantly higher oocysts shedding and greater growth shortfalls at 6 months post infection in children with *C. hominis* in Brazil (29) and a longer duration of symptoms, higher rate of asymptomatic infection and lower CD4+ cell count occurred in HIV-infected patients with *C. hominis* infection compared to *C. parvum* infection in Tanzania (94). A recent study showed that infection with *C. hominis*, but not *C. parvum* was associated with an increased risk of non-intestinal sequelae in immunocompetent adults and children (97). Another recent study in Peru showed that zoonotic species like *C. canis*, *C. felis*, and subgenotype Id of *C. hominis* were associated with diarrhea, and infection with *C. parvum* was associated with chronic diarrhea and vomiting in the HIV infected (35).

Some association between *Cpgp40/15* subgenotypes and transmission and disease has also been described. Studies on waterborne outbreaks showed an association with *C.hominis* subgenotype Ib indicating that it was potentially more infectious or virulent (51). Therefore identifying the species and subgenotypes to differentiate between anthroponotic and zoonotic strains and documenting the molecular epidemiology of cryptosporidiosis will help plan interventional measures to prevent and treat cryptosporidial infections.

***Cryptosporidium* in children in developing countries** - Diarrhea is one of the most common childhood illnesses with every child aged below 5 years in the developing world experiencing at least 3 episodes of diarrhea per year (121). In addition to the mortality and morbidity associated with diarrhea due to dehydration, chronic and recurrent diarrhea leads to poor absorption of nutrients resulting in stunting and developmental delay (25). Amongst the more than 20 viral, bacterial and parasitic causes of diarrhea identified till date, the most frequently reported etiological agents in children in the developing world include rotavirus, diarrheagenic *Escherichia coli*, *Campylobacter jejuni*, *Shigella spp*, non-typhoidal *Salmonella*, *Giardia lamblia*, *Cryptosporidium spp*. and *Entamoeba histolytica* (172). Astrovirus, enteric adenovirus and calicivirus have also been reported, but previous studies have indicated a lower relative contribution to the total number of cases due to higher rates of bacterial and parasitic infections in these countries (172).

In developing countries, cryptosporidiosis, is seen more frequently in malnourished than well-nourished children and the consequences are more severe in the former than the latter, possibly because of impaired T cell responses. Studies of Haitian children with cryptosporidiosis reported that malnourished children have increased levels of systemic and fecal proinflammatory cytokines (119). Watery diarrhea, vomiting, anorexia and weight loss are the commonest symptoms of cryptosporidiosis, with persistent diarrhea frequently reported from developing countries. Studies done in Brazil suggest that even a single episode of cryptosporidiosis predicts a subsequent increased risk of diarrheal disease (86).

Diarrhoea is the cause of death in approximately 23% of Indian children who die before the age of 5 years (107). *Cryptosporidium spp*. are a leading cause of infectious diarrhea in children in India. A number of studies have reported *Cryptosporidium spp*. in diarrheal stool samples of children by microscopy with positivity rates ranging from 1.1% to 18.9. Many studies also examined asymptomatic or "control" children (i.e. without diarrhea) and reported positivity rates between 0% and 9.8% (Table 3.3). These studies differed in their methods of patient recruitment and stool examination, but only 3 studies conducted till date have used molecular techniques for identification and typing indicating that actual infection rates may be significantly higher. In a hospital based study from Calcutta, of 40 microscopy positive samples identified over 5 years, 35 were positive for *C. hominis*, 4 were positive for *C. parvum*, and 1 was positive for *C. felis* (57). A recent hospital based study from Secunderabad on adults and children also found that *C. hominis* (69%) was the most common genotype (158). A study from Vellore revealed that *Cryptosporidium* along with rotavirus, enteric adenovirus and Group B *Salmonella,* was a common cause of nosocomial diarrhea in children aged < 3 years (109). In more recent studies in Vellore, this parasite was also the commonest cause of parasitic diarrhea among children attending the hospital and also had a high pathogenecity index. When conventional and molecular methods were compared for the detection of *Cryptosporidium spp*. among the children with diarrhea, it was found that the detection rates were significantly higher when a combination of molecular and conventional methods were applied as compared to the conventional method of microscopy alone (6).

Table 3.3: Cryptosporidiosis in Children in India

Place	Year	Reference	Age (years)	% symptomatic	% asymptomatic
Vellore	1985	Mathan (140)	<3	13.1	9.8
Chandigarh	1987	Malla et al(137)	<12	1.3	
Calcutta*	1987	Das et al (56)	<12	5.9	
Calcutta	1988	Sengupta et al(205)	<5		
				6.1	1
Varnasi	1988	Singh et al(213)	0.5-3	3	0
Mumbai	1989	Pherwani (184)	<5	4.4	0
Bhubaneswar	1989	Subramanyam et al(224).	<8	13	0
Calcutta	1989	Pal et al(175).	<5	5.6	1.2
Idukki	1989	Reinthaler et al(193).	<10	6	3
Delhi	1991	Kaur(114)	<2	5	0
New Delhi	1991	Uppal(234)	<10	4.9	
Calcutta	1993	Das et al(58)	<12	5.5	1.1
Varanasi	1993	Nath et al(160).	<5	3.8	1.8
Amritsar	1995	Jindal (106)	<3	1.3	
Manipal	1995	Shetty(210)	<5	1.8	
Chandigarh	1999	Sethi et al.(206)	<12	1.4	-
Secunderabad	2001	Nagamani et al(159)	0.25 - 3	6	
Vellore*	2001	Kamalaratnam(109)	<3		7.2
Manipal	2002	Ballal (19)	<5	15.6	3
Delhi	2002	Kaur et al(115).	<5	18.9	-
Calcutta	2005	Palit(176)	<12	-	2.3
Calcutta	2006	Das et al(57)	<5	4.6	1.2
Secunderabad	2007	Nagamani et al (158)	<12	7.6	

*data based on analysis of results in the publication

Although diarrhea in itself is a leading cause of morbidity and mortality among children in developing countries, the actual effects of repeated and chronic diarrhea on child development and future productivity as adults is grossly under-estimated (87). Recurrent and persistent diarrhea has been consistently associated with stunting in children (194). In studies on children living in slums in Brazil and Peru, early childhood diarrhea (defined as episodes of diarrhea in the first 2 years of life (86)) negatively correlated with tests of cognitive function, verbal fluency and physical fitness and resulted in long term growth faltering (86, 134, 166, 178).

Children who develop cryptosporidiosis are at risk of further nutritional compromise during the course of the infection. Studies from Peru suggest that both symptomatic and asymptomatic cryptosporidiosis in children are associated with growth faltering in the month after infection and recovery is slower in children with symptomatic infection (25). In these studies, diarrhea due to *Cryptosporidium spp*. were frequently found to be associated with lower cognitive function scores and stunting, and the risk increased with the number of episodes per year (25, 86). Studies in other countries like Malaysia (8), Turkey (37) and Bangladesh (225) have also implicated diarrhea with *Cryptosporidium spp* (44, 45, 86, 166) as an independent predictor of stunting. The role of cryptosporidial infections on cognitive function and growth in Indian children has not been assessed.

Cryptosporidiosis in HIV - Diarrhea is the most common gastrointestinal symptom reported in HIV infected patients with the incidence increasing when CD4 counts drop below 200 cells/mm (162). In India, recent sentinel surveillance estimated that there were

around 2.47 million people living with HIV/AIDS (PLHA) and the four states of Andhra Pradesh, Maharashtra, Tamil nadu and Karnataka contributed to 63% of all reported cases (157). The common enteric opportunistic pathogens described are Cytomegalovirus, *Cryptosporidium spp*., *Isospora belli*, Microsporidium *spp*., *Mycobacterium avium intracellulare* and more recently, *Clostridium difficile. Cryptosporidium spp*. and *I. belli* have been reported as the most common causes of diarrhea, however, most studies have focused only on protozoan etiology (17, 22). Patients can have chronic watery diarrhea that can last for more than 2 months and shed oocysts in stool during the entire period, resulting in severe dehydration, weight loss and malnutrition, extended hospitalizations, and mortality(3, 98). Other symptoms are abdominal cramps, anorexia, nausea, vomiting, fatigue and low-grade fever. AIDS patients with cryptosporidiosis also have a significantly shorter duration of survival from the time of diagnosis (138). In the immunocompromised host, *Cryptosporidium spp* is also the most commonly isolated pathogen in the biliary tract in patients with AIDS-cholangiopathy (47). There have been also been a few reports on other sites of infection, involving the pancreas and lungs (133).

In India, there have been reports from the mid 1990s on the prevalence of symptomatic cryptosporidiosis in HIV infected adults from different parts of the country ranging from as low as 0.7% to 81% (Table 2.4). A high prevalence of around 80% was reported from a study in Imphal (5) and another in Maharashtra (79) but both had very small sample sizes. Most of these studies were carried out on HIV infected adult patients and employed

modified acid fast staining of concentrated stool samples. The data on prevalence is highly varied and could reflect geographical differences, as well as differences in the populations being studied especially with respect to socioeconomic status and access to potable water. A few recent studies that also carried out ELISA and PCR for detection (102, 116) found a higher sensitivity for PCR and lower for antigen detection ELISA, compared to microscopy also contributing to variability in prevalence rates. In children with HIV in India, chronic diarrhea has been reported in 6.8 to 42 % of cases but very few studies examined the specific etiology of diarrhea (207).

Several studies in India have also documented mean CD_4 counts in HIV infected patients with most studies showing that symptomatic cases had CD_4 counts < 200 cell/mm^3 and asymptomatic cases had CD counts > 300 cell/mm^3 reinforcing the importance of CD_4 T cells in mediating resistance to this pathogen (17, 61, 80, 156). A study from Delhi that stratified patients based on their CD_4 counts showed that *Cryptosporidium* was the commonest parasite seen in 46% patients with counts < 200 cell/mm^3. The prevalence of cryptosporidial diarrhea (56%) was also significantly higher when CD_4 counts were below 200 compared to patients with higher CD_4 counts (40%) (199) (Table 3.5).

Table 3.4: Cryptosporidiosis in HIV Infected Patients in India

Place	Year	Reference	Cases	% symptomatic	% asymptomatic
Chennai	1995	Kumarasamy et al (126)	Adults	16	-
Northern India*	1995	Giri et al(80)	Adults and children	4	-
Manipur	1996	Anand et al(10)	Adults (iv drug users)	46.6	-
Karad, South Maharashtra	1996	Ghorpade et al(79)	-	83.3 †	-
Mumbai*	1996	Lanjewar et al (127)	Adults	31	-
Manipur*	1997	Anand et al(11)	Adults (asymptomatic iv drug users)	-	57.8
Chennai	1997	Ananthasubramaniam et al (12)	-	6.5	0
Imphal	1998	Agarwal(5)	Adults	81.8†	-
Vellore	1999	Mukhopadhya et al (155)	Adults	9.8	6
Lucknow	2000	Prasad (185)et al	Adults, chronic diarrhea	11.5	-
Chandigarh*	2002	Mohandas(144) et al	Mostly adults	22.6	1.49
Mumbai	2002	Joshi(108) et al	-	8.5	-
Chennai	2002	Kumar et al(124)	Adults	13.7	8
New delhi	2003	Vajpayee et al(235)	Adults	18.6	-
Mangalore	2003	Shenoy et al(209)	-	17.5	-
Manipal	2003	Singh et al(211)	Adults	-	43
New Delhi*	2004	Sharma et al(208)	Adults	9.3	-
Vellore	2005	Banerjee et al(22)	-	15.5	-
Manipal	2005	Ballal et al(18)	-	18.57	-
Delhi	2005	Sadraei et al(199)	Adults	42.1	38.1
Nepal	2006	Adhikari et al(4)	Adults		5.2
Vellore	2006	Muthusamy et al(156)	Adults	25.2	4.7
Varanasi	2006	Attili et al(16)	-	5.71	-
Bangalore	2007	Becker et al(24)	Adults	0.7	2
Delhi	2007	Dwivedi ey al(61)	Adults	66.6	8

*data based on analysis of results in the publication

† very small sample sizes

Although cryptosporidiosis is endemic in tropical countries, only a limited number of isolates have been typed from developing countries, especially from HIV-infected people. Data on genotypes and species in HIV infected patients in India is limited to a single study from Vellore which showed a prevalence of 25% among HIV seropositive individuals with diarrhea and 4% in those without diarrhea. Although *C. hominis* was the most common species identified comprising 64% of positive cases**,** a strikingly high prevalence of potentially zoonotic species was seen including, *C. parvum, C. meleagridis, C. felis* and *C. muris* in symptomatic as well as asymptomatic HIV-infected adult patients, but no significant animal contact was found in the cases with potentially zoonotic infections.

In developed countries, zoonotic infections are usually due to contact with domestic pets but studies from endemic countries like Thailand and India have shown a high prevalence of zoonotic species of up to 50% with no significant contact with pets in these cases (75, 156). A recent study from Delhi has documented history of contact with animals in 87% of patients with diarrhea and in 32% of cases with cryptosporidiosis, however, genotyping to identify zoonotic cryptosporidial species was not carried out (61). Studies on bovine cryptosporidiosis in Calcutta and Punjab have found prevalence rates ranging from 26% to 50% in diarrheic and 8.5% to 25.7% in non diarrheic calves with the predominant species being *C. parvum* (179, 201) with a high mortality rate of 35 % (212). These studies indicate that, in addition to causing disease and death in livestock

Table 3.5: CD_4 counts Associated with Symptomatic and Asymptomatic Cryptosporidiosis in Indian patients

Place	Year	Author	CD4 counts (cells/mm^3)	
			diarrhea	no diarrhea
Northern India	1995	Giri et al(80)	56	-
New Delhi	2003	Vajpayee et al(235)	227	-
New Delhi	2004	Sharma(208)	61	-
Vellore	2006	Muthusamy et al(156)	145	312
Varanasi	2006	Attili et al(16)	255.2	366.9
Delhi	2007	Dwivedi et al (61)	125	301

and other animals, the high prevalence and asymptomatic cryptosporidial infections in these animals could serve as a reservoir of infection to susceptible human hosts in India.

Antiretroviral therapy also greatly influences the outcome of cryptosporidiosis both indirectly by immune restitution and increase in CD_4 counts (204) and also by the direct effect of protease inhibitors on oocyst shedding (142) resulting in a sustained therapeutic effect after follow-up. However, despite the use of HAART, HIV-infected patients can still present with coccidian diarrhea, possibly due to noncompliance with medications, viral drug resistance or decreased drug bioavailability (149). Relapses after discontinuation of HAART have also been documented (251). In India, recently HAART has become available at an affordable cost or through the government's ART roll out programme in 2004 and a recent study from Chennai on the current NNRTI based therapy initiated when CD_4 counts were < 250 cells/mm^3 showed a substantial increase in life expectancy (67). Another study examining the effect of HAART on the incidence of OIs in India has found that there has been a decrease in both OIs and tuberculosis in patients on therapy. Hence, increased access to HAART has impacted the natural history of HIV infection (125). Although western data indicates a shift in etiology of diarrhea in HIV infected patients (33) and there seems to be a decrease in OIs in a single study from India, the effect of HAART on the occurrence of cryptosporidiosis and etiology of diarrheal disease in Indian patients remains to be examined.

Among the other coccidian parasites, *Isospora belli* has also been reported frequently with prevalence rates ranging from 2.5 to 60% in patients with diarrhea. Most recent studies in India show lower prevalence rates than *Cryptosporidium* (probably due to prophylactic treatment with trimethoprim-suphamethoxazole)(17, 61, 116, 156, 191) but a few have recorded higher prevalence rates (89, 237). *Cyclospora*, on the other hand has been isolated very infrequently from HIV infected patients in India with low prevalence rates ranging from 0.98 to 6.5% in symptomatic cases (144, 155, 156, 208).

Other susceptible populations in India- There have been few studies documenting cryptosporidiosis in transplant patients in India. A previous study on patients undergoing allogeneic bone marrow transplantation in Vellore identified *Cryptosporidium spp*. in 7 of 65 cases, both in the pre- and post-transplantation period and also found a higher mortality in patients with enteric pathogens than those without. The other enteric pathogens identified were rotavirus, adenoviruses, *Clostridium difficile* and diarrheagenic *E. coli* (112). In more recent studies from the same center, *Cryptosporidium* was identified in 2.9% of all allogeneic bone marrow transplant recipients and 1.7 % of pediatric allogeneic BMTs (77, 78). Rates of infections in these patients were similar to those in the West, possibly attributable to good infection control strategies. A study on renal transplant recipients in North India identified cryptosporidial diarrhea in 16.6% of cases (232). Only one study from Varanasi, evaluating risk factors for cryptosporidiosis in the geriatric age group has been carried out, in which 18.3% of cases had cryptosporidiosis among which 66% had a history of close contact with animals (71). Isolation of *Cryptosporidium* in this study increased during the rainy season.

Immune response - Immune responses involved in mediating resistance to infection with this parasite are not well understood, but evidence obtained from immunodeficient animal models have shown that a Th_1 response involving primarily TCR αβ+ CD_4+ lymphocytes, IFN-γ and IL-12 play a major role in the control of C. *parvum* infection (62). The susceptibility of HIV/AIDS patients to this pathogen and resolution of cryptosporidiosis following immune restitution underscores the importance of CD_4 T cells (204). In addition, a number of studies have shown that the cytokine IFN-γ is critical in protection from and clearance of the infection (195). In human volunteer studies, IFN-γ expression could be detected in jejunal biopsies of *Cryptosporidium*-infected adults and was associated with the presence of anti-*Cryptosporidium* serum antibodies and absence of oocyst shedding (240). Peripheral blood mononuclear cells (PBMCs) from infected humans proliferate in response to recombinant and crude preparations of *C. parvum* antigens (83) The cytokines IL-4, IL-10, IL-13 and IL-15 have also been shown to be important (128).

Increasing evidence for the protective effect of an initial innate response has also been documented. Decreased levels of mannose binding lectin in Haitian children and HIV infected adults correlated with susceptibility to cryptosporidiosis (120). Toll like receptors 2 and 4 mediated response to infection via MyD88 and NFκB in both mice models and in epithelial cell lines (49). NFκB then results in the expression of proinflammatory cytokines. IL-15 mediated elimination of parasites by NK cells (55) and IL-18 potentially mediated secretion of antimicrobial peptides like β-defensin (141).

The role of antibodies in resistance to infection and protective immunity is less clear. Humoral immune response to oocyst lysates, as well as to specific glycoprotein antigens, have been characterized in volunteer studies and in seroepidemiological studies (195). Many of these have been seroprevalence studies which have reported a wide range of seropositivity depending on age, geographic location, living and environmental conditions. In general, IgA, IgM and IgG rise during an infection but IgG levels persist. In adult human volunteer studies, serological response to cryptosporidial antigens was coincident with resolution of symptoms and preexisting antibodies associated with decreased severity and duration of infection, but these antibody responses may only be markers of other cell mediated protective responses (195). *Cryptosporidium*-specific fecal IgA antibody responses in human volunteer studies correlated significantly with the presence of active or recent infection (54). In children in developing countries, antibody titers increase with age (53) but children with persistent diarrhea in Bangladesh showed a decrease in IgA and IgM titers (118).

Immunodominant antigens - Immunoblot analyses of sera from infected humans have identified antibody responses to several antigens, most consistently to groups of 15-17 kDa (also called gp15 or Cp17) and 23-27 kDa proteins (also called Cp23). Both gp15 and Cp23 are largely conserved among *C. hominis* and *C. parvum* isolates. The most immunodominant is the 15-17 kDa antigen which has been consistently identified and used to assess antibody response in a number of studies (195). A study in human volunteers showed that pre-existing antibodies to the 15/17kDa antigen were associated

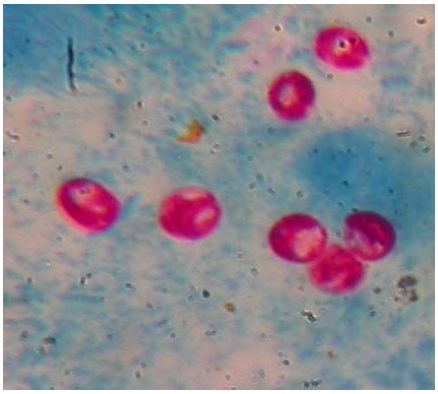
with protection from diarrhea. The study in children in Peru also reported higher IgG levels against the 15 kDa antigen in children with asymptomatic infection, also possibly indicating protection. In a recent study from a birth cohort of children in Peru, serum IgG to partially purified native 15 kDa and recombinant 27kDa antigen were used to identify cryptosporidial infection in children and found an increase in serum IgG levels with age. Children with asymptomatic infections had higher antibody levels than those with symptomatic infection (187). A recent study reported IFN-γ production in response to *C. hominis* gp15 in immunocompetent adults with prior cryptosporidial infection (186).

Cp23 plays an important role in cell mediated immunity. The antigen stimulated specific proliferative response by splenocytes and mesenteric lymph node cells from infected IFN-γ knockout BALB/c mice, and also induced TNF-α, IL-2, and IL-5 mRNA production by spleen cells from infected animals (28). A study on Haitians showed that IgG responses were greater among persons who exhibited T cell responses to Cp23 (216). The presence of serum antibodies to Cp23 was associated with protection from diarrhea in HIV-infected subjects (18). Additionally, studies have shown that Cp23 induces a proliferative T cell response in immunocompetent adults (8, 38). Further, DNA encoding Cp23 was shown to elicit protective immune responses in mice (14).

These studies suggest that Cp23 and gp15 may be potential vaccine candidates and have been used in early studies with positive results. Calves receiving colostrum from cows

that were injected with recombinant Cp23 did not develop diarrhea when exposed and also had a significant decrease in oocyst production. A recent study using DNA vaccine vector expressing Cp23 showed clearance of infection and antibody response to the antigen in C57BL/IL-18 KO mice (244). Similar animal studies using gp15 also showed high serum and colostrum antibody responses in gp15 plasmid injected sheep (103). Nasal immunization of pregnant goats also resulted in less oocyst shedding in the suckling kids (200). Another group recently tested a recombinant plasmid containing both antigens. The fusion protein given to BALB/c mice protected them from infection (93).

Figure 3.1: Cryptosporidial Oocysts in Stool Samples Stained by Modified Acid Fast Method



Diagnosis – Microscopy has remained the 'gold standard' for diagnosis of intestinal cryptosporidiosis in humans. The most commonly used method is the modified acid fast stain which is carried out on concentrated stool specimens, collected on 3 consecutive days. Cryptosporidial oocysts appear acid fast and irregularly stained and in the size range of 4-6µ (Figure 3.1). Other non-morphology based tests include IFA, ELISA and immunochromatography for detection of oocysts or antigen in stool (203). Flow cytometry based tests using standardized methods are used to detect oocysts in large volume samples such as water or sewage, but are available only in developed countries. PCR based tests are also used in surveillance and environmental samples (129).

Treatment - Therapeutic approaches for cryptosporidiosis in the past have included macrolide antibiotics, paramomycin, rifaximin, octreotide and immunotherapy, among others. Although nitazoxanide has been recently licensed for treatment of cryptosporidiosis in children (9) and immunocompetent individuals in most countries including India, a recent meta-analysis found it to be ineffective in HIV patients (3). Unlike other coccidian causes of diarrhea like *Isospora spp*. and *Cyclospora spp*., which can be treated with co-trimoxazole, there is still no effective treatment for cryptosporididosis in HIV infected persons.

- **CHAPTER 4**

SCOPE AND PLAN OF THE STUDY

A vaccine for prevention of cryptosporidiosis in children and HIV infected adults in developing countries is necessary in the light of the fact that there is still no efficient antimicrobial agent to treat this infection. The characterization of immune responses to *Cryptosporidium spp*. in a developing country setting where diarrhea is known to be an important cause of morbidity and mortality is vital to facilitating development of interventions appropriate to the communities in which cryptosporidiosis is common.

There have been few studies on the molecular epidemiology of cryptosporidiosis in children in India (73), all hospital based and two previous studies on HIV infected adults (117), including one from our center (156). Previous birth cohort studies in the community on cryptosporidiosis have been in South America (26, 29) and Africa (181, 229) but none from the Indian subcontinent. This study will provide detailed information on the circulating species and subgenotypes in the country in these 2 susceptible populations. Cryptosporidiosis in children will be studied in two settings - the community in Vellore and among hospitalized children in Delhi, Vellore and Trichy. Cryptosporidiosis among HIV-infected adults attending the hospital in Vellore will also be studied. These data will help elucidate the transmission pattern and epidemiology of cryptosporidiosis in the country. It will help delineate whether this is truly an anthroponotic disease or whether a significant zoonotic component exists. Factors like sociodemographic status, housing, number of siblings, water supply, contact with animals etc will also be studied to identify risks for acquiring cryptosporidiosis. In addition, any seasonal effect on incidence of cryptosporidiosis will also be studied. This information is vital to formulate effective control measures.

Humoral immune response to cryptosporidial antigens will be studied in children and HIV infected adults. Previous studies on immune response to cryptosporidiosis in India are limited to one study from Chandigarh (117). However, this study used a crude antigen preparation to document the cytokine profile. In this study, we plan to use 3 well characterized immunodominant antigens, gp15, gp40 and Cp23 to study immune response. Studies on the immune response and genotyping will lead to identification of the correlates of protective immunity and form the basis for future studies on vaccine development and other preventive measures.

- **CHAPTER 5**

MATERIALS AND METHODS

Study Populations

This study recruited participants from three different settings – children in the community in Vellore who were followed up from birth, children with diarrhea who were hospitalized at Vellore, Delhi and Trichy and HIV infected adults attending a tertiary care hospital at Vellore.

The birth cohort - The subjects in this study were part of a community based birth cohort of 452 children recruited to study rotaviral diarrhea in the semi-urban slum areas of Ramnaikapalayam, Chinnallapuram and Kaspa in Vellore, South India between April 2002 and July 2003, who were followed up for 3 years (20) (Figure 5.1). The study area had a population of approximately 33,390 and all households in the area were mapped using Geographic Information Systems (GIS) (Figure 5.3). Garmin GPS V (GARMIN International Inc., Kansas, USA) was used for collecting waypoints and trackpoints (latitude, longitude). These were then downloaded using GPS Utility 4.10.4 (GPS Utility Ltd., Southampton, England) and mapped using ArcView GIS 3.3 software (Environmental Systems Research Institute Inc., California, USA). Data on sociodemographic, environmental, and clinical characteristics were collected during the study and included information on the composition of the household in terms of residents, family structure, occupation and socio-economic status. Data on housing structure, arrangements for eating, sleeping, water supply and toilet facilities were also recorded. Each child in the cohort was visited at home twice a week to record any morbidity or diarrhea and stool samples collected fortnightly (Figure 5.2).

Figure 5.1: Birth Cohort Study Area - Semi-Urban Slum Areas of Ramnaikapalayam, Chinnallapuram and Kaspa

Figure 5.2: Twice Weekly Follow Up Visits of Children in the Birth Cohort

Figure 5.3: GIS Map of Households in the Birth Cohort Study Area

Diarrhea was defined as the passage of three watery stools in a 24 hour period. In children less than 6 months of age, a change in number or consistency of stools reported by the mother was considered indicative of diarrhea. An episode was defined as at least 1 day of diarrhea, preceded and followed by 2 or more days without diarrhea. The episode was considered to have ended on the day bowel movements returned to normal (152). Any child who had diarrhea was assessed clinically and treated appropriately. Fecal samples were collected from the child and the child was followed up daily until cessation of diarrhea. Laboratory testing was done on the diarrheal stool sample to identify bacterial, parasitic or viral agents of gastroenteritis. Serum and fecal samples were collected in the neonatal period. A serum sample was also collected from the mother at the time of delivery. Serum samples were then collected every 3 months during the first 2 years of life and every six months during the third year. Diarrheal samples were screened for *Cryptosporidium spp*. by microscopic examination of fecal smears stained by modified acid fast stain. Children with no cryptosporidial diarrhea were defined as children who did not have *Cryptosporidium spp*. detected by microscopy or PCR in any diarrheal stool sample up to 2 years of age. For this study, maternal sera collected at delivery, sera from children with cryptosporidial diarrhea and without cryptosporidial diarrhea at 3.5, 9 and 24 months of age as well as sera from cases collected 2 to 12 weeks before (pre-infection) and after (post-infection) the first episode of cryptosporidial diarrhea were tested. Informed consent was obtained from the parent prior to enrollment in this study and the study was approved by the institutional review board.

Hospital based study on children with diarrhea- This study was carried out with stool samples originally collected for a multi-center rotavirus surveillance programme called the Indian Rotavirus Strain Surveillance Network. Samples from 3 centers – Christian Medical College, Vellore, St. Stephen's Hospital, Delhi and Child Jesus Hospital, Trichy were available for this study representing both south and north India. Children aged less than 5 years presenting to one of the three study hospitals with acute gastroenteritis and requiring hospitalization for rehydration for at least 6 hours were enrolled in the study. A detailed clinical evaluation of the episode of diarrhea including duration and frequency of diarrhea and vomiting, fever and degree of dehydration was recorded and severity of the diarrheal episode was assessed. Informed consent was obtained from the parent/guardian before sample collection and the study was approved by the Institutional Review Board of Christian Medical College, Vellore. All stool samples collected at CMC, Vellore were transported within two hours to the laboratory and stored at 4ºC till testing. Samples from St.Stephen's Hospital, Delhi and Child Jesus Hospital, Trichy were stored at 4ºC upon collection and were transported in boxes with ice-packs at 15 day intervals to CMC. All samples were processed on the same day of receipt and aliquots of samples were stored at -70ºC for further characterization studies. Stool specimens collected from each child enrolled in the study were tested for *Cryptosporidium spp*. by modified acid fast staining and microscopy. In addition, a few children found to have cryptosporidial diarrhea at the CMC, Vellore center were followed up and stool collected for up to 3 weeks to determine duration of oocyst shedding.

HIV infected adults – HIV-infected adults who presented to the outpatient unit or who were admitted to the Department of Medicine Unit – I at Christian Medical Hospital, Vellore were enrolled in this study after obtaining informed consent. A stool sample and 5 ml of blood was collected at enrollment. CD_4 T cells were counted for most patients if no CD_4 count was available within 3 months prior to enrollment. CD_4 counts were carried out by flow cytometry using the Guava Technologies PCA instrument with Easy CD_4 software in the Department of Clinical Virology. Details on the current diarrheal episodes as well as any other co-infection, history of diarrhea and anti retroviral treatment were collected. Data on concurrent opportunistic infections was also collected by reviewing case records. For patients with diarrhea, detailed clinical information on frequency, duration, and associated symptoms such as weight loss was collected. In this study, all stool samples were screened for *Cryptosporidium* by PCR and serum samples were tested for anti cryptosporidial antibodies. Diarrhea was defined as three or more stools per day for at least 72 hours. Acute diarrhea was defined as diarrhea of less than 14 days duration. Persistent diarrhea was defined as diarrhea for 14 or more days. The study protocol, questionnaires and consent forms were approved by the institutional review board.

Laboratory Methods

Microscopy - Stool samples were concentrated by formalin-ether sedimentation method and smears prepared from the deposit. The dried, fixed smears were then stained by the modified Ziehl-Neelsen staining technique which involves staining with strong carbol-fuchsin followed by decolorization with acid alcohol and counter staining with methylene

blue (72). *Cryptosporidium* oocysts appear acid-fast, round or slightly ovoid and 4.5-6.0 μm in diameter with variable degrees of staining.

DNA extraction– DNA was extracted from stool samples by the QIAamp DNA stool mini kit for DNA (Qiagen Inc, Valencia, CA). In this protocol, around 180-220 mg or, if liquid, 200 μL stool was weighed in a 2 ml microcentrifuge tube and placed on ice. Around 1.4 ml of buffer ASL was added and the suspension thoroughly vortexed and heated for 5 min at 95°C and stool particles was pelleted by centrifugation. The supernatant was pipetted into a new microcentrifuge tube and the pellet discarded. The InhibitEX (Qiagen Inc, Valencia, CA) tablet was added to each sample, vortexed until the tablet was completely suspended and then incubated for 1 minute at room temperature to allow inhibitors in the sample to adsorb to the InhibitEX matrix. The samples were then spun down twice to pellet the inhibitors. Around 200 μL of the supernatant was pipetted into a new microcentrifuge tube containing 15 μL Proteinase K to which 200 μL of Buffer AL was added and vortexed to form a homogeneous solution. This was incubated at 70°C for 10 min following which 200 μL of ethanol (96-100%) was added to the lysate and mixed. This was added to a QIAmp spin column which was then centrifuged at full speed for 1 minute and placed in a new collection tube and the filtrate discarded. 500 μl of Buffer AW1 and AW2 were added in succession to the spin column and centrifuged. Finally, the spin column was placed into a new, labeled 1.5 mL microcentrifuge tube and 200 μL of Buffer AE pipetted directly onto the membrane. This was incubated for 1 minute and then centrifuged at full speed for 1 minute to elute DNA. The eluted DNA was aliquoted at -20°C till further testing.

PCR for screening and typing of cryptosporidial DNA - DNA from microscopy positive and negative samples were screened for *Cryptosporidium spp*. by a nested SSU rRNA (Small subunit RNA) PCR (105) (Figure 5.4). Samples that were microscopy positive but SSU rRNA PCR negative were also screened by COWP (*Cryptosporidium* oocyst wall protein) and TRAP C1 (thrombospondin related adhesive protein) PCR (105). The primers and cycling conditions used for each of these PCR reactions is summarized in table 5.1. Samples that were PCR positive were then speciated by SSU rRNA PCR RFLP and subgenotyped by *Cpgp40/15* PCR RFLP.

RFLP – PCR amplicons from the *SSU rRNA* PCR (Figure 4.4) were subjected to a restriction digestion using the enzymes *SspI* (New England Biolabs) and *VspI* (Promega) for species determination (Table 4.2) (Figure 5.5) (105). In this protocol, 20-30 µl of the second round PCR product was incubated with 2 µl enzyme and 5 µl of the 1X RE buffer. The volume was made up to 50 µl with sterile water and incubated in a 37°C water bath for 1 hour. PCR amplicons from the *Cpgp40/15* PCR (Figure 5.6) were subjected to a restriction digestion using the enzymes *AluI* and *RsaI* (New England Biolabs) for subgenotyping (Figure 5.7) (Table 5.2) (130). Ten µl of the second round PCR product was incubated with 1µl enzyme and 2 µl of the 1X RE buffer and the volume was made up to 20 µl with sterile water and incubated in a 37°C water bath for 1 hour.

Table 5.1: PCR Primers and Cycling Conditions used for Screening and Identification of *Cryptosporidium spp*.

Locus	Primers and primer sequence	Cycling conditions	Amplicon size
SSU rRNA (nested)	SSU 3: 5' TTC TAG AGC TAA TAC ATG CG 3' SSU 4: 5' CCC TAA TCC TTC GAA ACA GGA 3' SSU 5: 5' GGA AGG GTT GTA TTT ATT AGA TAA AG 3' SSU 6: 5' AAG GAG TAA GGA ACA ACC TCC A 3'	95°C 2 min, 72°C 1 min 94°C 45 sec 55°C 45 sec } 35 cycles 72°C 1 min 72°C 10 min	820 bp
COWP	C0WP 1: 5'- GTAGATAATGGAAGAGATTGTG -3' COWP 2:5'- GGACTGAAATACAGGCATTATCTTG -3'	95°C 2 min, 72°C 1 min 94°C 60 sec 55°C 30 sec } 34 cycles 72° C 50 sec 72°C 10 min	600 bp
TRAP	TRAP1: 5– GGATGGGTATCAGGTAATAAGAA -3 TRAP2: 5- CCATTCTCTCCCTTTACTTC -3	Similar to COWP PCR	500 bp
Cpgp 40/15 (nested)	7 - 5'-ATGCAAAAATACGTGGACTGGG-3' 8 - 5'-TCGCACGAAAGATTTCCATTG-3' 9 - 5'-TTACTCTCCGTTATAGTCTCCGCTG-3' 10 - 5'-cgaataaggctgcaaagattgc-3'	95°C 15min 94°C 40 sec 55°C 50 sec } 40 cycles 72°C 1min 30s 72°C 10 min	~ 1kbp

Figure 5.4: Nested PCR at *SSU-rRNA* Locus for Identification of *Cryptosporidium spp*. (amplicon size of 840 bp)

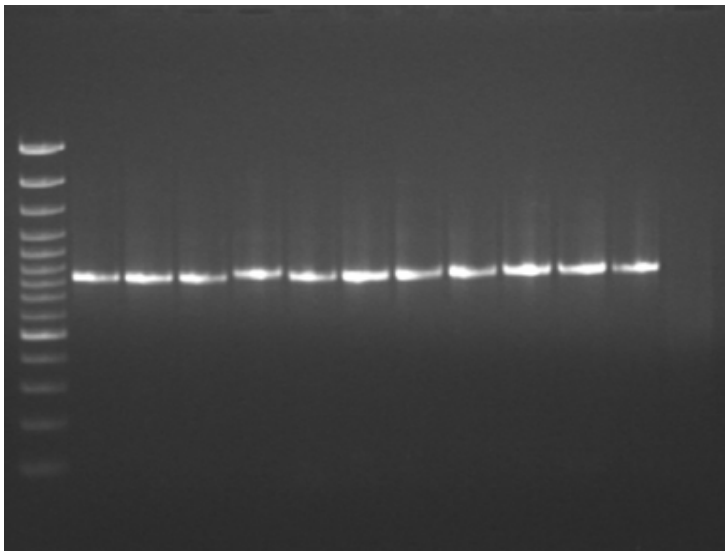
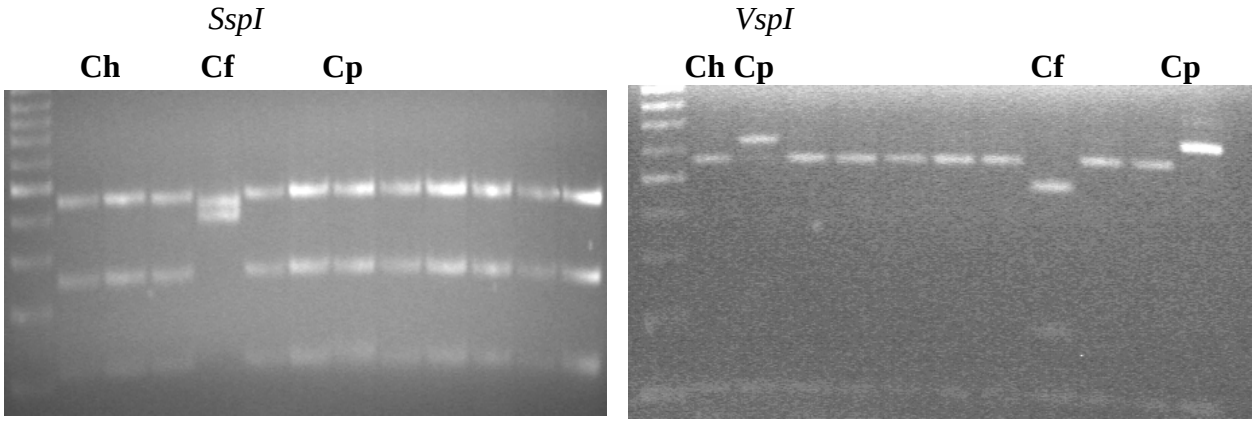


Figure 5.5: SSU rRNA PCR RFLP with *SspI* and *VspI* Restriction Endonucleases for Speciation of *Cryptosporidium*



Ch – *C. hominis*, Cp – *C. parvum*, Cf – *C. felis* with 1 kb marker

Figure 5.6: Nested PCR at *Cpgp40/15* Locus for Identification of Cryptosporidium *spp*. Subgenotypes (amplicon size of ~ 1kbp)

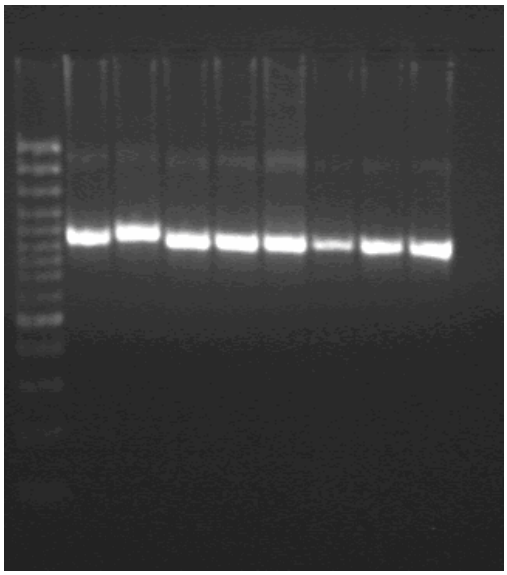


Figure 5.7: ***Cpgp40/15* PCR RFLP with *AluI* and *RsaI* Restriction Endonucleases for Subgenotyping of *Cryptosporidium***

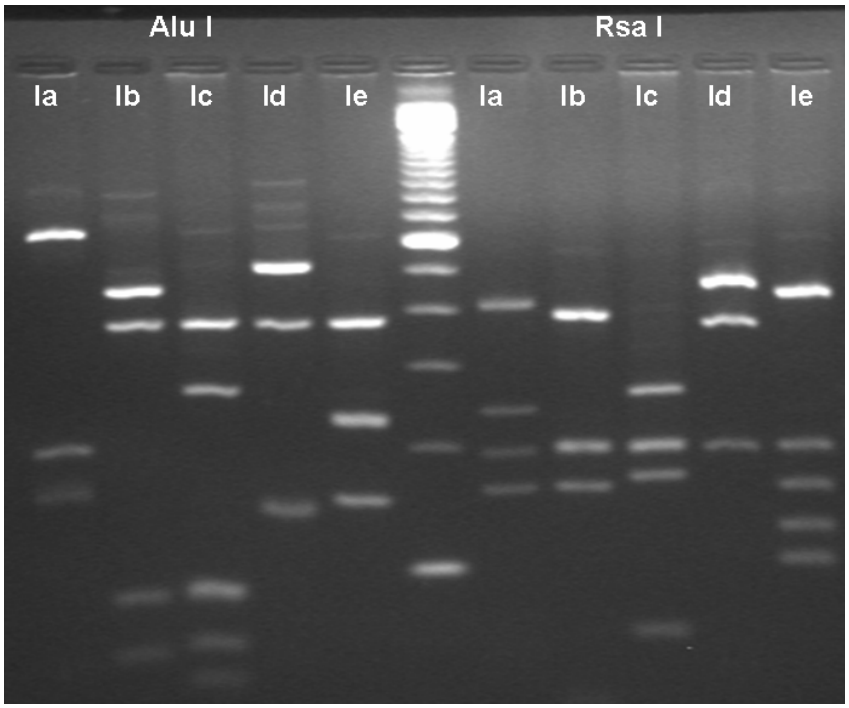


Table 5.2: RFLP Patterns for Common *Cryptosporidium* Species and *Cpgp40/15* Subgenotypes

SSU rRNA PCR RFLP (104)		
Species	***SspI***	***VspI***
C.hominis	450,267,111	561,115
C.parvum	450,267,108	629,115
C.meleagridis	450,267,108	457,171,115
C.felis	426, 404	659, 115
C.canis	417, 267, 105	624, 115
C.muris	449, 398	732, 115
***Cpgp40/15* PCR RFLP** (243)		
Subgenotype	***AluI***	***RsaI***
C.hominis Ia	587, 201, 146	391, 241, 159, 143
C.hominis Ib	457,328,75, 56	379,200,159,143
C.hominis Ie	328, 232, 143	424, 159, 134, 129, 106
C.parvum IIa	258, 242, 206, 81,60, 36	329, 199, 177, 143, 35
C.parvum IIc	325, 278,232, 36	356, 256, 143, 81, 35

Gel electrophoresis - PCR amplicons were resolved by gel electrophoresis using 1% Agarose gel (Medox) containing ethidium bromide (0.5 mg/ml) in 1X TBE (Tris – Boric acid – EDTA, Sigma) buffer. The RFLP was resolved by electrophoresis using a 2% agarose gel in 1X TBE buffer. All gels were then recorded under UV light using a gel documentation system (BioRad, United Kingdom). For RFLP, the bands were visualized under UV and based on the digestion pattern obtained, the species or subgenotype was determined.

Nucleotide Sequencing, Cloning and Phylogenetic Analysis - In some *C. hominis* samples and in zoonotic isolates, when results obtained by PCR-RFLP at the *Cpgp 40/15* locus could not be resolved into any of the known subgenotypes, the PCR products were purified using a QIAquick kit (Qiagen Inc, Valencia, CA) and sequenced by the Big dye-terminator method using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) in an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, USA), and the electropherograms were analyzed using the sequencing analysis software (version 1.01, Applied Biosystems).

The secondary PCR products of two *C. felis* isolates were cloned into the pCR 2.1-TOPO vector (Invitrogen Corp., Carlsbad, CA) and the inserts sequenced. Briefly, the *Cpgp 40/15* PCR product was purified using the QIAquick gel extraction kit (Qiagen Inc, Valencia, CA) followed by post amplification addition of 3′ A-overhangs. The TOPO

cloning reaction was set up using pCR II-TOPO vector followed by the transformation reaction using One Shot® Chemically Competent *E. coli* that were heat-shocked for 30 seconds at 42°C. 10-50 µl from each transformation reaction was spread on a pre warmed selective LB plate containing ampicillin and X-gal and incubated overnight at 37°C. Five white or light blue colonies were then selected and cultured in LB medium with 50 µg/ml ampicillin. The cells were then pelleted and the plasmid DNA purified using Qiaprep Spin Miniprep kit. The cloning reaction was then checked by *EcoRI* digestion as per the TOPO cloning protocol. Samples that contained the insert were then sequenced using the M13 primers.

Nucleotide sequence similarity searches were performed using the National Center for Biotechnology Information (NCBI - National Institutes of Health, Bethesda) basic local alignment search tool (BLAST) server on the GenBank database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Multiple sequence alignment was then carried out with the ClustalW alignment algorithm of the AlignX program of Vector NTI, Suite 8 (Informax, North Bethesda, MD) or the BioEdit Sequence Alignment Editor version 5.0.0 (91) by including selected nucleotide sequences representative of the known subgenotypes obtained from Genbank. Phylogenetic analysis was performed using the Maximum Likelihood method (PhyML or Vector NTI) or Neighbor Joining methods with BioEdit Sequence Alignment Editor version 5.0.0 (91). Tree construction with carried out with Treeview.

Real time PCR –Real time PCR for quantification of parasite burden was performed using a Chromo4 real time cycler (MJ Research, BioRad, Hercules, CA) or ABI 7500 cycler (Applied Biosystems, Foster city, CA) using primers designed to amplify a 114 bp fragment from the conserved gp15 region of the *Cpgp40/15* gene. The primer sequences were: gp15-F, 5' CTTTCTATACCGGCGCAAACAG 3' and gp15-R, 5' CAGAGGAACCAGCAT CCTTCAA 3'. The master mix contained 12.5 µL of QuantiTect SYBR Green PCR Master Mix (Qiagen Inc, Valencia, CA) and 10 pmol of each primer to which 1 µL of DNA was added. The PCR cycling conditions were: 95°C for 15 minutes, followed by 39 cycles of 94°C for 30 seconds, 60°C for 1 minute, and 72°C for 30 seconds. Purified plasmid DNA containing the 1 Kb sequence of the *Cpgp40/15* gene from the *C. parvum* IOWA strain cloned into the *T. gondii* expression vector pHLEM (171). was used for generating the standard curve. Dilutions of the standards ranging from 10^6 to 10 copy numbers were included in each run. Each sample was run in triplicate. Negative controls with no template DNA were included for each reaction series.

ELISA- Sequences encoding gp40 from subtypes II (*C. parvum,* GCH1) and Ia (*C. hominis,* TU502), gp15 from subtype II (*C. parvum,* GCH1) and a control protein containing the fusion tags (39) were cloned into the pET32Xa/LIC vector (Novagen), the recombinant (r) proteins over expressed in *E. coli* AD494 (DE3) cells and purified by metal-affinity chromatography. Serum IgG levels to rgp15, rChgp40 Ia and rCpgp40 II were quantified by ELISA. Ninety six-well micro titer plates were coated with

recombinant antigens rgp15 or rChgp40 Ia or rCpgp 40 II or the control protein at a concentration of 0.4μg protein/well. Excess antigen was washed off with PBS-0.05% Tween 20 and non-specific binding was blocked with 1% bovine serum albumin (BSA) in PBS. Wells were then incubated with serum diluted 1:100 in PBS with 1% BSA for 1 hour at 37°C. After washing three times, wells were incubated with 50 μL alkaline phosphatase-conjugated goat-anti-human γ chain-specific IgG, diluted 1:5000 in 0.25% BSA/PBS. After washing, wells were incubated with 50 μL substrate solution (100mM Tris-HCl, pH 9.5, 100mM NaCl, 5mM $MgCl_2$) containing *p*-nitrophenyl phosphate (1 mg/ml) at room temperature, the reaction was stopped after 15 min and absorbance read at 405 nm (A_{405}nm). The same positive and negative sera were run on each plate to control for plate to plate variation. Since all samples were run in triplicate and the mean A_{405}nm was determined (118).

- **CHAPTER 6**

MOLECULAR AND SPATIAL EPIDEMIOLOGY OF CRYPTOSPORIDIAL DIARRHEA IN CHILDREN– RESULTS FROM A BIRTH COHORT IN A SEMI URBAN SLUM COMMUNITY

Introduction

Cryptosporidiosis is a major cause of diarrhea in children with and without HIV infection in developing countries (101, 147, 180, 220, 228). In these countries, cryptosporidial infection in early childhood has been reported to be associated with subsequent impairment in growth, physical fitness and cognitive function (86, 166). In India, *Cryptosporidium spp*. are a leading cause of infectious diarrhea in children with reported positivity rates ranging from 1.1% to 18.9% (115, 193, 206, 224). The epidemiology of cryptosporidiosis in humans is not completely understood due to the existence of multiple transmission routes with person-to-person, animal-to-person, waterborne, food borne and possible airborne transmission (30). More recently, genetic characterization of isolates at polymorphic loci has facilitated the development of molecular approaches to study the epidemiology of cryptosporidiosis (247, 249). Extensive polymorphisms in the *Cpgp40/15* gene have been widely used to define at least 8 allelic subgroups or subgenotypes of the parasite (130, 243). The majority of human infections including those in developing countries are caused by *C. hominis* (130, 151, 156, 226, 229, 245). There have been several studies on the prevalence rates of cryptosporidial infections in children in India but there are only 2 reports on the species or genotypes of *Cryptosporidium spp*. from children in India (73, 158), and none from southern India. Further, there have been no studies on genetic characterization of *Cryptosporidium spp*. from children in a well-defined community setting.

Geographical Information Systems (GIS) have previously been used to map the location of residence of sporadic cases as well as in ecosystem studies of cryptosporidiosis (96,

113). The use of GIS to study the spatial distribution of cases has been found to be useful in identifying geographical variation, but not necessarily for identifying the reasons for this variation. In this study we determined the species, genotype and subgenotype of *Cryptosporidium spp*. isolates from a well-defined cohort of children in a semi-urban community in Vellore, South India. A spatial analysis of children with cryptosporidial diarrhea was also carried out in order to study the transmission dynamics of cryptosporidiosis in this community.

Materials and Methods

Study population, sample collection and screening - The study subjects are part of an ongoing birth cohort of 452 children recruited for a study on rotaviral infections from a semi-urban slum area with a population of approximately 33,390 in Vellore in south India. The study was approved by the Institutional Review Board of Christian Medical College and informed consent was obtained from the parents. The children were enrolled at birth and followed up for three years on a twice weekly basis. Demographic and birth details were recorded at baseline. In this study, an episode was defined as at least one day of diarrhea (three or more watery stools in a 24 hour period), preceded and followed by two or more days without diarrhea. The episode was considered to have ended on the day bowel movements returned to normal (152). Children with diarrhea were assessed clinically and details on the number of stools passed per day, any associated fever or vomiting and treatment given were recorded daily until cessation of diarrhea. Fecal samples collected from 1989 episodes of diarrhea over 14,584 child-months (the sum of months of follow-up of all children) of follow up from April 2002 to January 2006 were

screened for *Cryptosporidium spp*. by microscopic examination of modified acid fast stained smears and these samples were also tested for other bacterial and parasitic diarrheal pathogens by culture and microscopy and for rotavirus by ELISA (Dako Cytomaton Rotavirus IDEIA, Ely, UK).

DNA isolation, speciation, genotyping and subgenotyping- Briefly, DNA extracted from fecal samples that were positive for *Cryptosporidium spp*. by microscopy with the QIAamp Stool DNA minikit (Qiagen Inc, Valencia, CA) was subjected to PCR-RFLP at the *SSU rRNA* locus using the enzymes *SspI* and *VspI* for species determination (246) and PCR-RFLP at the *Cpgp40/15* locus using enzymes *AluI* and *RsaI* for subgenotyping (130) as previously described. Samples negative by *SSU rRNA* PCR were analyzed by PCR-RFLP at the *COWP* and *TRAP-C* loci (218, 219). In samples where ambiguous results were obtained by PCR-RFLP at the *Cpgp 40/15* locus, the PCR products were purified using a QIAquick kit (Qiagen Inc, Valencia, CA), sequenced by the dye-terminator method and compared with sequences of known subgenotype using the ClustalW alignment algorithm of the AlignX program of Vector NTI, Suite 8 (Informax, North Bethesda, MD). The secondary PCR products of two *C. felis* isolates, one obtained from an HIV infected adult from a previous study (156) and one from a child in this study, were cloned into the pCR 2.1-TOPO vector (Invitrogen Corp., Carlsbad, CA) and the inserts sequenced. The *Cpgp 40/15* PCR product of the *C. parvum* (mouse) isolate was also sequenced and analyzed. Phylogenetic analysis was performed using the Maximum Likelihood method with BioEdit Sequence Alignment Editor version 5.0.0 (91).

Spatial analysis- The Poisson probability model (122) was used to identify any spatial or space-time clusters for high rates with the maximum spatial cluster size of 50% of the total population. Spatial analysis was carried out for cryptosporidial infections from birth till January 2006 to detect any clustering of episodes using SaTScan version 6.0 (123). The streets and the study houses of the urban slum area were previously mapped and geo-referenced using ArcView GIS 3.3 (Environmental Systems Research Inc., California, USA). The way-points and trackpoints were collected using a GPS Garmin V and downloaded as layers using GPS Utility 4.10.4. (GPS Utility Ltd., Southampton, England).

Statistical analysis - Data generated during this study were double entered using Epi Info 6.4. software (CDC, Atlanta, GA, USA) and analyzed using STATA version 9.0 (Stata Corp., College Station, TX, USA). Statistical comparisons were made using Fisher's exact and Chi-square tests for categorical variables and Mann Whitney U test for continuous variables.

Sequences from this study were deposited in Genbank with accession numbers, DQ 848995-97.

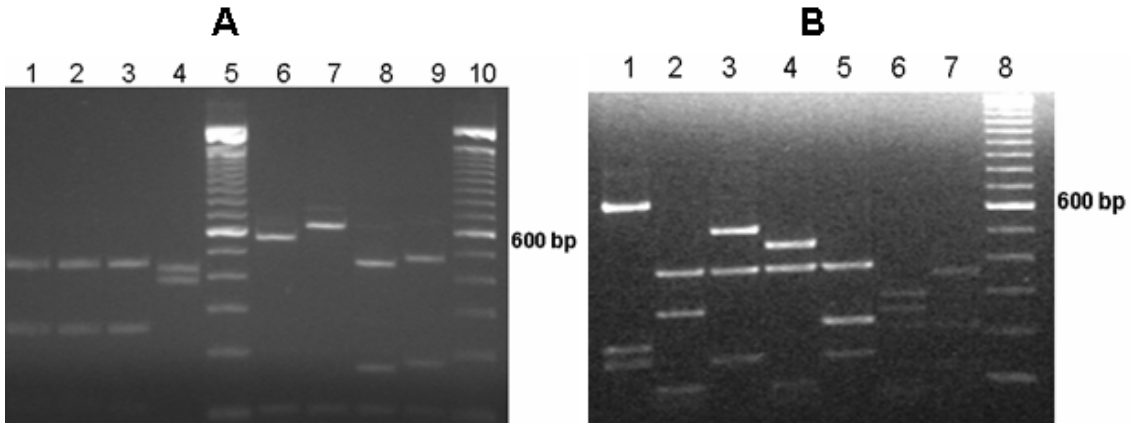
Results

Cases – Of the 1949 diarrheal episodes screened, one or more diarrheal agents was detected in 591 episodes. *Cryptosporidium spp* alone accounted for 7.61% of episodes and was the third most common aetiologic agent, after rotavirus and *Giardia.* Fifty-eight diarrheal episodes associated with *Cryptosporidium spp*. were identified in 53 children,

with 5 children having 2 episodes each. Mixed infections with rotavirus, *Shigella flexneri, Vibrio cholerae* or *Giardia spp* were identified in 4 children. In the children with repeated episodes of cryptosporidial diarrhea, the duration between the episodes ranged from 10 days to more than a year. Fifty four of these episodes were treated with oral rehydration. None of the children with cryptosporidial diarrhea were hospitalized or required intravenous rehydration. In 7 of the 58 episodes the child was found to be dehydrated clinically based on assessments carried out in the field. A total of 5 of 452 children in the cohort died during the follow up period , with 1 death each due to 3 congenital anomalies and a seizure disorder, and three deaths due to diarrhea. The diarrheal episodes were not associated with cryptosporidiosis.

Species and subgenotypes - The most common species identified was *C. hominis* (47/ 58, 81%) followed by *C. parvum* in (7/58, 12.1%) and *C. felis* (3 /58, 5.2%). One sample, which was negative by SSU (18 S) rRNA PCR was identified by COWP PCR. On comparison of the three PCR-based techniques in microscopy positive samples, others have found a sensitivity of 97, 91, and 66% for the 18S rRNA, COWP, and TRAP-C1 gene fragments, respectively(105). Given this sensitivity, it is possible that one of 58 samples, tested twice, could be negative by SSU PCR but positive by COWP PCR. One isolate of *C. parvum* (mouse genotype) was also identified (Figure 6.1 A). Subgenotyping of the 47 *C. hominis* isolates at the *Cpgp 40/15* locus showed that subgenotype Ia was most common (35 /47, 74.5%), followed by Id (8 /47, 17%), Ie (3/47, 6.4%) (Leav et al) and Ib (1/58, 1.7%).

Figure 6.1: Speciation and Subgenotyping of Cryptosporidial Isolates



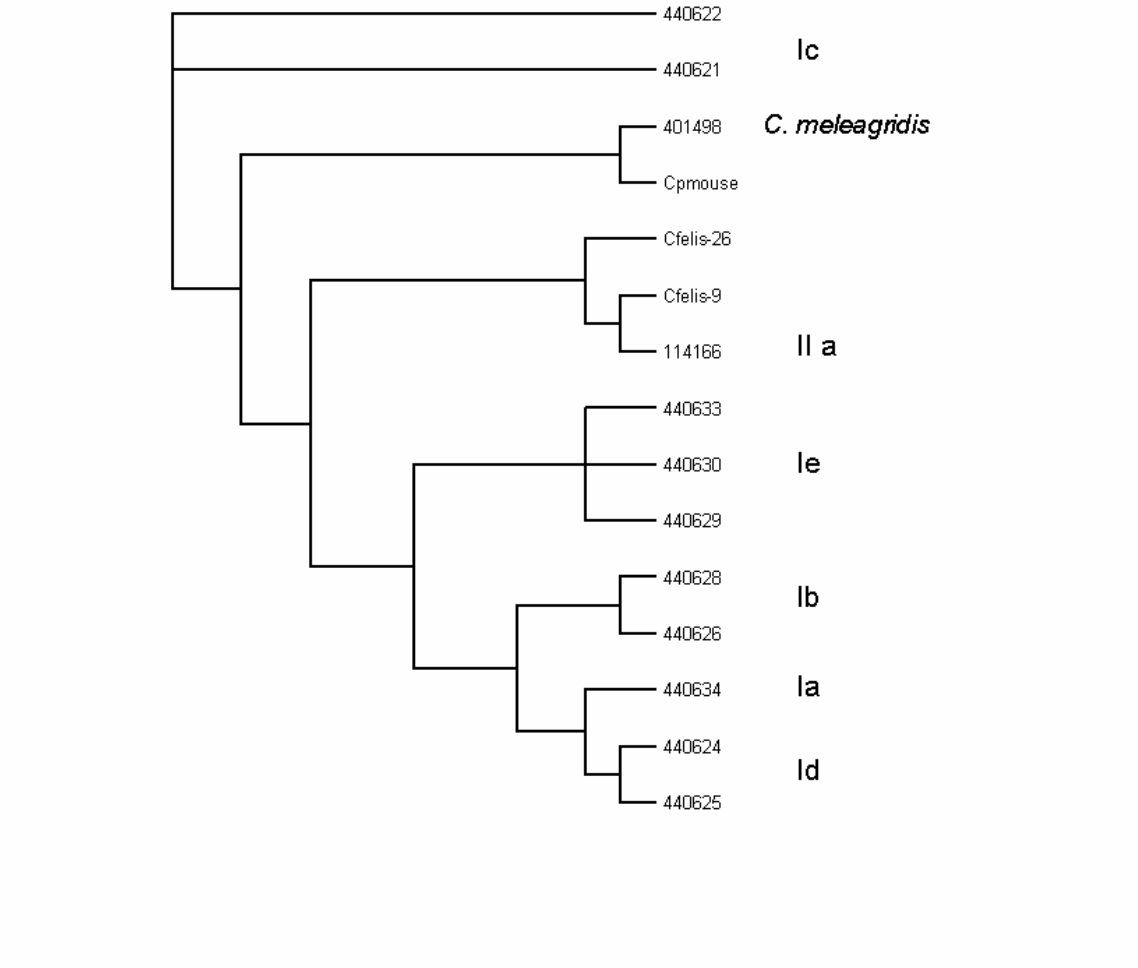
A) Restriction fragment length polymorphism patterns at the following loci for *Cryptosporidium spp*. after nested PCR amplification A) *SSU rRNA* locus and digestion with *SspI* (lanes 1-4) and *VspI* (lanes 6-9). Lanes 1 & 6, *C. hominis*; lanes 2 & 7, *C. parvum*; Lanes 3 & 8, *C. parvum (mouse)*; Lanes 4 & 9, *C. felis*; Lane 10, 1 kb marker.

B) *Cpgp 40/15* locus and digestion with *AluI* Lane 1, *C. hominis* Ia; Lane 2, *C. parvum* Ic; Lane 3, *C. hominis* Id; Lane 4, *C. hominis* Ib, Lane 5, *C. hominis* Ie (Leav) Lane 6, *C. felis*; Lane 7, *C. parvum* (mouse); Lane 8, 1 kb marker.

Subgenotyping of the *C. parvum* isolates revealed that all 7 were subgenotype Ic (Figure 6.1 B). In the 5 children with repeat infections, both infections were caused by *C. hominis* subgenotype Ia in 4 children and the remaining child had *C. felis* and *C. hominis* Ia infections.

The *C. felis* and *C. parvum* (mouse genotype) isolates could not be classified into any previously described subgenotypes by PCR-RFLP at the *Cpgp 40/15* locus (Figure 6.1 B). Phylogenetic analysis of the *Cpgp40/15* sequences from these isolates revealed that the C. *felis Cpgp 40/15* allele *Cpgp 40/15* alleles was most closely related to the *C. parvum* IIa subgenotype (Identities - 0.759 / 100% similarity over 795 bp), (Figure 6.2) while the *C. parvum* (mouse genotype) isolate was most closely related to the *C. meleagridis* allele (Identities-0.611/ 96% identity over 326 bp).

Figure 6.2: Phylogenetic Analyses of *C.felis* and *C. parvum* (mouse) *Cpgp 40/15* Allele



Phylogenetic analyses of *C.felis* (isolates 9 and 26) and *C. parvum* (mouse) *Cpgp 40/15* allele using the Maximum Likelihood method performed with BioEdit Sequence Alignment Editor Version 5.0.0. (The isolates from this study have been compared with the following sequences obtained from Genbank - AF440621-22, AF440624-26, AF440628-30, AF114166, AF440633-34, AF 401498.)

Association of clinical characteristics with *Cryptosporidium* species and subgenotypes - There were no significant differences in demographic (age, gender, birth order, educational status of the mother or occupation of the father) or clinical (nutritional status assessed by weight and height for age, vomiting, fever and hospital visits) characteristics between *C. hominis*-infected children and those infected with other species (Table 6.1) or between subgenotype Ia infected children and those infected with other subtypes. However, *C. hominis*-infected children had significantly greater severity of diarrhea ($p<0.05$, Fisher's exact test). There was also a trend towards a longer average duration of diarrhea in *C. hominis*-infected children ($p=0.09$) compared to those infected with other species (Table 6.1). However, there was no increase in severity of diarrheal symptoms in children who were co-infected with rotavirus, *Shigella flexneri, Vibrio cholerae* and *Giardia*.

Diarrhea associated with subgenotype Ia, the most common subgenotype isolated, was not more severe than that associated with the other subgenotypes ($p = 0.77$). In the 5 children with repeated episodes of cryptosporidial diarrhea, the severity of diarrhea was increased in one, decreased in 2 cases and remained the same in the remaining two children during the second episode.

Table 6.1: Association of Demographic and Clinical Features with *Cryptosporidium* Species

	C. hominis (n=47)	Other species (n=11)	p
Demographic features			
Age at diarrhea *	14.4 (9.6-23.1)	10.3 (7.3-24.4)	0.17
Gender			
Male	22(47)	7 (64)	0.50
Female	25(53)	4 (36)	
Birth order			
First	11 (23)	2 (18)	1.0
Others	36 (77)	9 (82)	
Education of the mother			
None	12 (26)	6 (55)	
Primary	17 (31)	4 (36)	0.11
More than Primary	18 (38)	1 (9)	
Occupation of the father			
Unskilled	17 (36)	5 (45)	0.73
Skilled	30 (64)	6 (55)	
Clinical features			
Associated fever			
	4 (9)	2 (18)	0.33
Associated vomiting			
No	41 (87)	9 (82)	0.64
Yes	6 (13)	2 (18)	
Malnutrition (Weight for age<-2SD)			
	14 (30)	4 (36)	0.72
Malnutrition (Height for age<-2SD)			
Yes	22 (47)	3 (27)	0.32
Diarrhea			
Severity**			
1-3	5	5	
4-5	12	4	**0.005**
>=6	29	2	
Duration ***	3 (2-5)	2 (2-3)	0.09

*Median (25^{th}-75^{th} percentile) no. of months: Mann-Whitney U test, **Maximum no. of stools/day (percentage): Fisher's exact test, ***Median (25^{th} – 75^{th} percentile) no. of days: Mann-Whitney test, the numbers in parentheses are percentages except for age (months).

GIS analysis- There were two significant space-time clusters of cryptosporidial diarrhea, one during February-March 2005 and the other during June-August 2003. The most likely space-time cluster involved 6 cases within an area of 0.07 km radius during the time frame of 1st Feb 2005 to 31st March 2005 (RR-133.8, p value-0.002). The second likely cluster involved 8 cases during the time frame of 1st June 2005 to 31st August 2005 (RR-22.9, p value-0.002) (Figure 6.3 A). Calendar time distribution of cryptosporidial diarrhea during the follow up period against the total diarrheal episodes experienced by the cohort also showed a peak in February 2005 with 16% of all diarrheal episodes in that month associated with *Cryptosporidium spp* (Figure 6.4).

When children with *C. hominis* subgenotype Ia alone were analysed, a similar pattern was found, with the most likely space-time cluster involving 8 cases within an area of 0.26 km radius during the same time period (RR-48.9, p value-0.001) and the second likely cluster to be 6 cases during 1st June 2005 to 31st August 2005 (RR-62.4, p value-0.001) (Figure 6.3 B). There was no clustering when the other subtypes were analyzed. No clustering was seen with the 7 children with *C. parvum* infection. In order to identify potential zoonotic transmission of infection, the households as well as neighbors of the children with *C. parvum* and *C. felis* infections were questioned for the presence of cattle or domestic pets but no history of exposure to these animals was found.

Figure 6.3 (A): Spatial Analysis with SaTScan Version 6.0 of Cases with *Cryptosporidium* Infection Revealing 2 Space Time Clusters in the Community

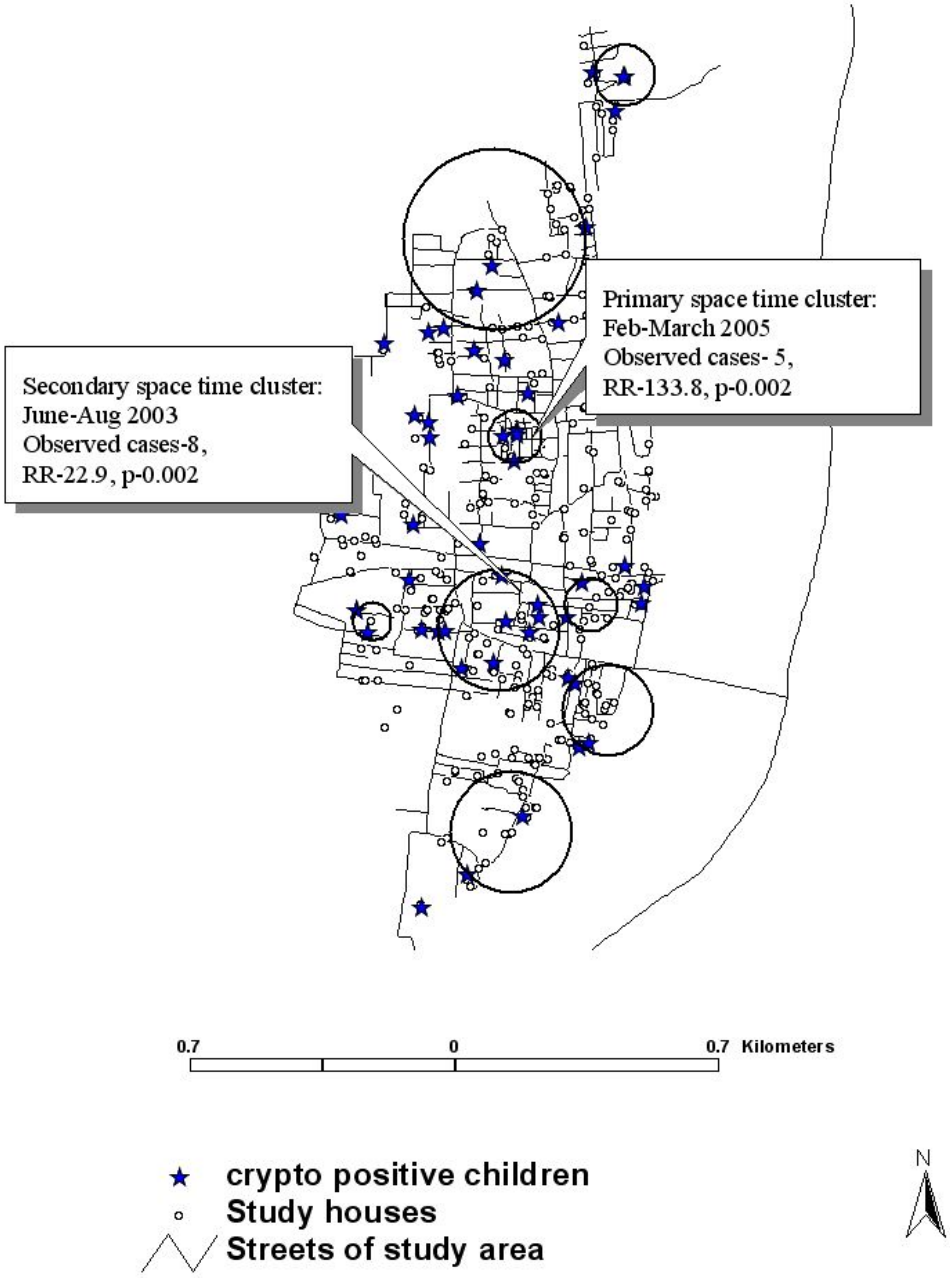


Figure 6.3 (B) : Spatial Analysis with SaTScan Version 6.0 of Cases with *C. hominis* Ia Infection Revealing 2 Space Time Clusters in the Community

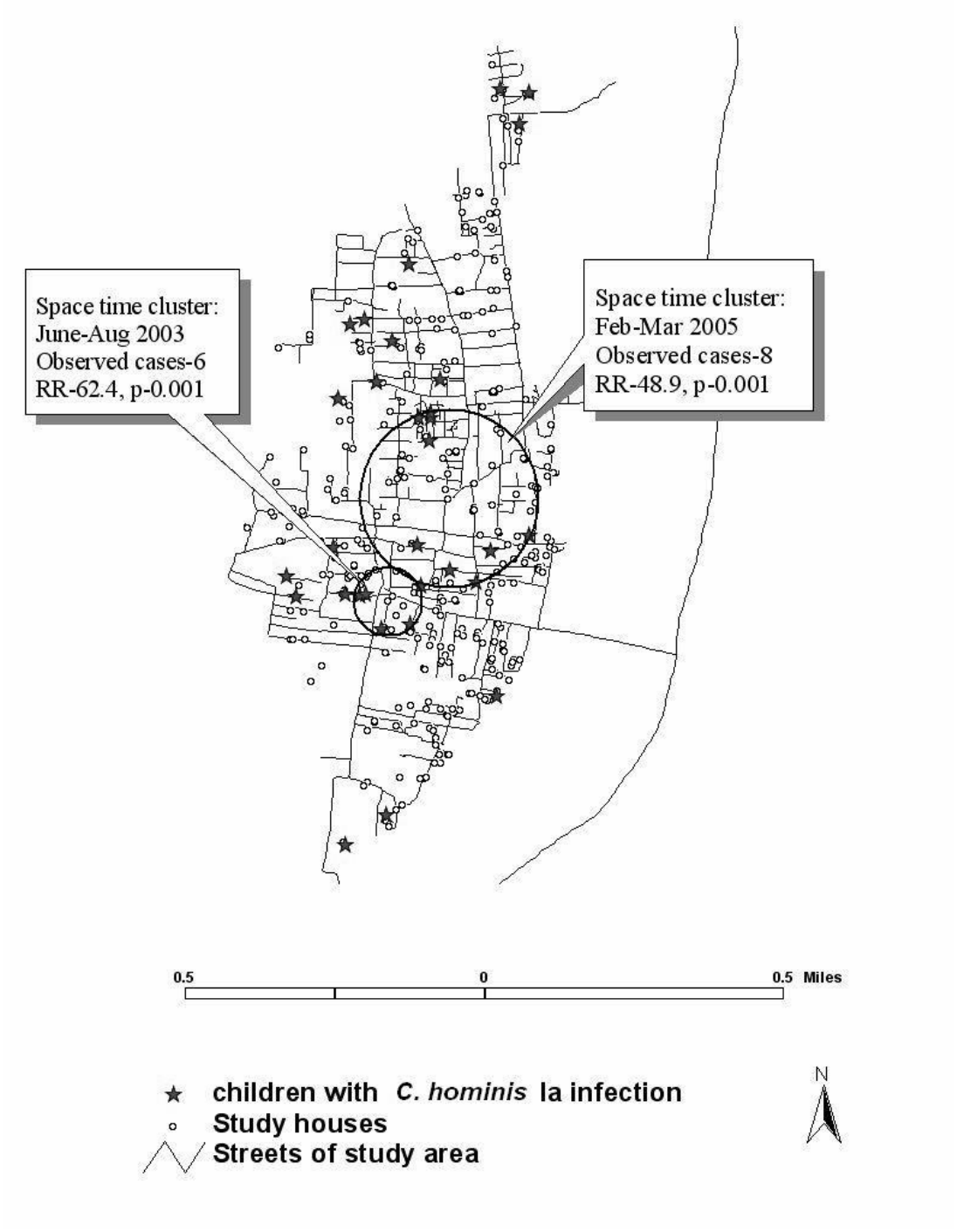
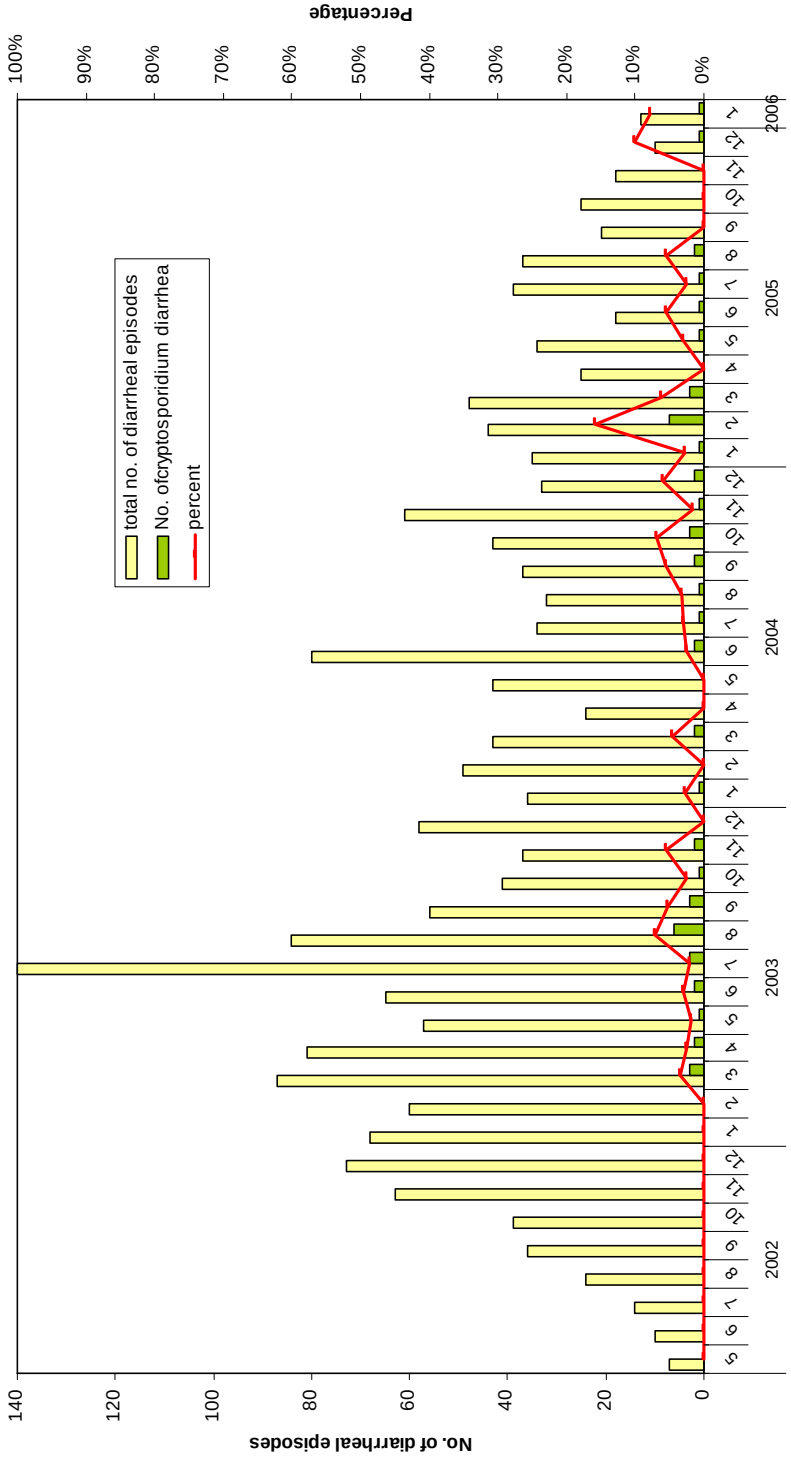


Figure 6.4: Calendar Time Distribution of Cryptosporidial Diarrhea



The association between cryptosporidial diarrhea and water source was also studied. In the community, 5 different overhead tanks supplied water to different areas and the data was available for 395 children in the cohort. The tank specific infection rates (Table 6.2 A and B) revealed that 7 of the 27 households which were supplied by a particular tank (tank 1) had cases with *C. hominis* Ia, which was significantly higher than in areas supplied by the other tanks (Chi square test, $p<0.001$) (Table 6.2 B). There was also a tank (tank 2) that was associated with only one episode of cryptosporidial diarrhea (Table 6.2 A). Water samples from the area were tested for coliforms and the most probable number/ml (MPN) assessed. Of the 3 samples collected from the Kaspa region during Jan-March 2005, two had MPN > 360 indicating a very high level of contamination suggesting a possible water related transmission for this cluster. The technology and reagents for testing water samples for cryptosporidial oocysts are not readily available in India and this was not carried out.

Discussion

This is the first study to genetically characterize *Cryptosporidium spp*. isolated from children and compare the association of species and subgenotype with clinical features in a community setting in India and it is also the first study to carry out spatial and temporal analysis of cryptosporidial diarrhea in India. When compared to our previous hospital-based study on *Cryptosporidium spp*. isolates from HIV infected adults in the same area (156), there was a significant difference in the proportion of potentially zoonotic species in the two populations ($p<0.001$,Chi square test) with fewer isolates of zoonotic species in children in the community compared to HIV-infected adults in the hospital.

Table 6.2 (A): Tank Specific Infection Rate for Cryptosporidial Diarrhea

Tank Number	Children with no cryptosporidial diarrhea	Children with cryptosporidial diarrhea	Total
1	20	7	27
2	27	1	28
3	206	28	234
4	54	6	60
5	37	9	46
Total	344	51	395

Table 6.2 (B) : Tank Specific Infection Rate for *C. hominis* Ia Diarrhea

Tank Number	Children with no *C.hominis* Ia diarrhea	Children with *C.hominis* Ia diarrhea	Total
1	20	**7**	27
2	28	0	28
3	219	15	234
4	57	3	60
5	40	6	46
Grand Total	364	31	395

The children in this study were not tested for HIV; however, none had risk factors for or developed clinical features suggestive of an immunosuppressive disease process.

The *Cpgp 40/15* subgenotypes were also more diverse among the HIV-infected adults than in children. The predominant subgenotypes among the *C. hominis* isolates were Ib and If in HIV infected adults whereas Ia predominated in children. All *C. parvum* isolates from children were identified to be the anthroponotic Ic/ subgenotype at the *Cpgp 40/15* locus, whereas several different subgenotypes including IIa, IIb and Ic/IIc were seen in *C. parvum* isolates from HIV infected adults. These data suggest differences in transmission patterns or susceptibility to disease in these two populations which merit further study of potential sources of infection in a developing country. In a previous report, a probability proportional to size cluster survey on approximately 3000 samples from individuals in the 15-40 years age group was carried out to estimate the community prevalence of antibodies to human immunodeficiency virus in rural and urban Vellore. This study showed that the prevalence of antibodies to HIV in the population was 0.66% (111). The prevention of HIV transmission from parent to children program has been in place since 2000, with all HIV infected pregnant women through out the state receiving anti-retroviral therapy (http://www.unicef.org/india/hiv_aids_278.htm). Even in the absence of such a program, expected rates in children, who could possibly have had vertical transmission of infection, would be less than a third of the adult population or 0.22% in children, or one child in the cohort of 452 recruited. Therefore, we do not believe that immunosuppression due to HIV could have been a significant risk factor for cryptosporidiosis in the 53 infected children or for recurrent diarrhea in the cohort.

Data on subgenotype distribution is scarce and derived mainly from hospital-based studies and from outbreaks (51, 81, 130, 156); this is the first community-based study to subgenotype isolates in a developing country. This is also the first non-outbreak study to show predominance of one subgenotype, Ia, in the community. A previous study from Malawi involving community acquired diarrhea in children showed the presence of 6 different subtypes among *C. hominis* isolates (180). In the present study, diarrhea due to *C. hominis* was also found to be more severe than diarrhea due to other species in this community. A study from Peru reported longer duration of symptoms and increased periods of oocyst shedding in *C. hominis* infected patients as well as association with other symptoms like nausea, vomiting and malaise (34, 245) and another from Brazil reported greater oocyst shedding than other species but there has been no previous report on association with severity of diarrhea.

The *C. parvum* isolates were all Ic, a subgenotype associated with anthroponotic transmission (247). In children infected with these isolates, no potential sources of zoonotic transmission were found. The finding that all *C.parvum* isolates that were subgenotyped as Ic at the *Cpgp40/15* locus had discordant alleles at the *SSU rRNA* locus has been reported previously (130, 156, 180) and it has been suggested that this allele should be renamed IIc. These data collectively suggest a predominantly anthroponotic transmission cycle of *Cryptosporidium spp*. in this community although the sources of infection were not determined.

The application of spatial and temporal analysis revealed two significant clusters of infections due to subgenotype Ia in children, which had not been identified on routine surveillance. Previous studies involving GIS and *Cryptosporidium spp.* have focused on the epidemiology of sporadic cases of cryptosporidiosis in the north west of England and Wales (96) and to demonstrate contamination sites in watershed environments in Kenya and Ecuador (113). This is the first study to apply GIS technology to document the epidemiology of cryptosporidiosis in a well defined community where the disease is endemic. Additionally, an area of the community supplied with water from a particular overhead tank (Tank 1) was found to have significantly higher incidence of cryptosporidiosis. Previous studies on water borne outbreaks have found an association with *C. hominis* subgenotype Ib (51, 81, 252) . In contrast, the subgenotype seen in the children potentially acquiring cryptosporidiosis from Tank 1 was Ia. Only one of the 47 *C. hominis* isolates subgenotyped in this study was Ib, suggesting that water borne transmission of subgenotype Ib may not be a significant mode of acquisition of disease in this area and that there may be a geographic variation in genotypes associated with water borne transmission. Alternatively, it is possible that subgenotype Ib is less prevalent in India than in other transmission sites due to other as yet undefined reasons. These data provide new evidence to support the need for the use of molecular and geo-spatial tools to investigate potential sources of infection and study transmission patterns in the community in order to apply relevant interventional measures for prevention of disease.

- **CHAPTER 7**

EFFECTS OF MULTIPLE SYMPTOMATIC AND ASYMPTOMATIC CRYPTOSPORIDIAL INFECTIONS IN CHILDREN IN THE COMMUNITY

Introduction

Cryptosporidium spp. is a leading cause of diarrhea in children in developing countries where it primarily affects children under the age of 5 years. Diarrhea due to this parasite has been associated with long lasting effects like cognitive function deficits and stunting. The risk of stunting increases with the number of episodes per year (86). Studies from Peru found that both symptomatic and asymptomatic cryptosporidiosis in children were associated with growth faltering following an infection but recovery was slower in children with symptomatic infection (25). In India, while several studies have reported *Cryptosporidium spp*. in diarrheal stool samples of children, some studies have also reported up to 9.8% (140) positivity in asymptomatic children.

In the previous chapter, studies on a well defined birth cohort of children in a semi urban slum in Vellore, South India, identified 41 children who had cryptosporidial diarrhea at or below 2 years which increased to 53 children at or below 3 years of age by microscopic examination of stools. The most common species associated with diarrhea was *C. hominis*. PCR RFLP at polymorphic *Cpgp40/15* locus used as a subtyping tool by several groups, (51, 130, 250), identified the most common subgenotype as Ia. Five of the 53 children were identified to have 2 episodes of cryptosporidial diarrhea by microscopy. In 3/5 cases who had repeat episodes identified by microscopy, the second infection occurred <1 month later and was of the same subgenotype of *C. hominis* (Ia) probably indicating relapse, not re-infection. In a study on hospitalized children with diarrhea, it was found that PCR detected more than 3 times the number of cases detected by microcopy (6). In this study, we aimed to characterize the natural history of

cryptosporidial infections among a subset of these children in the birth cohort previously identified to have cryptosporidial diarrhea and to identify the effect and frequency of cryptosporidial infections, both symptomatic and asymptomatic using PCR as a more sensitive screening tool.

Methods

The birth cohort – As previously described, 452 children were recruited in a community based birth cohort study from a semi-urban slum area in Vellore, South India and followed up twice weekly. Any child who had diarrhea was assessed clinically and treated. An episode of diarrhea was defined as at least 1 day of diarrhea (with the occurrence of 3 or more watery stools in a 24-h period) preceded and followed by 2 or more days without diarrhea. Diarrheal stools were screened for *Cryptosporidium spp*. by modified acid fast staining and also subjected to culture and rotaviral ELISA (Dako). Surveillance stool samples were collected fortnightly.

Study subjects and samples screened- Twenty of the 41 cases identified to have cryptosporidial diarrhea below the age of 2 years were randomly selected. DNA extraction (Qiagen Inc, Valencia, CA) and a screening PCR at the *SSU rRNA* locus (246) was carried out on all diarrheal and surveillance samples collected in the first 2 years of life by pooling 3 sequentially collected samples. Samples from positive pools were then re-extracted individually and the infecting species and subgenotype identified by PCR RFLP at the *SSU rRNA* locus and *Cpgp40/15* locus respectively (130) using previously described protocols.

Table 7.1: PCR Amplification Targets for Other Enteric Pathogens

Organism	Locus (Primers)	Amplicons size	Reference
Adenovirus	Adl1/Adl2	482 bp	(183)
Rotavirus	VP6	382 bp	(100)
Norovirus (genogroup 2)	NIE3	113 bp	(84)
Norovirus (genogroup 1)	SG1/D1	150 bp	(168)
Sapovirus	R80/JV33	320 bp	(148)
Campylobacter (*coli* and *jejuni*)	16S rRNA	854 bp	(131)
Diarrhoeagenic *E. coli*	(multiplex)		(236)
	Stx1	348bp	
	Stx2	584bp	
	Eae	482bp	
	Bfp	300bp	
	Lt	218bp	
	St	129bp	
	Vir F	618bp	
	Ipah	933bp	
	Dae	542bp	
	AafII	378bp	

In a few of the diarrheal samples, further testing for other agents of diarrhea to identify co-infections was carried out using methods previously described. This included norovirus genogroups I and II, *Campylobacter*, diarrheagenic *E. coli* (6), adenovirus (183) and sapovirus by PCR (148) as well as rotavirus (Dako Cytomation IDEIA,UK) by ELISA. The locus and product size of PCR amplicons is described in Table 7.1.

Statistical analysis – Comparison of diarrheal severity and duration was carried out between pools that were positive for *Cryptosporidium* and those that were not using the Mann Whitney U test. Nutritional status and maternal antibody status of children with single and multiple episodes were compared at 24 months and 36 months was analyzed by Mann Whitney U test; Fisher's exact test was used to compare the proportion of seropositive mothers in both groups.

Results

Episodes of cryptosporidiosis - A total of 1019 (surveillance and diarrheal) samples in 341 pools from these 20 cases were screened. 12/20 (60%) cases had more than one positive pool. There was no difference in severity (p=0.307) or duration of all causes of diarrhea (p=0.794) between pools containing diarrheal samples that were or were not positive for *Cryptosporidium* (Table 7.2). Using the definition of an "episode of cryptosporidiosis" as a consecutive set of samples that are positive for *Cryptosporidium* by microscopy or PCR and having no intervening negative samples, 36 episodes were seen (Figure 7.1). Two children had a gap of more than 1 month between 2 available positive samples which were assumed to belong to a single episode (second episode for

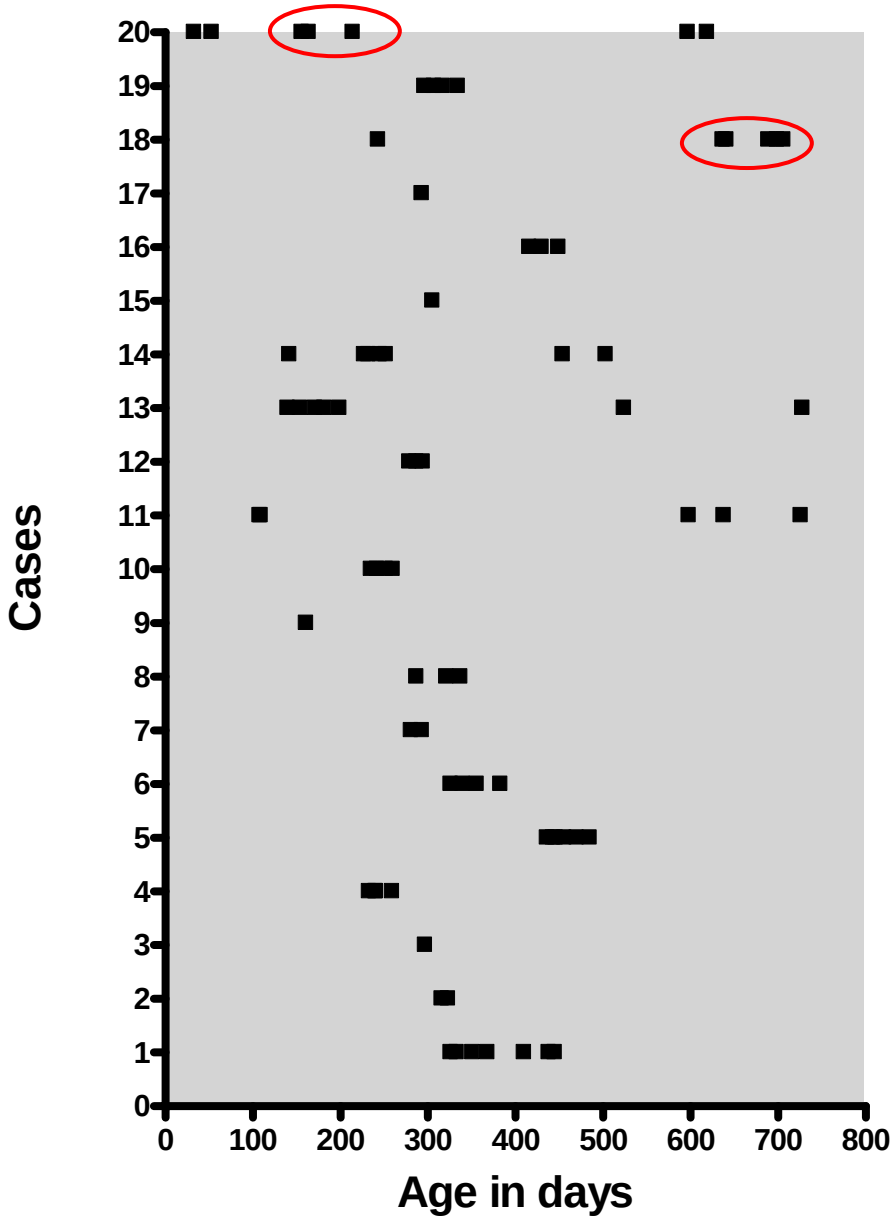
child 18 and 20, Figure 7.1) while one case dropped out at 10 months (child 19, Figure 7.1). Episodes were classified as symptomatic if at least one sample in the episode was a diarrheal stool (Figure 7.2). Twenty five episodes were symptomatic while the remaining 11 episodes were not asymptomatic. Among the 11 children with asymptomatic cryptosporidiosis, one episode was preceded by a diarrheal episode less than a month earlier, which was also due to *Cryptosporidium* (child 6). A majority of both symptomatic and asymptomatic episodes were due to *C. hominis* (33/36) while 1 episode of *C. parvum* and 1 infection with C. *meleagridis* was detected. One weak positive sample could not be speciated. Only 22 of the *C. hominis* could be subgenotyped and most were Ia (13/22) as previously documented in this birth cohort followed by Id (6/22). Two isolates of Ie and one of Ib were also identified. The *C. parvum* isolate was the anthroponotic IIc subgenotype.

In 15 of the symptomatic episodes, asymptomatic shedding was detected for more than 2 weeks (range 7 to 65 days) after the documented episode of cryptosporidial diarrhea while 6 episodes were preceded by asymptomatic oocyst shedding (range 7 to 22 days). Among the 6 children (child 1, 4, 10, 13, 16 and 19 in Figure 7.2) who had asymptomatic shedding prior to the onset of diarrhea, the first diarrheal sample in that episode of cryptosporidiosis was screened for other causes of diarrhea as described in the methods section (Table 7.1). Four samples were found to be positive for other enteric pathogens including, rotavirus, enterotoxigenic *E. coli* and adenovirus which could potentially be the actual cause of diarrhea superadded onto an asymptomatic episode of cryptosporidiosis (Table 7.3).

Table 7.2: Comparison of diarrheal severity and duration in pools positive for *Cryptosporidium* and those not

Median (IQR)	**Cryptosporidial diarrhea**	**Non cryptosporidial diarrhea**	p value
Severity	6 (5 – 8)	6.5 (5 – 9)	p=0.307
Diarrheal duration	7 days (6 – 10.5)	7 (6 – 12)	p=0.794

Figure 7.1: Episodes of Cryptosporidiosis in 20 Children followed up over 24 months



Although an intermittent pattern of diarrhea has been described for cryptosporidiosis, in child 5 and child 6 (figure 7.2) who had intermittent diarrhea in this study, the recurrent diarrheal samples were also screened for other enteric pathogens. Child 5 was found to be co-infected with adenovirus and *Giardia spp* while in child 6 was co-infected with enteroaggregative *E. coli.*

Multiple episodes - Multiple episodes of cryptosporidiosis occurred in 40% (8/20) of children below the age of 2 years. Among the 8 children who had multiple episodes of cryptosporidial diarrhea, 2 children had 3 episodes and 3 had 4 episodes each. Almost all secondary infections occurred more than a month later (Median 86 days, range 7-489). One child (child 8) was identified to have a recurrent cryptosporidial diarrhea within a week of the previous episode with the same subgenotype and so could represent a relapse rather than recurrence. In 5 children with cryptosporidial diarrhea in whom no recurrent cryptosporidial episode was detected, a relapse of diarrhea was seen within 2 months (5-48 days) of cryptosporidial infection. The median duration between a diarrheal and asymptomatic infection was 325 days (range 20-489). Nearly all repeat episodes were due to *C. hominis* with only 1 child having a *C. meleagridis* infection.

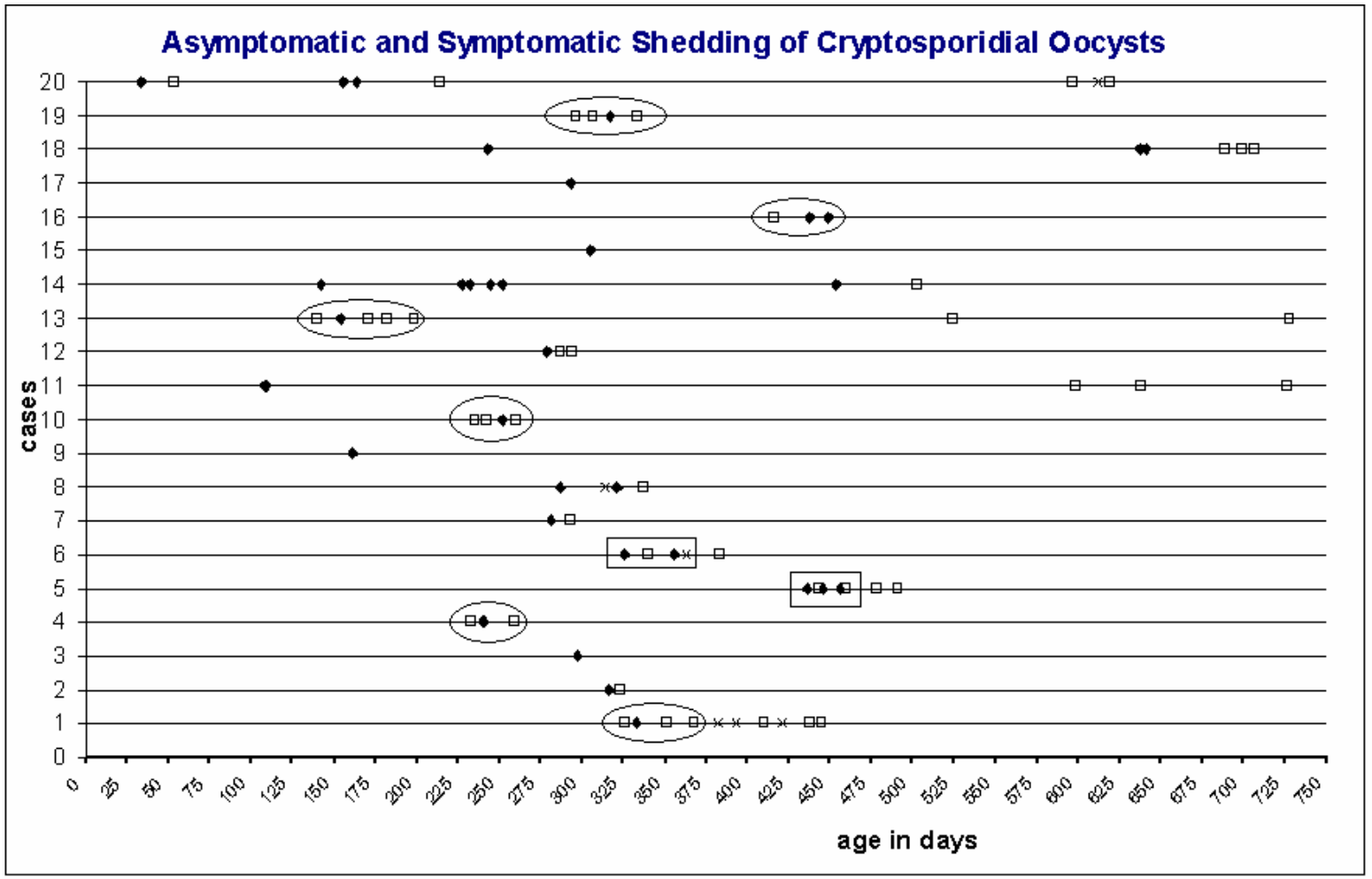
In all cases, there was a decrease in duration and severity of diarrhea or occurrence of asymptomatic infections (indicated by severity and duration =0) after the first episode (Figure 7.3 and table 7.4). In 2 children (child 14 and child 20), the 2nd diarrheal episode was more severe than the first episode and were investigated for other causes (table 7.3).

They were found to be co-infected with multiple pathogens during the second episode of cryptosporidiosis.

The maternal antibody (explained in more detail in chapter 9) levels to the immunodominant cryptosporidial antigen gp15 was compared between children with single and multiple episodes. Although there was no significant difference between proportion of seropositive mothers between the 2 groups (p=0.335), when the median (IQR) of OD values was compared among seropositive mothers in the 2 groups (0.300, 0.176 - 0.307 and 0.081, 0.003 - 0.158 for single and multiple episode groups,respectively) the difference was near significant.

When the nutritional status of 11 children with a single episode and 8 with multiple episodes were compared, no difference in birth weight was seen. However, there were significant differences in WAZ and HAZ scores between the 2 groups at 24 months (p, 0.013 and 0.021). The difference in WAZ persisted at 36 months (p = 0.069) (Figure 7.4).

Figure 7.2: Symptomatic and Asymptomatic Episodes of Cryptosporidiosis



(● - positive diarrheal sample, "□" – positive surveillance sample and "x" intervening negative sample)

Table 7.3: Screening of Diarrheal Samples in for Other Enteropathogens

child	Adenovirus	Norovirus	Diarrheagenic *E. coli*	*Campylobacter*	Stool culture and microscopy	Rotavirus (ELISA/PCR)
Children with asymptomatic oocyst shedding prior to diarrhea						
1	-	-	-	-	-	-
4	-	-	-	-	-	Positive
10	-	-	-	-	-	-
13	Positive	-	-	-	-	-
16	-	-	ETEC	-	-	-
19	Positive	-	-	-	-	-
Children with intermittent diarrhea during a cryptosporidial episode						
5	Positive	-	-	-	*Giardia*	-
6	-	-	EAEC	-	-	-
Children with more severe second episode of cryptosporidial diarrhea						
14 a	-	-	-	-	-	Positive
14 b	Positive	-	ETEC	-	-	-
20 a	-	Positive	EAEC	-	-	-
20 b	Positive	-	-	-	-	Positive

Figure 7.3: Severity and Duration of Cryptosporidial Episodes Among 8 cases with Multiple Episodes

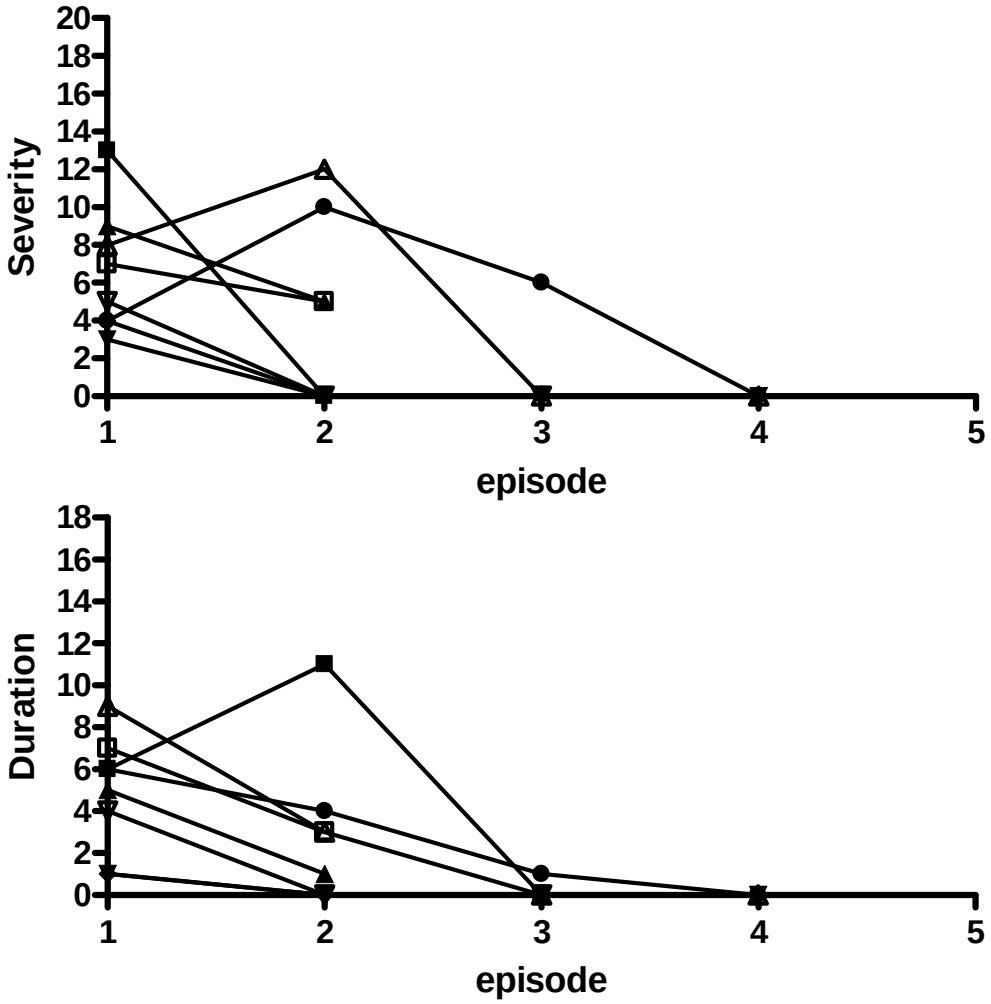
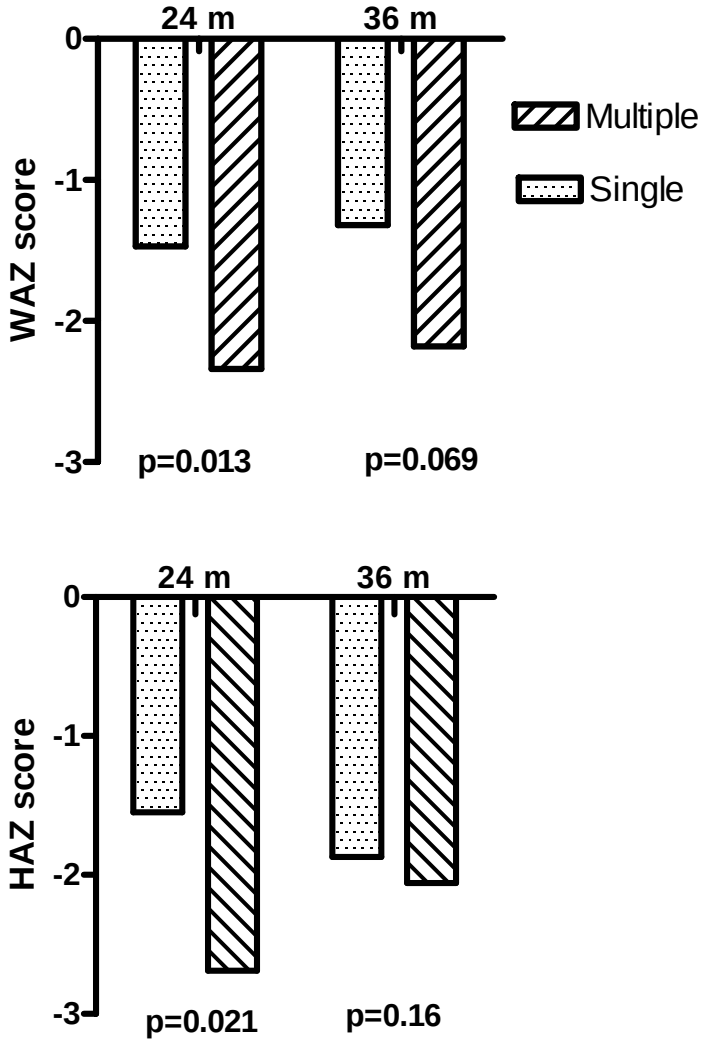


Table 7.4: Eight Cases with Multiple Episodes of Cryptosporidiosis with Gap between Episodes and Episode Type

child	1st episode	Interval	2nd episode	Interval	3rd episode	Interval	4th episode
11	acute	489	asymptomatic	40	asymptomatic	88	asymptomatic
14	acute	85	acute	202	acute	49	asymptomatic
20	acute	103	acute	441	asymptomatic	22	asymptomatic
13	acute	375	asymptomatic	204	asymptomatic		
1	acute	42	asymptomatic	28	asymptomatic		
6	acute	20	asymptomatic				
8	acute	7	acute				
18	acute	394	asymptomatic				

Figure 7.4: Effect of Multiple Episodes of Cryptosporidiosis on Weight for Age and Height for Age at 24 and 36 Months



Discussion

There have been previous studies on cryptosporidiosis in Africa and South America carried out on birth cohorts of children but this longitudinal study is unique because it used molecular tools to delineate episodes of cryptosporidiosis and describe the natural history of cryptosporidiosis in children in the first two years of life.

Multiple episodes of cryptosporidiosis were found to occur in 40% of children studied and there were more episodes associated with diarrhea (symptomatic) (69%) than without diarrhea. A greater prevalence of symptomatic than asymptomatic infections has also been seen in longitudinal studies from Brazil (164) and Israel (66) and cross sectional studies in Mexico and Uganda (143, 229) as well as in a hospital based study at Vellore that used PCR to identify the parasite (6). Both longitudinal studies from Brazil and Israel, as well as those from Guinea Bissau (146) and Uganda (229) also identified more persistent diarrhea than acute diarrhea but this was not seen in our study where most infections were acute. In contrast, a longitudinal study from Peru consistently identified more asymptomatic infection than symptomatic infections (26, 34, 45).

This study also documented a much higher rate of recurrent infections possibly due to PCR being a more sensitive tool. Other studies documented recurrent infections in 14 (Israel) to 22% (Peru) of cases (26, 34, 66). This occurrence of multiple infections including repeated symptomatic infections could be due to the multiple subgenotypes of *C. hominis* with limited cross protectivity. However, as noted in this study, there was a decrease in severity and duration with repeat infection. Also, among the 2 cases with

more severe recurrent episodes, other co-infecting enteropathogens were documented and could have resulted in the increased severity. There was no difference in duration or severity of diarrhea due to *Cryptosporidium* compared to non- cryptosporidial diarrheal episodes in this study. A pattern of intermittent diarrhea was also seen in a few cases and this has also been documented in children in other birth cohorts but could be attributed to the presence of co-infection with other pathogens.

In developing countries, cryptosporidiosis is more common in malnourished than well-nourished children and the consequences are more severe in the former. Low birth weight, malnutrition, stunting and lack of breast feeding are some of the factors that have been found to predispose to cryptosporidiosis (45, 164, 229). This study also documented prolonged asymptomatic oocyst shedding occurring both before and after cryptosporidial diarrhea in at least 50% of cases which could play an important role in both transmission of disease as well as long term effects on growth. Studies have shown that although decrease in weight gain was less than in symptomatic infections, asymptomatic infections could still play a major role in growth faltering (45). The shortfall in growth has been found to affect children who acquired the infection at age less than 1 year more significantly (45), and boys more than girls (66, 146). In a study from Brazil, decrease in HAZ scores were found to persist at 6 months following a *C. hominis* infection, rather than in *C. parvum* infection along with increased oocyst shedding (29), indicating that the species and subgenotypes of *Cryptosporidium* play a significant role in determining disease.

In this study, we also compared nutritional parameters in children with single and multiple infections. There was no difference in birth weight between the 2 groups but following the episodes of cryptosporidiosis, children with multiple episodes had significantly greater growth faltering than those with a single episode by the age of 24 months and the difference in WAZ persisted at 36 months. This is the first study to document a link between cryptosporidiosis and malnutrition in Indian children. A more detailed analysis will be required to identify whether the multiple episodes of cryptosporidiosis resulted in growth deficits, or whether more episodes of cryptosporidiosis occurred in malnourished children.

This study illustrates the importance of using molecular tools to estimate the true frequency of cryptosporidial infections in children in the community and to document the actual duration of oocyst shedding during early childhood cryptosporidial diarrhea. These data can then be correlated with nutritional and environmental parameters to determine risk factors for acquiring re-infections with *Cryptosporidium spp*.. The long term effects of repeated cryptosporidiosis on physical growth and cognitive function in these children remain to be estimated.

- **CHAPTER 8**

MOLECULAR CHARACTERIZATION OF CRYPTOSPORIDIAL ISOLATES IN HOSPITALIZED CHILDREN WITH DIARRHEA AND QUANTITATION OF OOCYST BURDEN WITH REAL TIME PCR

Introduction

Cryptosporidium spp. are an important endemic cause of parasitic diarrhea in children in developing countries (118, 228). In addition to causing symptoms of watery diarrhea, vomiting and weight loss, studies from South America have shown that early childhood cryptosporidiosis is associated with subsequent growth faltering (25, 86). *C. hominis* and *C. parvum* cause the majority of infections in children in developing countries (29, 52, 76), with occasional reports of infections with zoonotic species like *C. felis*, *C. canis*, *C. meleagridis* and *C. muris* (76, 245, 247). *C. hominis* has been found to be associated with heavier (29) and longer periods of oocyst shedding (245) and longer duration of symptoms (94) compared to *C. parvum* infected patients. In the community based study described in chapter 5, we showed an increased severity of diarrhea in *C. hominis* infected children compared to children infected with other species.

Cryptosporidium spp. have further been classified into several distinct subgenotypes based on extensive polymorphisms in the *Cpgp40/15* locus [also referred to as Cp17 (189), S60 (242) or GP 60 (247, 249)]. In most instances *Cryptosporidium* subgenotypes can be reliably identified by PCR-RFLP (51, 243) and this tool has been used in studies from several countries (1, 14, 51, 130, 169, 190, 241, 253) to investigate outbreaks (51), clinical correlates (130), geographic variations (41) and transmission patterns (7, 165) of cryptosporidiosis. Real time PCR has also been applied by several groups for detection and quantitation of *Cryptosporidium* oocysts in stool samples (92, 177), sewage and

water samples (90) and in animal and cell culture studies (82, 136) using a variety of probes and primers.

In India, a number of studies have reported *Cryptosporidium spp*. in diarrheal stool samples of children with positivity rates of up to nearly 20% (115) and asymptomatic cryptosporidiosis of 10% (140) using microscopy but few studies have used molecular techniques for identification of cryptosporidiosis in children (57, 73, 158), suggesting that actual infection rates may be significantly higher. A previous hospital-based study in Vellore found that PCR (15.2%) identified more than 3 times the number of cases of cryptosporidial diarrhea than microscopy (4.4%) (6). The aim of the present study was to identify the infecting *Cryptosporidium* species and *Cpgp40/15* subgenotypes associated with cryptosporidial diarrhea in hospitalized children from 3 centers in the country. In addition, we also performed real time PCR to quantitate parasite burden during episodes of cryptosporidial diarrhea in some of the children.

Methods

Study population and sample collection - This study was performed on stool samples originally collected for a multi-center rotavirus surveillance program called the Indian Rotavirus Strain Surveillance Network from December 2005 to December 2008. Samples from 3 centers representing both south and north India namely, Christian Medical College, Vellore, St. Stephen's Hospital, Delhi and Child Jesus Hospital, Trichy were available for this study. Children aged less than 5 years of age presenting to one of the 3 study hospitals with acute gastroenteritis and requiring hospitalization for rehydration for

at least 6 hours were enrolled in the Indian Rotavirus Strain Surveillance Network study. Children not requiring supervised oral or intravenous rehydration were excluded. A detailed clinical evaluation of the episode of diarrhea including duration and frequency of diarrhea and vomiting, fever and degree of dehydration was recorded and severity of the diarrheal episode was assessed using a modification of the Vesikari scoring system (110, 198). Informed consent was obtained from the parent/guardian and the study was approved by the Institutional Review Board of Christian Medical College, Vellore.

Laboratory procedures - One stool specimen was collected from each child enrolled and tested for *Cryptosporidium spp*. by acid fast staining and microscopy. Aliquots of positive samples were stored at -70 ºC for further characterization and all laboratory work was carried out at the Vellore center.

DNA extraction, PCR RFLP and sequencing – DNA extracted from the positive stool samples using the QIAamp DNA stool kit (Qiagen Inc, Valencia, CA) was then analyzed by PCR-RFLP at the *SSU rRNA* locus (246) and PCR-RFLP at the *Cpgp40/15* locus (Cohen, 2006) using previously described protocols. In samples where ambiguous results were obtained by PCR-RFLP at the *Cpgp 40/15* locus, purified PCR products were sequenced by the Big dye-terminator method. Multiple sequence alignment was carried out with ClustalW including selected nucleotide sequences representative of the known subgenotypes obtained from Genbank followed by phylogenetic analysis using the Maximum Likelihood method with PhyML (13, 88) and tree construction with TreeDyn (50) using default settings in the Phylogeny.fr server (Version 2) (59).

Real time PCR – Real time PCR for quantification of parasite burden was performed using a Chromo4 real time cycler (MJ Research, BioRad, Hercules, CA) or ABI 7500 cycler(Applied Biosystems, Foster city, CA) using primers designed to amplify a 114 bp fragment from the conserved gp15 region of the *Cpgp40/15* gene. The primer sequences were: gp15-F, 5' CTTTCTATACCGGCGCAAACAG 3' and gp15-R, 5' CAGAGGAACCAGCAT CCTTCAA 3'. The master mix contained 12.5 μL of QuantiTect SYBR Green PCR Master Mix (Qiagen Inc, Valencia, CA) and 10 pmol of each primer to which 1 μL of DNA was added. The PCR cycling conditions were: 95°C for 15 minutes, followed by 39 cycles of 94°C for 30 seconds, 60°C for 1 minute, and 72°C for 30 seconds. Purified plasmid DNA containing the 1 Kb sequence of the *Cpgp40/15* gene from the *C. parvum* IOWA strain cloned into the *T. gondii* expression vector pHLEM (171) was used for generating the standard curve. Each sample was run in triplicate. Negative controls with no template DNA were included for each reaction series. In 6 children from the Vellore center, stool samples collected at a median 2 (range 1-5) day intervals for a period of up to 24 days from the first day of admission were analyzed by real time PCR to determine parasite burden and shedding patterns. Follow up samples were also analysed by microscopy, *SSU rRNA* pcr and *Cpgp40/15* PCR.

Statistical Analysis – The clinical and laboratory data was entered into Microsoft Excel which was used for descriptive statistics and analysis of real time PCR data. Comparison of clinical features between species and subgenotype was performed using the Mann Whitney U test for duration of diarrhea and the Fisher's exact test for severity of diarrhea.

Results

Prevalence and seasonality of cryptosporidial diarrhea - Seventy of 2579 children enrolled in the study (2.7%) were found to have *Cryptosporidium spp.* in the stool by microscopy. Children in Delhi had a higher prevalence rate (34/970, 3.5%) than children from the 2 south Indian centers of Vellore (20/1018, 1.96%) and Trichy (16/591, 2.7%). Most children with cryptosporidiosis were aged less than 2 years of age (78.5%) with a median (IQR) age of 14 (9-24) months. The median age was lower in Delhi (11, 8-18) and Trichy (13, 8.75-19.5). However the median age of children hospitalized with cryptosporidiosis in Vellore was 17.5 (9-24) months, which was similar to that found in a community study (16 months). Most cases were male (67%). This was not found in the community study in Vellore, where there was an equal prevalence among male and female children. The median (IQR) duration of diarrhea was 3 (2-5) days (also similar to the community based study in Vellore) with 9 children having diarrhea for over a week, 2 of whom had diarrhea for more than 2 weeks. When assessed for severity of diarrhea based on maximum number of stools in a 24 hour period, most children had moderate to severe diarrhea (95%). Although the numbers of children in each center were small, the data were analyzed to determine if there was any seasonal variation. The prevalence of cryptosporidiosis over the 3 years of the study in each center is given in Figures 8.1 to 8.3 and the cumulative incidence for each month for all 3 centers in Figure 8.4. This analysis identified a decline in cryptosporidial diarrhea in the Delhi (Figure 8.1, 8.4) center between November to March and in the Vellore center (Figure 8.2, 8.4) from December to April. No seasonal pattern was discernable in Trichy (Figure 8.3).

Figure 8.1: *Cryptosporidium* Positivity in Diarrheal Stools in the Delhi Center

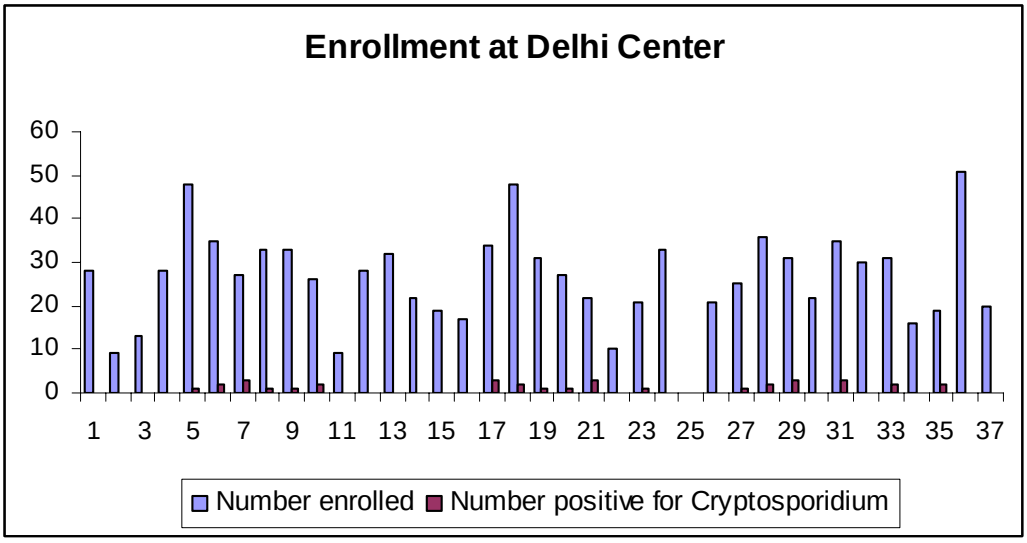


Figure 8.2: *Cryptosporidium* Positivity in Diarrheal Stools in the Vellore Center

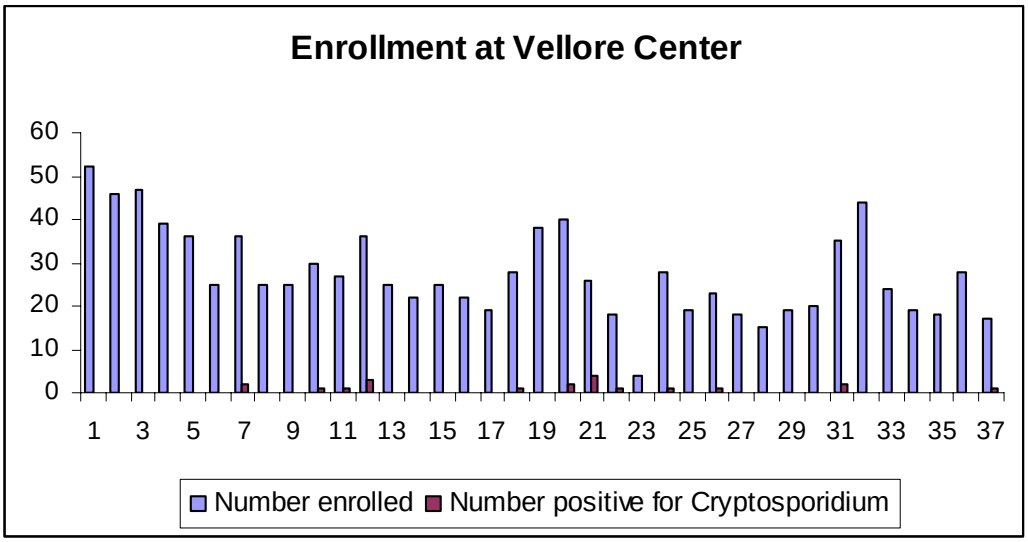


Figure 8.3: *Cryptosporidium* Positivity in Diarrheal Stools in the Trichy Center

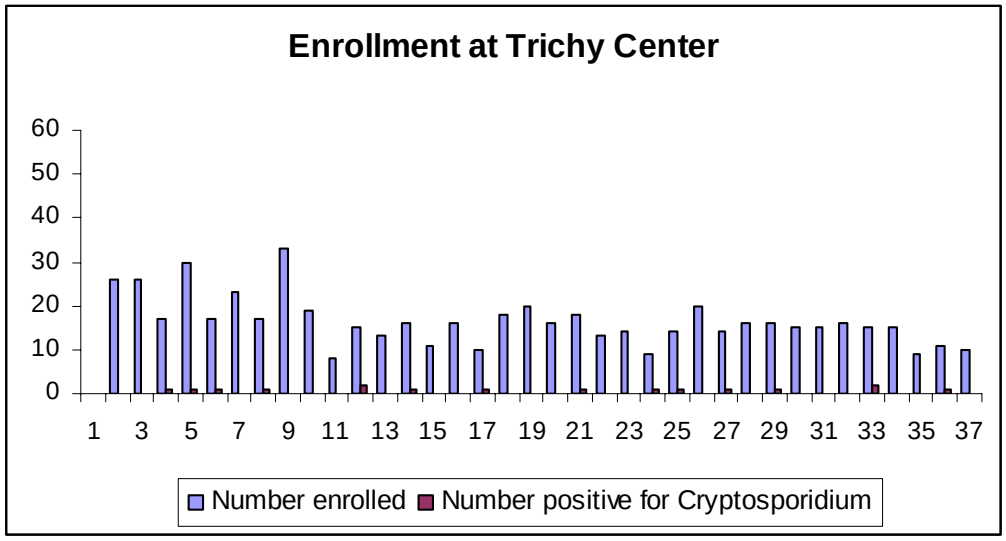
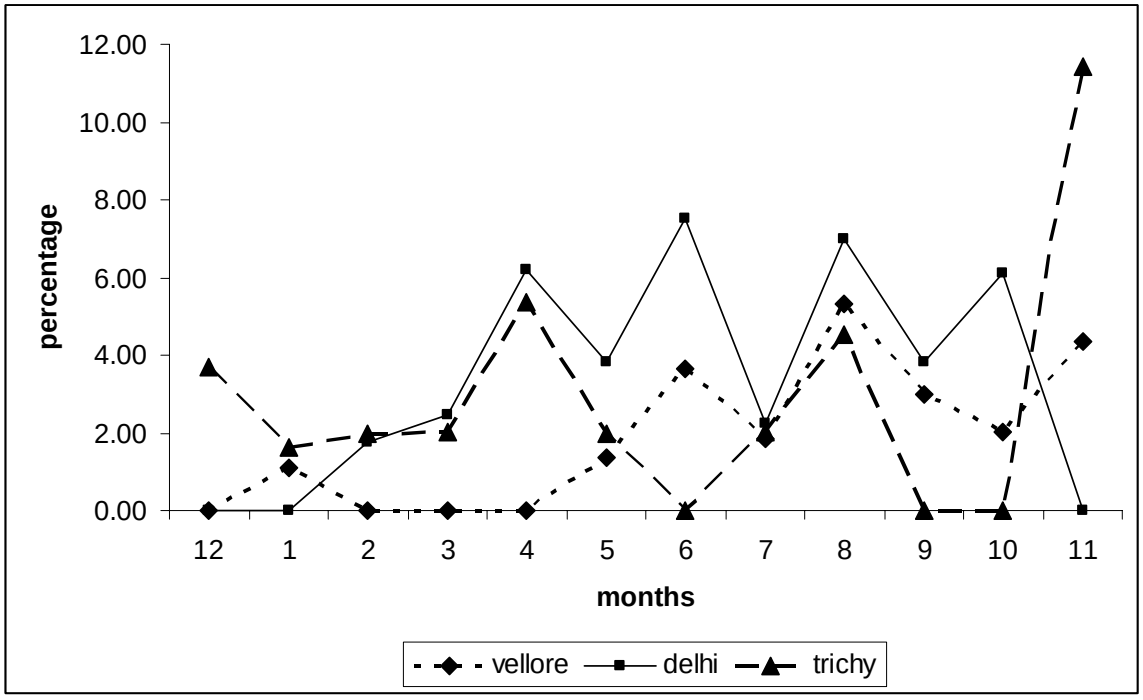


Figure 8.4: Cumulative Incidence of Cryptosporidiosis in all 3 Centers During the Study



Species and subgenotypes – PCR products for the SSU rRNA locus could be amplified from stool DNA in 67 of 70 children. *C. hominis* was the most commonly identified species in all 3 centers and was detected in 88.07% (59/67) of children followed by *C. parvum* seen in 7 (10.45%) children. Only one isolate of a zoonotic species, *C. meleagridis* was identified in a child from Trichy. Unlike the community study in Vellore, fewer zoonotic species were identified in hospitalized children.

PCR products for the *Cpgp40/15* locus could be amplified from stool DNA from 56 (56/59) of *C. hominis* and 6 (6/7) *C. parvum* isolates. Subgenotypes Ia and Ie (16/56 each) were the most commonly identified among the *C .hominis* and Ib (11/56) (Figure 8.5) and comprised the most commonly seen subgenotypes in all 3 centers. A mixed infection with Ia and If was also seen. The Id (7/56) subgenotype was also seen in all 3 centers. The If subgenotype (5/56), however, was seen mainly in Delhi with only one isolate seen in Vellore. Among the 7 *C. parvum* isolates, only one anthroponotic IIc subgenotype was identified in the Delhi center along with a IId isolate at the same center. Sequencing and phylogenetic analysis showed the presence of a newly described subgenotype IIm (previously identified in children from Bangladesh, Hira et al, unpublished data) in the south Indian centers of Vellore and Trichy (T415 and V740) and another novel subgenotype, tentatively described as IIn in accordance with current nomenclature conventions (217), was identified in the Vellore center (V416 and V 640) (Figure 8.6).

Figure 8.5: Prevalence of *C. hominis Cpgp40/15* Subgenotypes in all 3 Centers

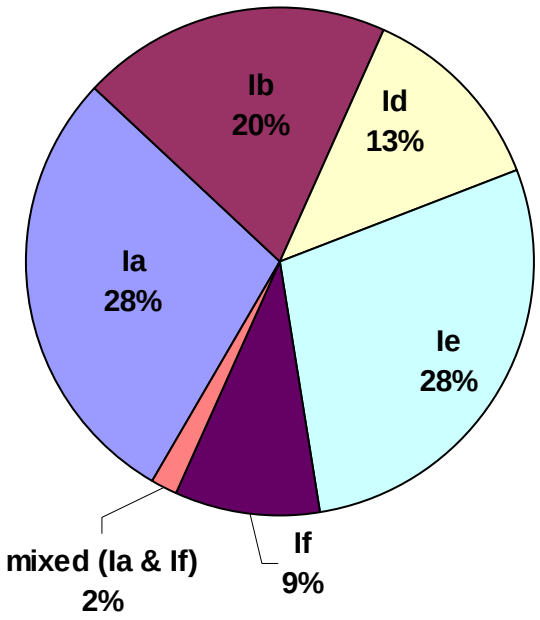
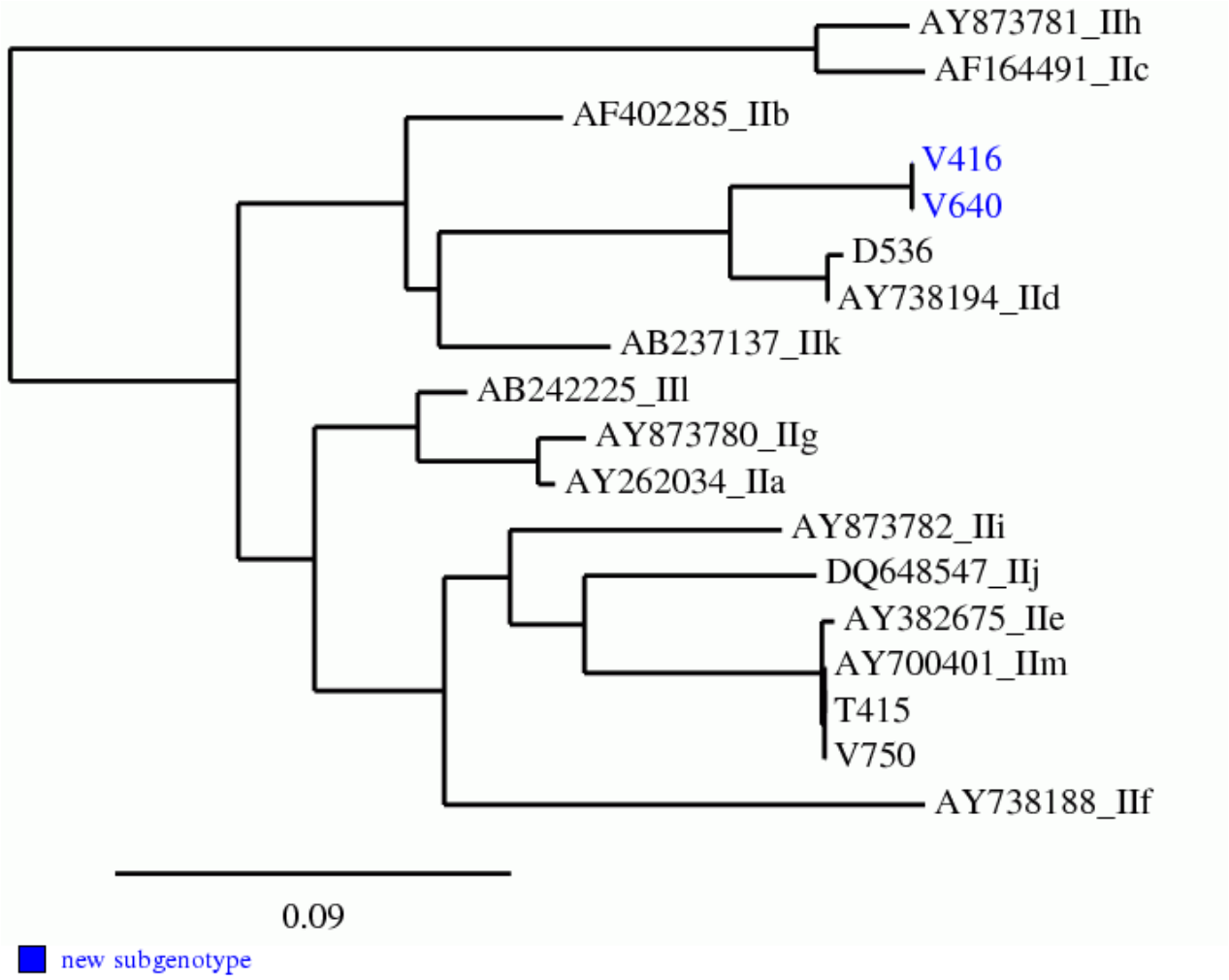


Table 8.1: Association of Clinical Features with Species and Subgenotype

Species	Median (IQR) duration of diarrhea	p	Median (IQR) episodes of diarrhea (24 hours)	P	Vomiting Present (%)	p
	n=61		n=62		n=62	
C. hominis	2 (2 - 3)	0.564	8 (6 - 15)	0.619	33 (60%)	1
C. parvum	2 (2 - 3)		9 (6 - 16)		5 (62.5%)	

Subgenotypes of *C. hominis*	Median (IQR) duration of diarrhea	Median (IQR) duration of diarrhea (other subgenotypes)	p	Median (IQR) episodes of diarrhea/24 hours	Median (IQR) episodes of diarrhea/24 hours (other subgenotypes)	P	Vomiting Present (%)	Vomiting present (other subgenotypes) (%)	p
	n=53			n=54			n=55		
Ia	4 (2 - 7)	3 (2 - 5)	0.31	9 (6 - 15)	8 (5 - 13)	0.641	10 (66.7%)	23 (57.5%)	0.758
Ib	4 (2 - 6)	3 (2 - 5)	0.698	9 (6 - 10)	8 (5 - 15)	0.858	6 (54.6%)	27 (61.4%)	0.739
Id	2 (2 - 3)	3 (2 - 7)	0.051	8 (5 - 15)	8 (6 - 15)	0.918	5 (83.3%)	28 (57.1%)	0.384
Ie	3 (2 - 7)	3 (2 - 5)	1	7 (5 - 15)	8 (6 - 12)	0.778	8 (53.3%)	25 (62.5%)	0.553
If	3 (3 - 5)	3 (2 - 6)	0.919	7 (6 - 10)	8 (6 - 15)	0.663	1 (20%)	32 (64%)	0.145

Figure 8.6: Phylogenetic Tree of Representative *C.parvum Cpgp40/15* Subgenotypes and Isolates Determined by Sequencing

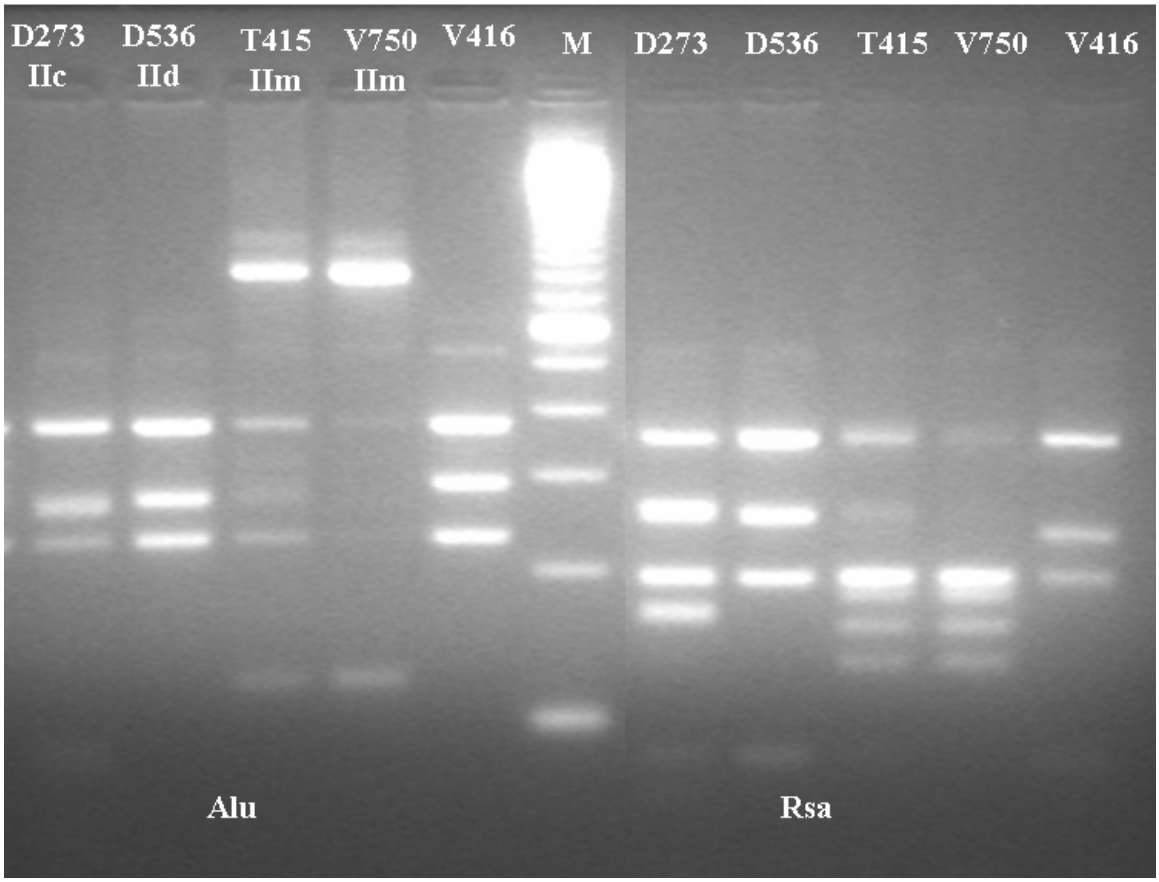


The subgenotypes IIm and IIn were also associated with a unique RFLP pattern that differentiated them from the other known subgenotypes (Figure 8.7). The diversity in the Vellore hospital isolates, with 7 different subgenotypes was very different from the community data that showed a greater than 60% predominance of a single subgenotype, Ia.

There was no difference in the severity or duration of diarrhea when children with *C. hominis* and *C. parvum* infections were compared. There was no difference in the severity of diarrhea among children infected with the common *C. hominis* subgenotypes Ia, Ib, Id and Ie. However, subgenotype Id was associated with a significantly lower duration of diarrhea than the other subgenotypes (p=0.051) (Table 8.1). There was no significant association between species or subgenotype and age at diarrhea.

Real time PCR – Sensitivity and linearity of the real time PCR assay was evaluated with serial 10-fold dilutions of plasmid DNA in 6 replicate runs which gave consistent and evenly spaced C_t values ranging from 15 to 32 (Figure 8.8) over 6 orders of magnitude with a correlation coefficient of 0.99. A single PCR product of the expected size (< 150 bp) was confirmed by agarose gel electrophoresis (Figure 8.9) and a single peak obtained by melting curve analysis (Figure 8.10), thus demonstrating specificity for the target

Figure 8.7: *Cpgp40/15* Subgenotypes of *C. parvum* Identified in the Study



gene. The limit of detection was 10 copies of the *Cpgp40/15* gene (Figure 8.9). Ct values greater than 37 were considered as negative. Real-time PCR amplified DNA from *C. hominis*, *C. parvum* and *C. felis* samples and none from *E. histolytica*, *Giardia spp*., *Cyclospora spp*. or *Isospora spp*. containing stool samples thus demonstrating specificity for *Cryptosporidium spp* (Figure 8.9). Data were expressed as copy numbers of the *Cpgp 40/15* gene. In 6 children from the Vellore center, stool samples collected between 16 to 24 days following admission were analyzed by real-time PCR (Figure 8.11) of whom 5 were identified to have *C. hominis* and 1 a *C. parvum* infection. In 3 of the 6 cases oocyst shedding stopped within 2 weeks of admission, while in 3 cases, even at 16, 18 and 24 days following admission, levels remained high, with up to 70 copies being shed per gram of stool. Real-time PCR was positive for all but 2 samples found to be positive by conventional *SSU rRNA* PCR. In 4 cases, real-time PCR recorded a longer duration of oocyst shedding than conventional PCR. Two cases (1 with *C. parvum* and prolonged shedding and 1 child with *C. hominis* in whom shedding stopped by 2 weeks) also showed intermittent shedding with levels dropping to zero (both real time PCR and conventional PCR were negative) at 10 -12 days following diarrhea, followed by an increase in copy number in the next sample.

Figure 8.8: Standard Curve for *Cpgp40/15* Real time PCR Generated from 6 Consecutive Runs of Standards with 10 Fold Dilutions

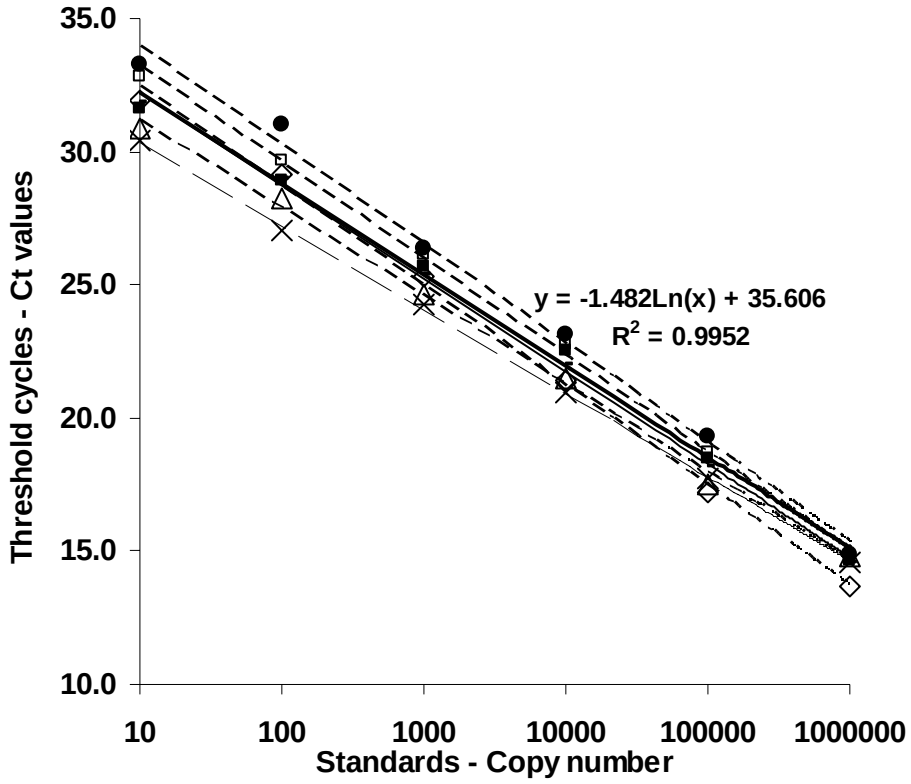


Figure- 8.9: Gel electrophoresis of the Amplicons of the Serially Diluted gp15 Standards and Samples Containing Other Parasites

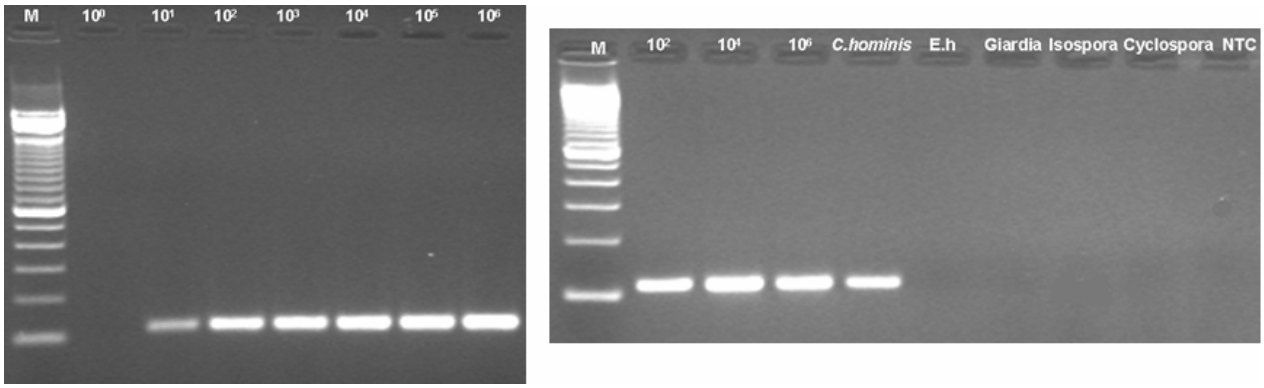
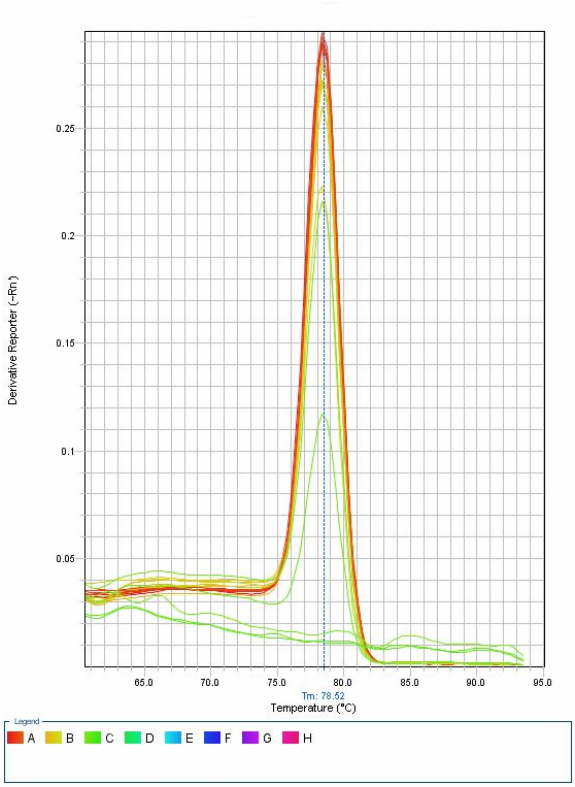


Figure 8.10: Melt Curve and Amplification plot for *Cpgp40/15* Real Time PCR



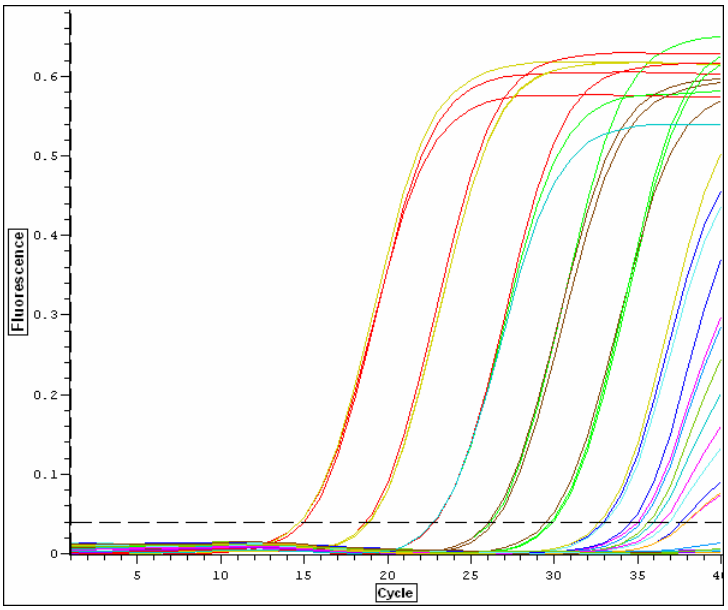
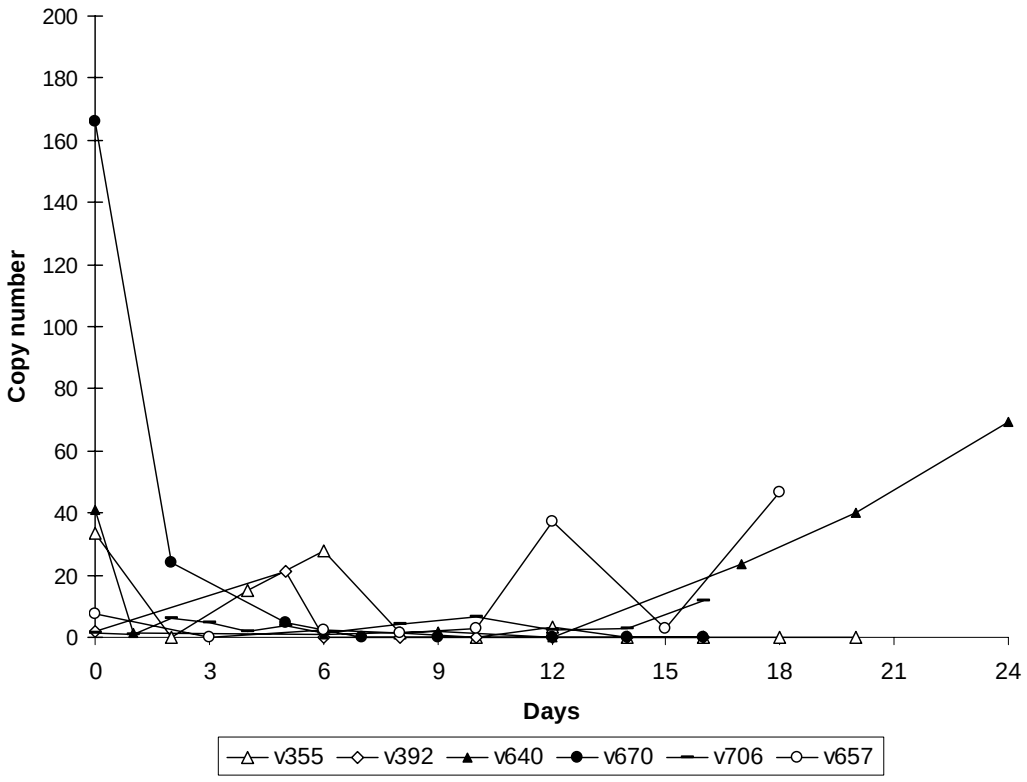


Figure 8.11: Real Time PCR on 6 Children with Cryptosporidial Diarrhea Followed Up



Discussion

In this study, cryptosporidial diarrhea identified by microscopic examination of stool samples in children in 3 different centers in India was described with differences in infecting species, subgenotype and seasonality. In all centers, *C. hominis* was the commonest species with very few zoonotic isolates among hospitalized children. In previous community based studies from South America, as well as based on results from the birth cohort study in Vellore, *C. hominis* has been found to be associated with more severe and longer duration of diarrhea (94), and therefore could results in more hospitalizations. Two other hospital based studies from Calcutta and Secunderabad also found that *C. hominis* was the most common species causing diarrhea in children (57, 158).

This study was the first to analyze any seasonal pattern in occurrence of cryptosporidiosis in India and found decreased prevalence in 2 centers in the months of November/December to March/April. Lack of any seasonal pattern in Trichy could possibly be due to less variation in temperature during the year. Seasonality in endemic cryptosporidiosis has been found in previous studies (99) and has been associated with the onset of rainy season in Guinea Bissau, Uganda and Brazil, the spring season in Korea and the summer and autumn months in Israel (40, 66, 164, 181, 229). A recent study from Kenya has also documented increased oocyst contamination of surface waters at the end of the rainy season, which was consistent with the timing of human infections in the region (154). In an alternative approach, a peak in incidence of cryptosporidiosis

has also been found to occur a little after a month after a peak in ambient temperatures in the United States (161). More detailed analysis including daily temperature and rainfall are required in this setting to determine association with incidence of cryptosporidiosis.

There was an increased diversity of *Cpgp40/15* subgenotypes among isolates from hospitalized children, unlike the previous community based study in the Vellore center where there was a predominance of a single subgenotype probably due to shared transmission pathways. However the most common subgenotypes (Ie, Ia, Ib and Id) were similar in all 3 centers and could reflect better host adaptation. An association between shorter duration of diarrhea and subgenotype Id was found. Although Cama et al, have reported an association of diarrhea with subgenotype Id along with other zoonotic species in HIV infected patients (35) no association was found between subgenotype and clinical features in children with diarrhea in Peru (34). Association of a clinical feature in children with subgenotype has been documented in a study in South Africa where those infected with subgenotype Ic were significantly older than those infected with other genotype I alleles (130).

Among the *C. parvum* isolates, a single zoonotic IId and anthroponotic IIc was identified. A more recently described subgenotype, IIm was found exclusively in the south Indian centers and a novel subgenotype was identified at the Vellore center. Interestingly, on phylogenetic analysis, the IIm subgenotype (Hira et al, unpublished data), was very

similar to the IIe subgenotype, also thought to be an anthroponotic strain (217). Once again, a much greater diversity of *Cpgp40/15* subgenotypes of *C. parvum* was identified in hospitalized children in Vellore compared to a previous community based study where only the anthroponotic IIc was identified.

Using real-time PCR, we were able to follow up children with cryptosporidial diarrhea and our findings indicate that cryptosporidial oocysts are shed for greater than 3 weeks following an episode of diarrhea, long after the disappearance of any symptoms. This asymptomatic shedding may in turn contribute to the long term effects of cryptosporidial infection, including stunting and growth retardation. In conclusion, this study documented the distribution of cryptosporidial species and subgenotypes in different regions of the country so that potential sources of infection and interventional measures can be identified.

- **CHAPTER 9**

SERUM IgG RESPONSES TO CRYPTOSPORIDIUM ANTIGENS IN A BIRTH COHORT OF CHILDREN IN A SEMIURBAN SLUM COMMUNITY IN SOUTH INDIA

Introduction

Cryptosporidium spp. is a frequent cause of infectious diarrhea in children in developing countries. In these countries, malnourished children are at greater risk of acquiring cryptosporidiosis and cryptosporidial diarrhea is more severe in malnourished than well-nourished children (85, 146, 164). Among the *Cryptosporidium* species, *C. parvum* and *C. hominis* cause most human infections. A few studies have shown that the most common species causing diarrhea in Indian children is *C. hominis* (57, 158).

The correlates of protective immunity to cryptosporidiosis are not well established. In adult human volunteers, serological responses to cryptosporidial antigens have been associated with partial protection from subsequent challenge and pre-existing antibodies associated with decreased severity and duration of infection (174, 233). Among the cryptosporidial antigens, one of the most consistently identified immunodominant antigens is the 15-17 kDa surface glycoprotein gp15 (also known as Cp17 or S16) (189, 195, 221, 242). The presence of pre-existing anti-gp15 antibodies has been associated with protection from diarrheal symptoms in naturally or experimentally infected adults (69, 195). Several workers have (39), (189, 221, 242) identified and cloned gp40/15 (also known as Cp60/45/15 or S60) a highly polymorphic gene, which encodes a precursor glycoprotein that is proteolytically cleaved to yield gp40 and gp15 (239). Gp40 is a mucin-like glycoprotein which associates on the parasite surface with the GPI-anchored gp15 fragment (170). Both gp40 and gp15 are implicated in mediating *Cryptosporidium* infection (39, 238). Although *Cpgp40/15* is highly polymorphic, particularly among *C. hominis* isolates, most of the polymorphisms are clustered in the

gp40 part of the molecule, while gp15 is relatively conserved (130, 223). This high degree of *Cpgp40/15* polymorphisms in isolates has been has been used as a subgenotyping tool by several groups, including ours (51, 130). In a study on a birth cohort of children in a semi urban slum in South India, it was found that the most common species in diarrheal samples was *C. hominis* with *Cpgp40/15* subtype Ia predominating.

In studies on cryptosporidiosis in a birth cohort of children in a semi urban slum community in South India presented in chapter 5, it was found that the most common species in diarrheal samples was *C. hominis* with *Cpgp40/15* subtype Ia predominating . The overall goal of this study was to assess the pattern of antibody responses to gp15 and gp40 in the same cohort of children in the first 2 years of life.

Methods

The birth cohort - The subjects in this study are part of a birth cohort of 452 children recruited for a study on rotaviral diarrhea in the semi-urban slum areas of Ramnaickapalayam, Chinnallapuram and Kaspa in Vellore, South India (20, 192). The study was approved by the Institutional Review Board of the Christian Medical College, and informed consent was obtained from the parents. Data on sociodemographic, environmental, and clinical characteristics were collected during the study. Details on collection of stool samples, and results of detection and molecular typing of *Cryptosporidium spp*. in diarrheal stools have been previously described in chapter 6.

Study subjects and samples- As mentioned earlier, the study subjects were part of an ongoing birth cohort of 452 children from a semi-urban slum area in Vellore in south India. In this study, an episode of diarrhea was defined as at least one day of diarrhea (three or more watery stools in a 24 hour period), preceded and followed by two or more days without diarrhea (152). Stool samples were collected during every episode of diarrhea as well as every 2 weeks. Blood samples were collected from the mothers at delivery or soon after recruitment (if the delivery was not institutionalized) and at approximately 3 month intervals from the children up to the age of 3 years. Since, it was found that most cryptosporidial diarrhea occurred before the age of 2 years (chapter 5), this study only included children ≤ 2 years of age. A total of 41 children with cryptosporidial diarrhea (defined as one or more episodes of diarrhea associated with the presence of *Cryptosporidium spp*. in the stool detected by microscopy and PCR) were identified in the cohort. Children with no cryptosporidial diarrhea (defined as one or more episodes of diarrhea with no evidence of *Cryptosporidium spp*. detected by microscopy or PCR in any diarrheal stool sample) were identified by screening 237 diarrheal stool samples from 123 randomly selected children in the cohort. The entire set of longitudinal serum samples was available for 39 of 41 children with cryptosporidial diarrhea and 90 children with no cryptosporidial diarrhea. Two children with cryptosporidial diarrhea dropped out of the study before completing 24 months of follow up. Maternal sera and sera from children collected at 3.5, 9 and 24 months of age were assayed for antibodies to gp15. Sera from the 39 children collected before (pre-infection) and after (post-infection) the first episode of cryptosporidial diarrhea were tested for antibodies to gp15 and gp40. The median (IQR) duration of collection of the pre-

infection sera was 5 (7) weeks prior to the first day of diarrhea, whereas for the post-infection sera it was 6 (6) weeks after the first day of diarrhea.

ELISA*:* Sequences encoding gp40 and gp15 from *C. parvum* subtype II, (GCH1 isolate), obtained from Dr. Saul Tzipori, Tufts Cummings School of Veterinary Medicine, N. Grafton, MA) and gp40 from *C. hominis,* subtype Ia (TU502 isolate), also obtained from Dr. Tzipori), and a "control" protein containing the His, Thioredoxin and S-tags alone (39) were cloned into the pET32Xa/LIC vector (Novagen), the recombinant (r) proteins over expressed in *E. coli* AD494 (DE3) cells and purified by metal-affinity chromatography by the Intestinal Microbiology Core laboratory of the GRASP Digestive Diseases Center at Tufts New England Medical Center, Boston as described previously (39). Serum samples were stored in aliquots at -80°C. Serum IgG levels to rgp15, rChgp40 Ia and rCpgp40 II were quantified by ELISA as previously described (118). Briefly, 96-well micro titer plates (Nunc, Rochester, NY) were coated with recombinant or control proteins at a concentration of 0.4μg protein/well. Excess antigen was washed off with 20mM sodium phosphate, 150mM sodium chloride, pH 7.2 (PBS) containing0.05% Tween 20 and non-specific binding was blocked with 1% bovine serum albumin (BSA) in PBS. Wells were then incubated with serum diluted 1:100 in PBS with 1% BSA for 1 hour at 37°C. After washing three times, wells were incubated with 50 μL alkaline phosphatase-conjugated goat-anti-human γ chain-specific IgG (Sigma, St. Louis, MO), diluted 1:5000 in 0.25% BSA/PBS. After washing, wells were incubated with 50 μL substrate solution (100mM Tris-HCl, pH 9.5, 100mM NaCl, 5mM $MgCl_2$) containing *p*-nitrophenyl phosphate (1 mg/ml) (Sigma, St. Louis, MO) at room temperature, the

reaction stopped after 15 min and absorbance read at 405 nm (A_{405}nm). The same positive and negative control sera (sera which were positive or negative for *C. parvum* rCpgp15 by ELISA and Western blot analysis) were run on each plate to control for plate to plate variation. All samples were run in triplicate and the mean A_{405}nm determined (118). For each sample tested, the final A_{405}nm value was calculated by subtracting the A_{405}nm in response to the 'control' protein.

Statistical analysis: Data was analyzed using STATA 10.1 for Windows (StataCorp LP, College Station, TX, USA). Cut-offs were generated for each time point by calculating the mean+2SD of the negative control for cases and controls and applying the higher cut off value to the data set. For the analysis of serum before and after an episode of cryptosporidial diarrhea the mean+2SD of the negative control for pre- and post-serum was calculated and the higher cut point applied. A particular sample was considered to be seropositive if there was a detectible antibody level to rCpgp15 or rChgp40-Ia or rCpgp 40-II above the cut-off determined (expressed in OD) and seronegative if below the cut-off level (Table 9.1).

The proportion of seropositive samples and the difference in the antibody titer values at different time points were compared between children with and without cryptosporidial diarrhea using the Chi-square test or the Fisher's exact test and the Mann Whitney U test respectively. A child was considered to have seroconverted at a particular time point

Table 9.1: Cut point values applied for each time point or data set based on mean+2SD of the negative control

	Cut point applied
Longitudinal sera	
Maternal sera (rgp15)	0.175
3.5 month sera (rgp15)	0.061
9 month sera (rgp15)	0.085
24 month sera (rgp15)	0.045
Pre and post infection analysis	
rgp15	0.097
rChgp40 Ia	0.070
rCpgp40 II	0.086

when the corresponding serum sample was positive, but the preceding serum sample was negative. The seronegative (N)/seropositive (P) ratio at the different time points were calculated. N/P ratio of 1 suggests equal proportions of seronegative and seropositive individuals, whereas an N/P ratio of less than 1 suggests higher number of seropositive children at that particular time point. A change in N/P ratio from greater than 1 to less than 1 suggests the time-point at which majority of children seroconvert, which was considered to be a proxy measure of exposure to cryptosporidial infection.

The transitional probabilities of having a particular serological status at a certain time point given the probability of having similar or a different serological status at a previous time point was calculated by considering it to be a first order Markov chain process. A first order Markov chain is a stochastic process wherein the transitional probability of a given state at time t+1 depends only on the present state at time t, and not on the previous states. Thus, the probability (P) of transition from state $x(t)$ to $x(t+k)$ for any value of t is a conditional probability of (k-1) independent states and follows the multiplicative law of probability:

$$P(x_t, x_{t+1}, \ldots, x_{t+n}) = P(x_t)*P(x_{t+1}|x_t) \ldots P(x_{t+n}|x_{t+n-1}).$$

The transitional probabilities were computed to evaluate the pattern of seropositive and seronegative stages among the children, and to determine the most prevalent transition pathway in the study population.

As mentioned earlier, the antibody levels to rChgp40Ia, rCpgp40-II and rCpgp15 before and after an episode of cryptosporidial diarrhea in children were also determined using

cut off calculated based on the mean+2SD of negative control for the pre and post serum (Table 9.1). The difference in the pre-post diarrhea titer levels for each antigen was compared using Wilcoxon signed-rank test. Among children that showed a rise in titer, the geometric mean (95% CI) of rise in antibody level (pre- to post-infection) was calculated by log transforming the antibody levels and calculating the difference between the log transformed values. To avoid negative values following log transformation, a negligible constant "1" was added to the original antibody level prior to transformation, resulting in a logged value of "0" [ln(1)=0]. The log transformed antibody responses were also compared using a two-tailed *t*-test.

Results

A total of 129 children with and without cryptosporidial diarrhea were included in this study. Sociodemographic status, standards of hygiene, duration of exclusive breast feeding, birth weight and presence of animals in the household were compared between the 39 children with cryptosporidial diarrhea and 90 children with no cryptosporidial diarrhea (Table 9.2) and no significant differences were found indicating that the 2 groups were comparable. The other causes of diarrhea during the first 2 years of life among the children enrolled in the study are listed in table 9.3 and children with cryptosporidial diarrhea had significantly more giardial, rotaviral and *Aeromonas* infections associated with an episode of diarrhea.

Table 9.2: Comparison of children with and without cryptosporidial diarrhea

Variable	Children with cryptosporidial diarrhea (n = 39)	Children without cryptosporidial diarrhea (n = 90)	p-value
Sex: Male[**]	18	45	0.688
Low SES[**]	15	35	0.964
Head of Family Education:[**]			
No formal education	7	20	
Standards 1-5	12	26	
Standards 6 and above	20	44	0.860
Maternal Education:[**]			
No formal education	10	24	
Standards 1-5	15	25	
Standards 6 and above	14	41	0.448
Maternal age at birth (years): Mean (SD)[*]	24 (3.52)	24.8 (4.63)	0.311
Family size: Mean (SD) [*]	5 (1.54)	5 (1.91)	0.561
No. of siblings: Mean (SD) [*]	3 (1.10)	2 (1.16)	0.393
Low birth weight[†]	1	14	0.065
Duration of exclusive breastfeeding (months)[*]	2 (1.51)	2 (1.30)	0.204
Presence of animals in the household[**]	5	13	0.807
Child defecates inside house (in toilet)	33	79	0.626
Mother washes hands before feeding the child[**]	15	27	0.346

[*]Two-tailed t-test; [**]Chi-square test; [†]Fisher's exact test; [‡]Data not available for 2 children

Table 9.3: Other Causes of Diarrhea in children with and without cryptosporidial diarrhea (n=129)

Pathogen	**Children with cryptosporidial diarrhea (%) (n = 39)**	**Children without cryptosporidial diarrhea (%) (n = 90)**	**p-value (Fishers exact test)**
Norovirus	2 (5.13)	2 (2.22)	0.584
Sapovirus	2 (5.13)	1 (1.11)	0.217
EPEC	2 (5.13)	0	0.090
Aeromonas	12 (30.77)	6 (6.67)	0.001
Giardia	15 (38.46)	6 (6.67)	<0.001
Shigella	3 (7.69)	4 (4.4)	0.431
Vibrio	1 (2.56)	1 (1.11)	0.515
Rotavirus	21 (53.85)	26 (28.89)	0.009

IgG levels to gp15 and gp40 before and after the first episode of cryptosporidial diarrhea – When serum IgG responses to rgp15, rChgp40 Ia and rCpgp40 II were assessed in 39 children before and after the first episode of cryptosporidial diarrhea, a significantly greater proportion of children were seropositive after the episode of diarrhea for all 3 antigens tested, increasing from 76.9 %, 46.2%, 56.4% for rgp15, rChgp40 Ia and rCpgp40 II respectively in the pre-infection sera to 100%, 76.9% and 84.2% respectively in the post-infection sera (Figure 9.1). The overall rise in titer (OD) when all 39 cases were considered was highly significant for all 3 proteins (Wilcoxon signed rank test, $p<0.001$) (Figure 9.2). For rCpgp15, all the 39 children showed an increase in levels of IgG post infection (geometric mean with 95% CI: 1.4, 1.29-1.51), 33 cases showed an increase in IgG levels to rChgp40 Ia and 31 to rCpgp40 II post infection (geometric mean with 95% CI, 1.15, 1.09-1.22 and 1.15, 1.09-1.21 respectively).

Since we had previously found that *Cpgp40/15* subgenotype Ia predominated in this area, we further analyzed the data to look for subtype specific responses. Twenty three children who had diarrhea associated with *C. hominis* Ia infection were included, among whom 19 children were seropositive post infection to Cpgp40 Ia and Cpgp40 II antigen. The rise in titer in OD values from all 23 cases was comparable for both to Cpgp40 Ia and Cpgp40 II ($p=0.36$, Wilcoxon signed rank test). Even when only cases demonstrating a rise in titer were compared (18 for rChgp40 Ia and 17 for rCpgp40 II) they were found to have comparable geometric mean titers.

Figure 9.1 Proportion of children with detectible antibody titers before and after the episode of cryptosporidial diarrhea (n=39)

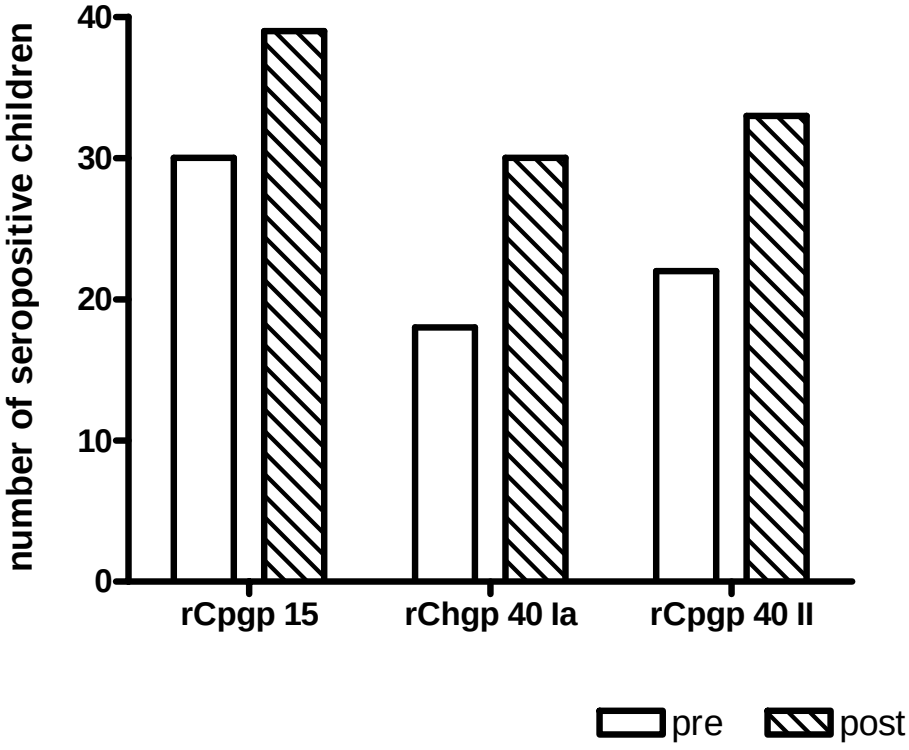
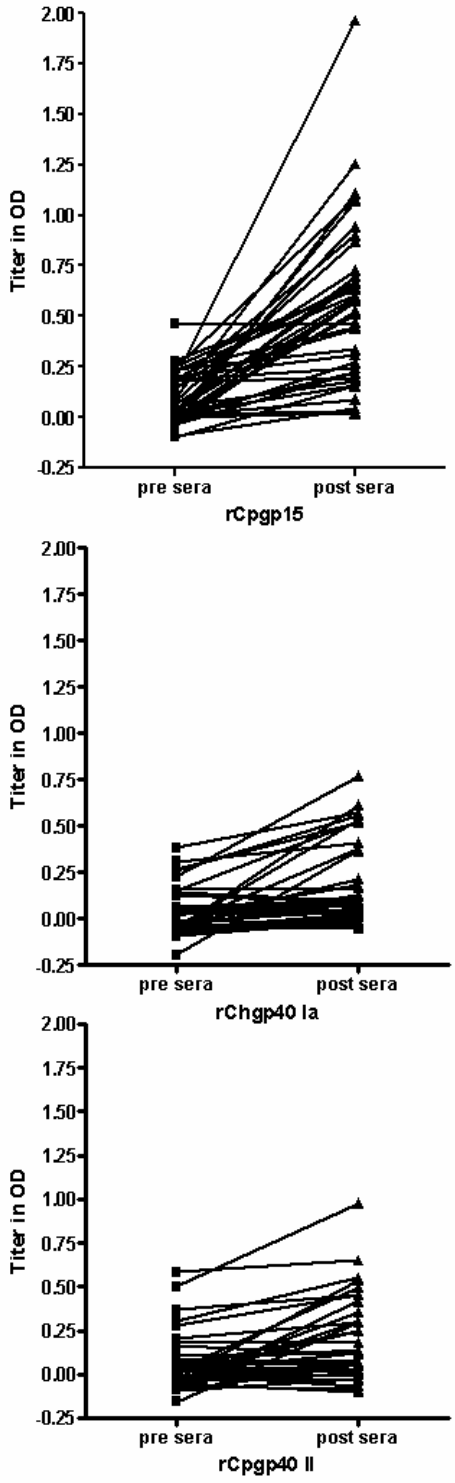


Figure 9.2 Serum IgG response to cryptosporidial antigens in children before and after an episode of diarrhea (n=39)



There was a significantly lower duration of diarrhea in children (median, IQR, 3, 2-4.5 days) whose pre-infection sera was positive for anti-rgp15 IgG than children who were not (6, 4-8 days) (Mann-Whitney U test, p=0.02). Presence or absence of anti-Cpgp40 Ia and Cpgp40 II IgG in pre-infection sera, however, did not have any effect on duration of diarrhea (Mann Whitney U test, p=1.00 & p=0.96 respectively). There was also no effect of anti-Cpgp15, Cpgp40 Ia and Cpgp40 II (Fisher's exact test, p=0.691, p=1.00 & p=1.00 respectively) IgG on severity of diarrhea when the maximum number of stools per 24 hour period were compared between children whose pre-infection sera was seropositive and seronegative.

Maternal antibody levels and longitudinal serum IgG responses to gp15 in children with and without cryptosporidial diarrhea - Out of a total of 129 maternal serum samples analyzed, 93 (72.1%) were seropositive. Only 43 (33.3%) of the 129 children were seropositive at the age of 3.5 months. However, by 24 months of age almost all children (127, 98.5 %) had seroconverted. In order to ascertain the time point at which the majority of children seroconvert, we calculated the N/P ratios at different time points. We found that although most children were seronegative at age 3.5 months (N/P ratio = 2), a large proportion of children seroconverted by 9 months (N/P ratio = 0.67) and almost all children were seropositive at 24 months of age (N/P ratio = 0.02).

When children with cryptosporidial diarrhea alone were considered, 24 (61.5%) of the maternal samples were seropositive. On the other hand, among the mothers of children with no cryptosporidial diarrhea a higher proportion were seropositive (69, 76.7 %)

(p=0.079). However, when the maternal antibody levels to gp15 were compared, mothers of the children with cryptosporidial diarrhea had significantly higher OD values than mothers of children without cryptosporidial diarrhea (p=0.004) (Table 9.4). At 3.5 months, children with no cryptosporidial diarrhea (33, 36.7%) were also more likely to be seropositive than children with cryptosporidial diarrhea (10, 25.6%) but this difference was not statistically significant (Mann Whitney U test, p=0.22). At 9 and 24 months, a similar proportion of children with and without cryptosporidial diarrhea were seropositive (p=0.2 and 0.5 respectively) (Table 9.4). When the N/P ratio at 9 months was analyzed separately for children with and without cryptosporidial diarrhea, seroconversion occurred earlier in children without cryptosporidial diarrhea (N/P ratio = 0.58) than in children with cryptosporidial diarrhea (N/P ratio = 0.95). However, among the seropositive children, at 9 months, children with and without cryptosporidial diarrhea had similar anti-gp15 levels (Mann Whitney U test, p=0.12); but by 24 months, the anti-gp15 levels of the children without cryptosporidial diarrhea were significantly higher (Mann Whitney U test, p=0.02) (Table 9.4).

Role of maternal antibodies in seroconversion status - The transitional probabilities between seropositive and seronegative status was calculated for children of seropositive (Figure 9.4a) and seronegative mothers (Figure 9.4b) separately to identify the role of maternal antibodies on seroconversion status of the child. The transitional probability for a particular node was estimated as the proportion of the number of individuals at a particular node to the number of individuals at the previous node. In order to estimate the

Table 9.4: Proportion of seropositive individuals and the distribution of antibody titres at different time points for children with (n=39) and without (n=90) cryptosporidial diarrhea

gp15 titers	Children with cryptosporidial diarrhea		Children without cryptosporidial diarrhea		*P*-value*†
	No. Pos (%)	Median (IQR) of OD values†	No. Pos (%)	Median (IQR) of OD values†	
Maternal	24 (61.5)	0.19 (0.18)	69 (76.6)	0.09 (0.14)	0.004
3.5 months	10 (25.6)	0.04 (0.07)	33 (36.7)	0.07 (0.12)	0.42
9 months	20 (51.3)	0.12 (0.2)	57 (63.3)	0.07 (0.1)	0.12
24 months	38 (97.4)	0.13 (0.17)	89 (98.9)	0.2 (0.25)	0.02

* Wilcoxon rank sum test

† Seropositive children only

commonest pathway, we computed a joint probability ("P") by multiplying the probabilities at a particular transitional node with that of the previous node. The pathway with the highest joint probability value at the end node was considered to be the most probable.

The commonest pathways for children of seropositive mothers were a seronegative child at 3.5 months who then seroconverted at 9 months and remained seropositive at 24 months ("P"=0.4). Children of seronegative mothers on the other hand tended to remain either seronegative until 9 months and seroconverted only by 24 months ("P"=0.3) or seroconverted by 9 months and remained seropositive at 24 months ("P"=0.39). When N/P ratios were analyzed, children of seropositive women seroconverted earlier (0.58 at 9 months) than children of seronegative women (1.0).

Figure 9.4 Transitional probabilities of serological status at different time points for (A) children of seropositive mothers

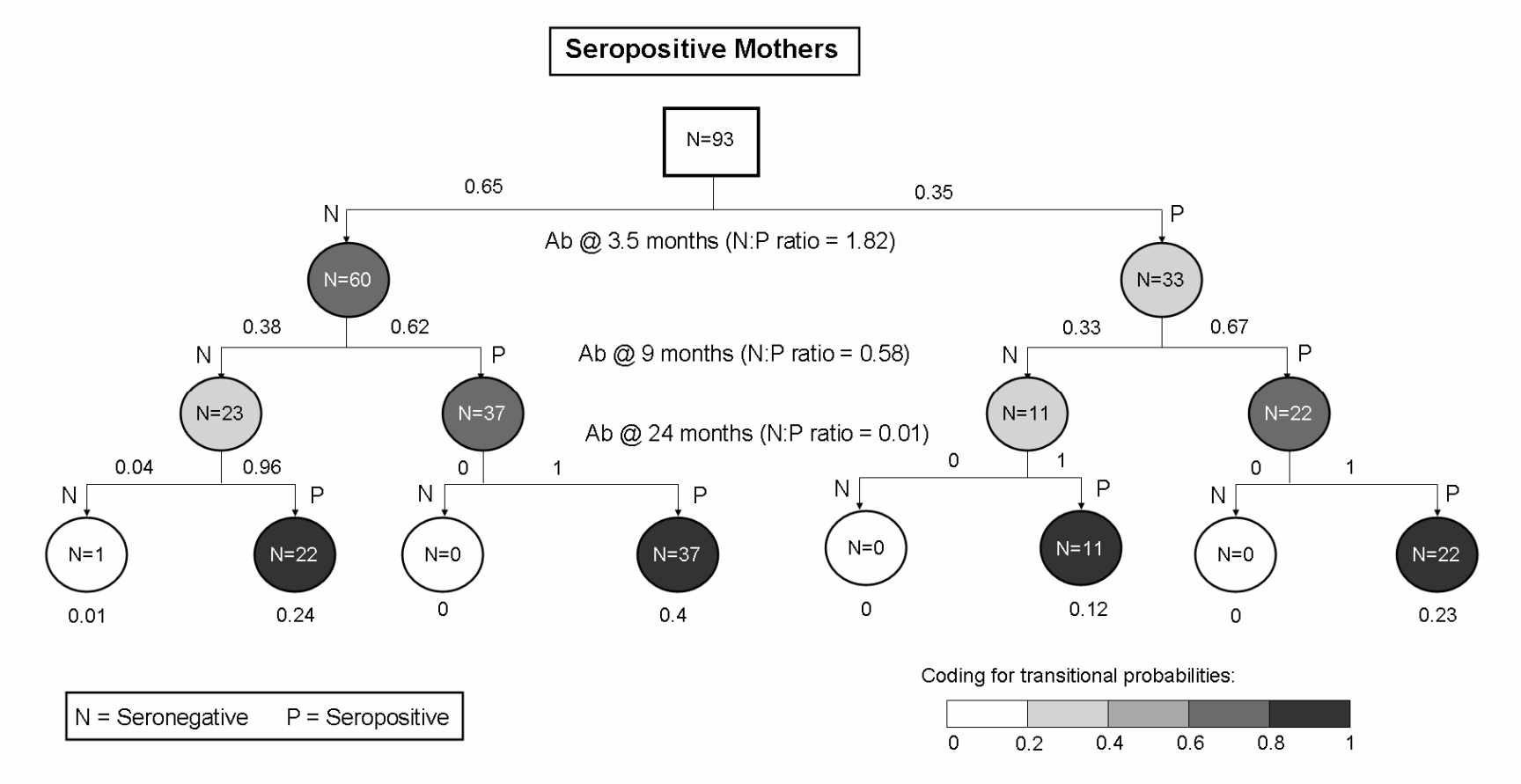
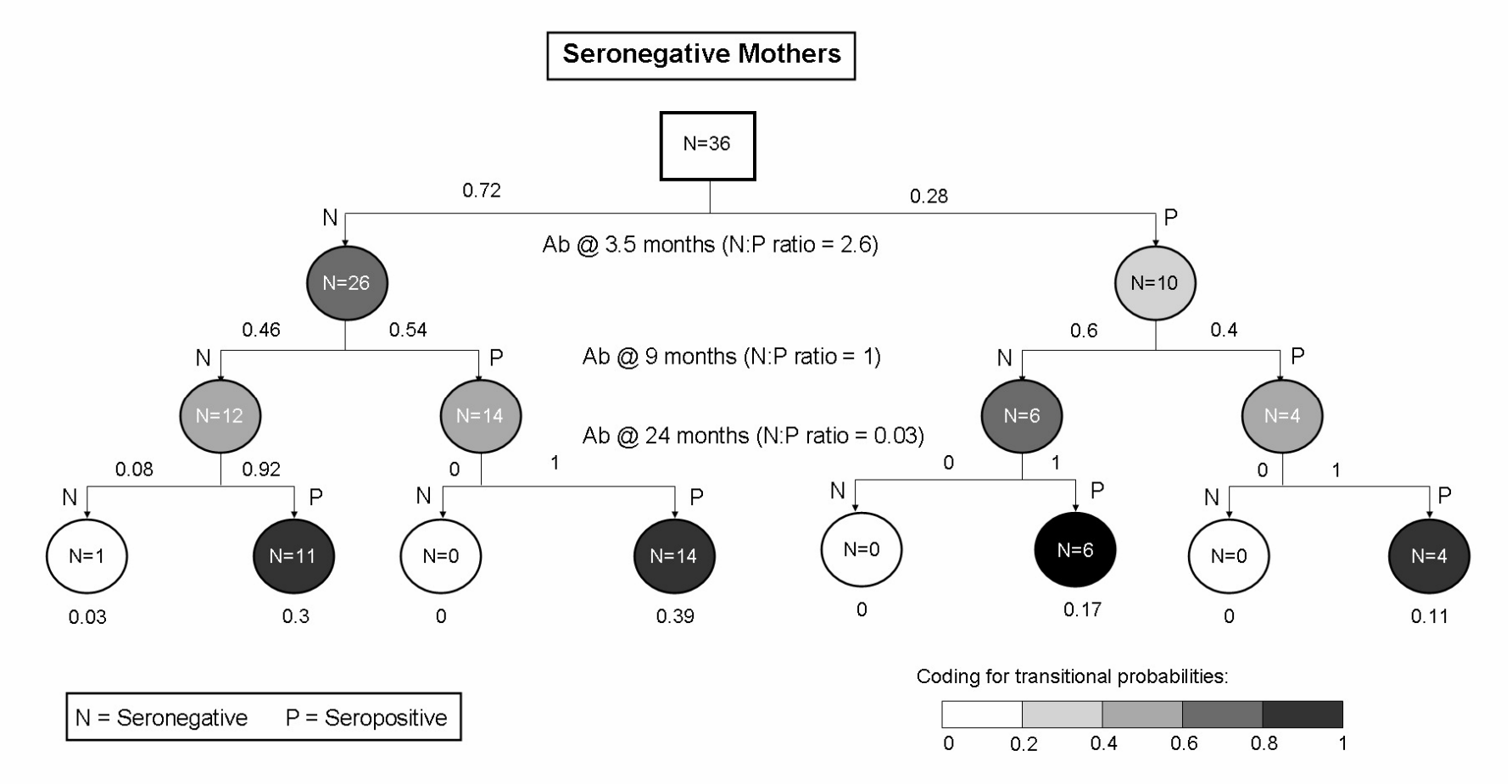


Figure 9.4 Transitional probabilities of serological status at different time points for (B): children of seronegative mothers



*The numbers at bottom represent joint probabilities for that particular end node

Discussion

This is the first study to provide an insight into humoral immune responses to immunodominant cryptosporidial antigens in Indian children. In this study, we have demonstrated a protective effect conferred by presence of maternal gp15 antibodies, the presence or absence of which influenced early seroconversion in their children. Children who had no cryptosporidial diarrhea seroconverted earlier than children with cryptosporidial diarrhea suggesting that they may have had asymptomatic or sub clinical infections that had a boosting effect on IgG levels. When seroconversion was used as a proxy measure of infection, all children appeared to have been exposed to the pathogen by the age of 2 years and for a child, the highest risk of exposure to *Cryptosporidium spp*. occurred between 3.5 to 24 months of age in our setting.

There have been very few studies on immune response to cryptosporidiosis in children in developing countries. Findings similar findings to ours have also been reported from a longitudinal study measuring *Cryptosporidium*-specific serum antibody levels in Israeli Bedouin children. In the study from Israel, *Cryptosporidium*-specific serum IgG levels in a cohort of Bedouin children were high soon after birth, dropped by 6 months of age and then continuously increased until 23 months of age with no differences detected between asymptomatic and symptomatic infections (196). A birth cohort study in Peru showed that serum IgG antibody levels to partially purified native Cp17 (same as gp15) and a recombinant 27kDa (also called Cp23) antigen increased with age, but differed from the Israeli study in that children with asymptomatic infections had higher antibody levels than those with symptomatic infections (188). The present study did not study serological

responses in asymptomatic infections but found that children without any documented cryptosporidial diarrhea had elevated antibody levels by 24 months, which is in agreement with a study in Peruvian children. Children with pre-existing antibodies to gp15 also had a shorter duration of diarrhea indicating a protective effect. This has been described previously in volunteer studies on healthy adults where those with pre-existing antibodies shed fewer oocysts and also tended to be asymptomatic (153) and also required higher oocyst doses to develop symptoms(43). Breast-feeding has been reported to be protective in some studies in developing countries (27, 119, 145) and not protective in others (45, 164, 182), including one study which found that *Cryptosporidium spp*. infection was actually more common in breast-fed children (163). There was no significant difference among the children in this study (Table 9.2). This study focused on longitudinal immune response to the cryptosporidial glycoprotein antigen gp15 as an antibody response to this protein has consistently been found in symptomatic infections and has been associated with protective immunity in several studies. Further more, since this conserved glycoprotein in association with the more polymorphic gp40, is actively involved in the process of attachment and invasion of sporozoites, it makes an attractive vaccine candidate (238).

This study has also documented immune response to subtypes of gp40 in children. *In vitro* studies on gp40 have shown some evidence of poor cross-reactivity between Chgp40 Ia and Cpgp40 II with antibodies to rChgp40 Ia reacting only with lysates from *C. hominis* but not *C. parvum* and antibodies to purified native *C. parvum* gp40 showing

weak reactivity with *C. hominis* isolates. However, in this study a similar proportion of children with *C. hominis* Ia diarrhea showed a rise in titer to the homotypic rChgp40 Ia antigen and to the rCpgp 40 II antigen. Because gp40 is a putative adhesion molecule (39), it may serve as a target for preventive or interventional modalities. Recent studies have demonstrated proliferative responses to recombinant gp40 in mesenteric lymph node cells of *C. parvum*-infected mice and IFN γ production in response to *C. hominis* gp15 in adult human CD4 and CD8 cells, indicating that these proteins induce cellular immune responses (214).

This study took advantage of an existing birth cohort on diarrheal disease, and incorporated molecular and epidemiological techniques to study immune response to cryptosporidial antigens over time among children in a developing country where *Cryptosporidium spp*. is endemic. A novel statistical approach involving probabilistic pathways was used to explore the immune response to *Cryptosporidium spp*. and seroconversion over time. This approach was very different from previous studies with threshold/baseline values defined based on standard deviations of the mean or seroconversion based on four-fold rise in titer (196, 220) or difference of 10% relative to the positive control (68). An important assumption in this study was that the rate of antibody transfer was uniform for all children, i.e. children with high maternal antibody levels had higher antibody levels at birth. A study by Zu et al in a rural community in China and Brazil found a good correlation in which antigen specific IgG levels between paired serum specimens from 30 matched mother-neonates showed transplacental transfer of IgG (254). However, transfer of antibodies is influenced by factors like

prematurity and gestational age, as the majority of the antibody transfer occurs in the third trimester. Data on cord-blood samples could have provided more robust estimates.

Our findings suggest that anti gp15 antibody production is associated with protection from cryptosporidial diarrhea in children. However, further studies are required to determine whether antibody responses are themselves protective or whether they are markers of cell mediated immunity. The potential of using epitopes on gp15 and gp40 as vaccine candidates also needs to be evaluated.

- **CHAPTER 10**

MOLECULAR EPIDEMIOLOGY OF AND IMMUNE RESPONSE TO CRYPTOSPORIDIAL INFECTION IN HIV INFECTED ADULTS AND QUANTITATION OF OOCYSTS IN PATIENTS WITH AND WITHOUT DIARRHEA

Introduction

Cryptosporidium and other pathogens like *Isospora belli*, *Microsporidium spp*., *Mycobacterium avium intracellulare* and *Clostridium difficile* have been documented to commonly cause diarrhea in HIV infected patients. Diarrhea is the most common gastrointestinal symptom reported in HIV infected patients with the incidence increasing when CD_4 counts drop below 200 cells/cu.mm (162).

In India, recent sentinel surveillance estimated that there were around 2.47 million people living with HIV/AIDS (PLHA) and the four states of Andhra Pradesh, Maharashtra, Tamil nadu and Karnataka contributed to 63% of all reported cases (157). The prevalence of symptomatic cryptosporidiosis in HIV infected adults in the country ranges from as low as 0.7% to 81%. Most of these studies were carried out on HIV infected adult patients and employed modified acid fast staining of concentrated stool samples. A few recent studies have also carried out ELISA and PCR for detection (102, 116). Patients can have chronic watery diarrhea that can last for more than 2 months and shed oocysts in stool during the entire period, resulting in severe dehydration, weight loss and malnutrition, extended hospitalizations, and mortality (3, 98). AIDS patients with cryptosporidiosis also have a significantly shorter duration of survival from the time of diagnosis (138).

There have been two previous studies on the molecular epidemiology of cryptosporidiosis HIV infected adults (117), including one from Vellore (156) but other than a study from Chandigarh (117) there have been no previous studies on immune

response to cryptosporidiosis in HIV infected individuals in India. This study from Chandigarh used a crude antigen preparation to document the cytokine response. In this study, we used 2 well characterized immunodominant antigens, gp15 and Cp23 to study immune response in patients with cryptosporidial diarrhea and asymptomatic infections. Studies on the immune response and genotyping will lead to identification of the correlates of protective immunity in this population.

Materials and methods

Study population - HIV-infected adults who presented to the outpatient unit or who were admitted to the Department of Medicine Unit –I at Christian Medical Hospital, Vellore were enrolled in this study after obtaining informed consent. A stool sample and 5 ml of blood was collected at enrollment. CD_4 counts were carried out on most patients if no CD4 count was available within 3 months prior to enrollment. Details on the current diarrheal episodes as well as any other co-infection, history of diarrhea and anti retroviral treatment were collected. Data on concurrent opportunistic infections was also collected by reviewing case records. All stool samples were screened for *Cryptosporidium* by PCR and serum samples were tested for antibody responses. Diarrhea was defined as three or more stools per day for at least 72 hours. The study protocol, questionnaires and consent forms had been approved by the institutional review board.

DNA extraction, PCR RFLP and sequencing – DNA extracted from the positive stool samples using the QIAamp DNA stool kit (Qiagen Inc, Valencia, CA) was then analyzed by PCR-RFLP at the small-subunit *SSU rRNA* locus (246) and PCR-RFLP at the

Cpgp40/15 locus (Cohen, 2006) using previously described protocols. In samples where ambiguous results were obtained by PCR-RFLP at the *Cpgp 40/15* locus, purified PCR products were sequenced and analyzed as mentioned previously.

Real time PCR – Real time PCR for quantification of parasite burden was performed using ABI 7500 cycler (Applied Biosystems, Foster city, CA) using primers designed to amplify a 114 bp fragment from the conserved gp15 region of the *Cpgp40/15* gene mentioned in chapter 8. Purified plasmid DNA containing the 1 Kb sequence of the *Cpgp40/15* gene from the *C. parvum* IOWA strain cloned into the *T. gondii* expression vector pHLEM (171) was used for generating the standard curve.

Real time PCR was also carried out using another pair of primers based on the 18S rRNA locus using previously described primers with an amplicon size of 212 bp (32). For this assay, genomic DNA extracted from *C. parvum* (IOWA strain) oocysts was diluted to obtain serial 10 fold dilutions and used to generate the standard curve. Patients with cryptosporidiosis, with and without diarrhea were compared. Ct values greater than 37 were considered as negative. Each sample was run in triplicate for both primers. Negative controls with no template DNA were included for each reaction series.

ELISA- The protocol for ELISA was similar to that described in chapter 9. Serum samples from all patients were stored in aliquots at -80°C. Serum IgG levels to rgp15 (along with the His tag protein) and Cp23 were quantified by ELISA as previously described in chapter 9 (118). The same positive and negative control sera (sera which

were positive or negative for *C. parvum* rCpgp15 by ELISA and Western blot analysis) were run on each plate to control for plate to plate variation. All samples were run in triplicate and the mean A_{405}nm determined (118). For each sample tested for rgp15, the final A_{405}nm value was calculated by subtracting the A_{405}nm in response to the 'control' protein. Cut-offs were generated for each protein tested by calculating the mean+2SD of the negative control for patients with and without diarrhea and applying the higher cut off value to the data set. A particular sample was considered to be seropositive if there was a detectible antibody level to rCpgp15 or Cp23 above the cut-off determined (expressed in OD) and seronegative if below the cut-off level.

CD_4 counts were carried out by flow cytometry using the Guava Technologies PCA instrument with Easy CD4 software in the Department of Clinical Virology.

Statistical analysis: Data was analyzed using STATA 10.1 for Windows (StataCorp LP, College Station, TX, USA). For the analysis of CD_4 counts and proportion of seropositive cases, Fishers exact test was used and comparison of antibody titers was carried out using Mann Whitney U test.

Results

A total of 87 HIV infected adults with diarrhea and 81 without diarrhea were recruited at the infectious disease clinic between October 2007 and February 2008. They had been diagnosed at a median (IQR) of 1.29 (0.25-3.44) years prior to enrollment. Twenty two cases with diarrhea and 27 without diarrhea were on HAART. Thirteen patients with

cryptosporidial diarrhea and five with asymptomatic cryptosporidial infections were detected by PCR. Among the 18 positive cases, only 10 were detected by microscopy. The median (IQR) age of patients with cryptosporidial diarrhea was 36 years (32-45) years and only 2 of them were on HAART. Other opportunistic infections were recorded in 67 of all patients enrolled and the other enteric parasites detected are given in Table 10.1. 88 cases had CD_4 counts above 200, 24 had counts between 100-200 and 32 had counts below 100. When CD_4 counts were compared between patients with (median, IQR, 196, 84-383) and without diarrhea (349, 188-479), there was a significant difference ($P<0.001$). There was also a significant difference in CD_4 counts between patients with (84, 37-154) and without (310, 135-462) cryptosporidiosis ($p<0.001$) (Figure 10.1). Among cases with cryptosporidial diarrhea, 92% had CD_4 counts below 200.

Species and genotypes – Among the cryptosporidial cases with diarrhea, 8 were infected with *C. hominis*, 2 with *C. parvum* and 1 patient was co-infected with both species. In addition, 1 case with *C. felis* and one with *C. meleagridis* infection was seen. All 5 asymptomatic cases were infected with *C. hominis*. When *Cpgp40/15* PCR RFLP was carried out to determine subgenotypes, 9/13 were typed. Among *C. hominis* isolates two isolates were If, 2 were Ib and one was Ia. The *C. parvum* isolates were IIc and IIm. The IIm isolate was identified by sequencing and phylogenetic analysis as described in chapter 8 (Figure 10.2). All but 1 asymptomatic *C. hominis* infection were identified as subgenotype Ie and the remaining isolate was Ia subgenotype. Details on the 18 *Cryptosporidium* positive cases are given in table 10.2.

Figure 10.1: CD_4 Counts in Enrolled Cases

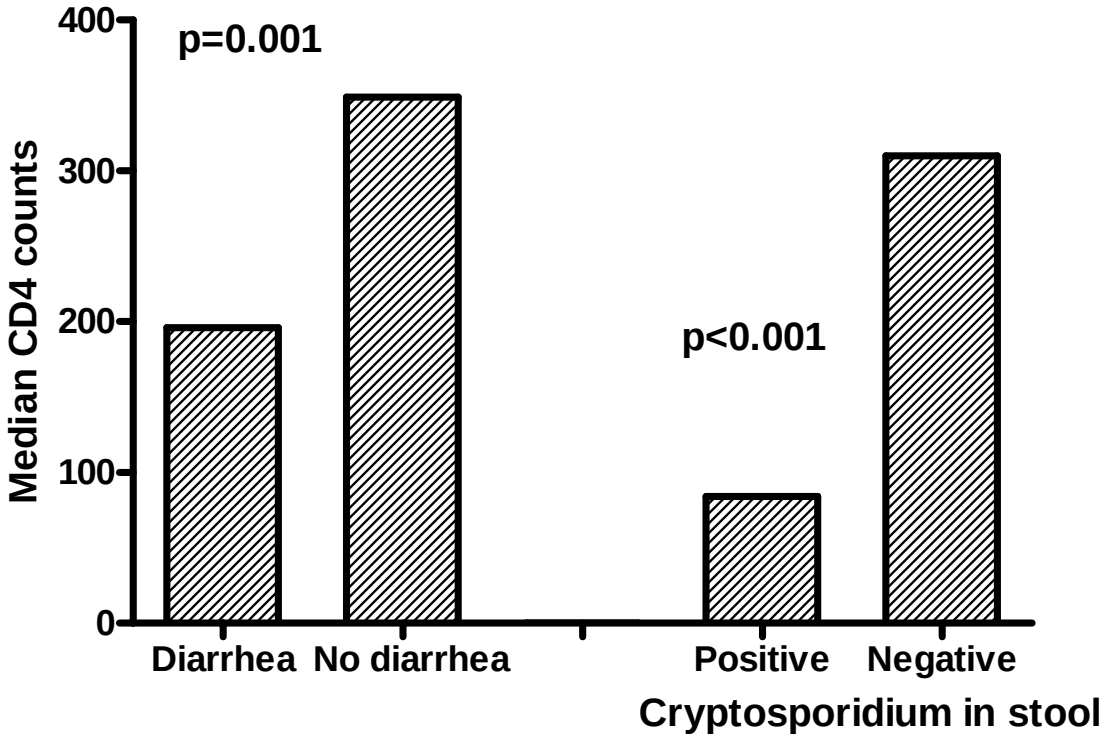


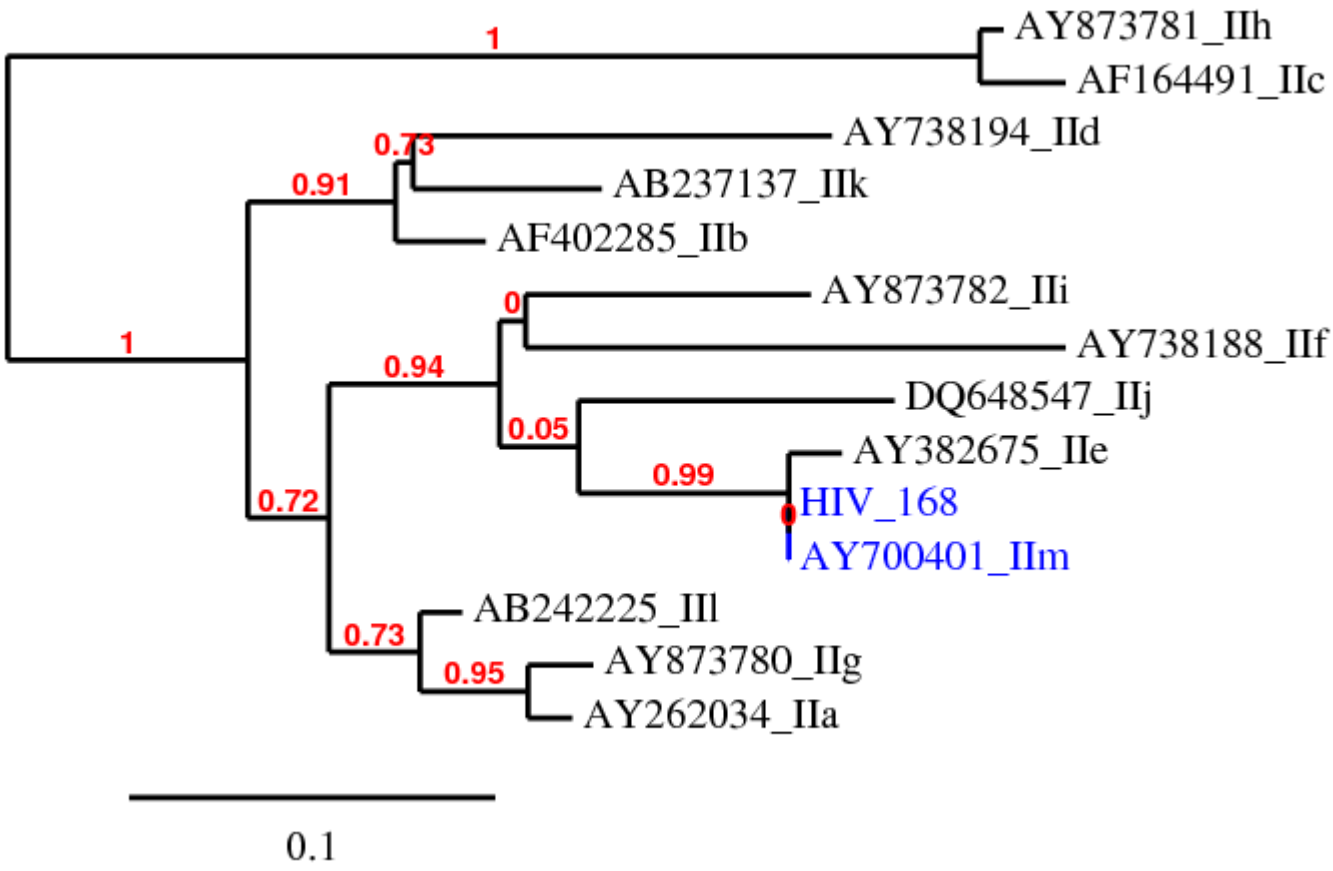
Table 10.1: Enteric Parasites Detected by Microscopy

	Diarrhea present	**Diarrhea absent**
Cryptosporidium	7	3
Giardia	1	2
Isospora	2	1
Hookworm	3	3
Strongyloides	5	-
Pinworm	1	-
H. nana	-	1

Table 10.2: HIV Infected Adults with Symptomatic and Asymptomatic Cryptosporidiosis

Patient number	Age	Sex	Microscopy	Species	Cpgp40/15 subgenotype	CD4 count	HAART	Opportunistic co-infections
Asymptomatic								
32	28	f	Positive	*C. hominis*	Ie	93	-	Oral candidiasis, furunculosis
85	32	m	positive	*C. hominis*	Ia		y	Tuberculosis, Candidiasis
165	53	f	Positive	*C. hominis*	Ie	30	n	n
58	27	f	Negative	*C. hominis*	Ie	244	-	
79	25	f	Negative	*C. hominis*	Ie	57	n	n
Symptomatic								
20	36	f	Positive	*C. parvum*	II c	228	n	n
99	37	m	Positive	*C. hominis*	If	143	n	CMV retinitis
128	45	m	Positive	*C. hominis*	Ib	84	n	Strongyloides, Isospora
131	32	m	Positive	*C. hominis* and *C. parvum*	Id and IIc	12	n	Cryptococci, Candidiasis
160	31	m	Positive	*C. felis*	-	42	-	-
168	35	m	Positive	*C. hominis*	Ib	166	n	n
170	45	m	Positive	*C. hominis*	Ie	37	n	Tuberculosis, Candidiasis
59	43	m	Negative	*C. hominis*	Ia	191	y	Tuberculosis, HSV
113	34	f	Negative	*C. meleagridis*	-	31	-	-
118	36	m	Negative	*C. hominis*	-	114	n	Tuberculosis
142	57	m	Negative	*C. hominis*	If	154	n	Tuberculosis, Candidiasis
166	32	m	Negative	*C. hominis*	-	70	n	HBV
168	50	m	Negative	*C. parvum*	IIm	25	n	Tuberculosis, Candidiasis, Syphilis

Figure 10.2: Phylogenetic analysis of HIV Isolate 168 *C. parvum* Cpgp40/15 Subgenotype IIm



Real time PCR for quantification of *Cryptosporidium* oocysts – Real time PCR was carried out to quantitate the copy number of *Cpgp40/15* gene in samples from patients with and without cryptosporidial diarrhea. Serial 10 fold dilutions of plasmid DNA in 6 replicate runs which gave consistent and evenly spaced C_t values ranging from 15 to 32 (chapter 8, Figure 8.8) over 6 orders of magnitude with a correlation coefficient of 0.99. The limit of detection was 10 copies of the *Cpgp40/15* gene (Figure 8.8). Real-time PCR amplified DNA from *C. hominis*, *C. parvum* and *C. felis* samples and none from *E. histolytica*, *Giardia spp*., *Cyclospora spp*. or *Isospora spp*. containing stool samples thus demonstrating specificity for *Cryptosporidium spp* (Figure 8.9).

The 18S rRNA real time PCR was standardized using serial 10 fold dilutions of genomic DNA and was confirmed on gel electrophoresis (Figure 10.3) and also gave consistent results with Ct values ranging from 18 to 32 with a correlation coefficient of 0.99 (Figure 10.4). When Ct values were compared between cases with (27.82) and without diarrhea (27.03) using the 18S rRNA primers, there was no significant difference in median Ct values. However for the gp15 primers, cases with cryptosporidial diarrhea (32.87) had a higher median Ct value than patients without cryptosporidiosis (27.9). The Ct values for the 2 primers for each of the samples tested are illustrated in Figure 10.5. These differences in Ct could be attributed to difference in copy number of each gene locus.

Figure 10.3: Gel Electrophoresis of Genomic DNA Standards used in 18S rRNA Real Time PCR

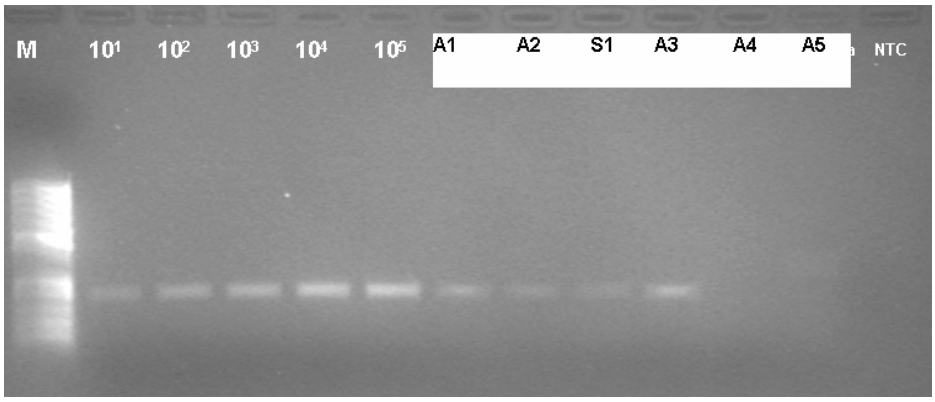


Figure 10.4: Standard Curve for 18S rRNA Real Time PCR using Genomic DNA

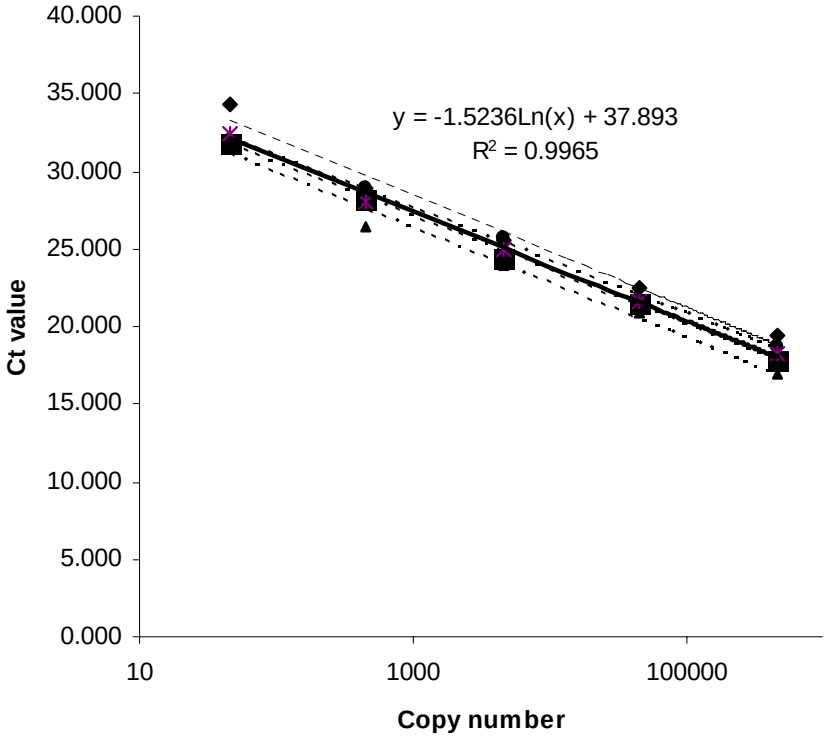
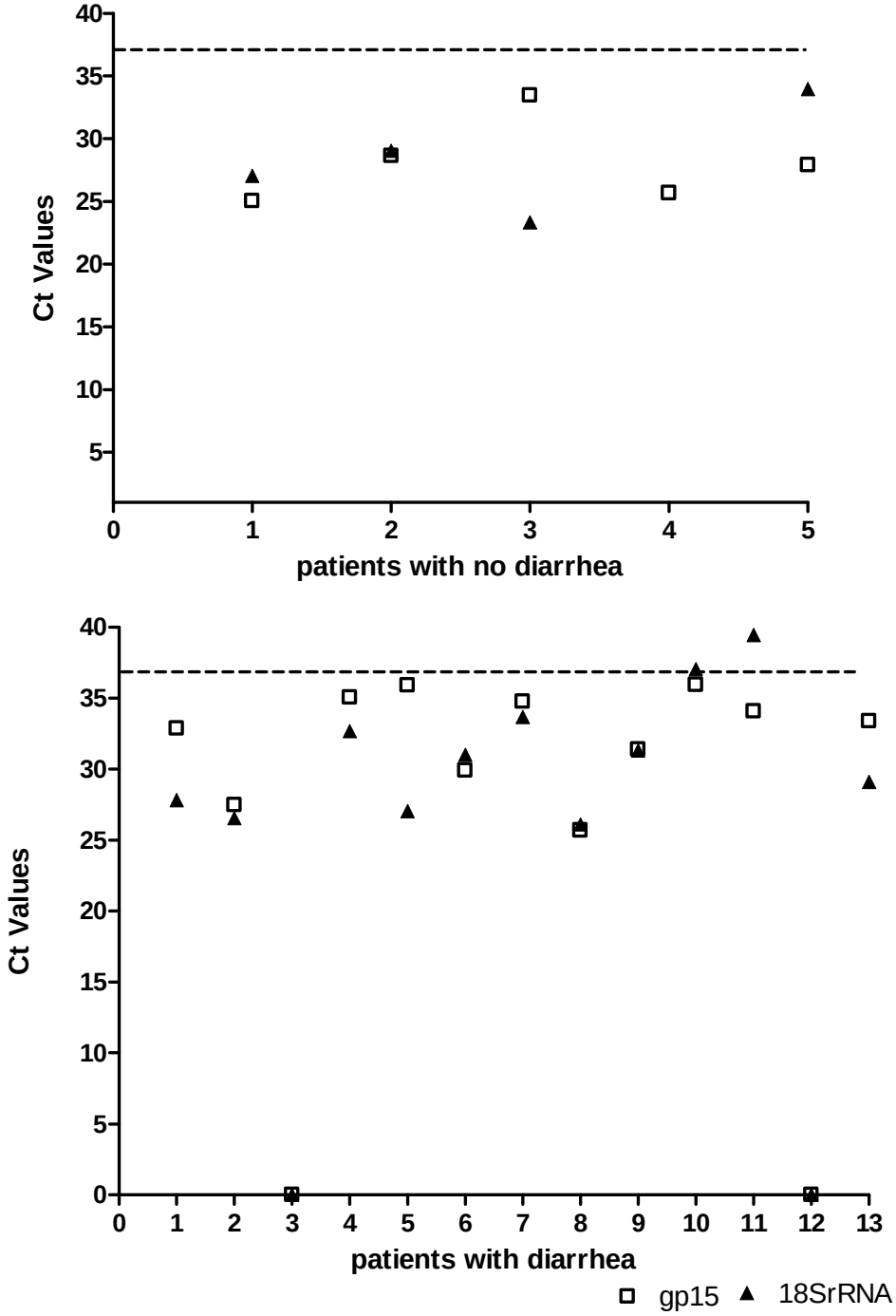


Figure 10.5: Comparison of Ct Values for Patients with and without Cryptosporidial Diarrhea



Anti gp15 and Cp23 IgG antibodies – IgG antibody titres to the 2 immunodominant antigens gp15 and Cp23 were determined in all enrolled patients. The cut off values determined by mean+2SD of the negative control sera were 0.053 and 0.083 respectively for gp15 and Cp23. Any serum sample with an OD value above this cut off was considered seropositive. Among the cases with diarrhea, 80/87 were seropositive for gp15 and 86/87 were seropositive for Cp23. Among cases without diarrhea, 77/81 and 80/81 were seropositive for gp15 and Cp23 respectively. There was no significant difference in proportion of seropositive cases between *Cryptosporidium* positive (18/18 for both antigens tested) and *Cryptosporidium* negative (139/150 and 148/150 for gp15 and Cp23 respectively) cases. However, when the median OD value between these 2 groups was compared there was a significant difference for both antigens (Table 10.3, Figure 10.6 and 10.7). This difference in OD values persisted when only cases with diarrhea were analysed (table 10.3). But no significant difference in OD values was seen when patients without diarrhea were analysed (table 10.3). When *Cryptosporidium* positive cases alone were analysed, all of whom were seropositive to both antigens as mentioned above, the median OD of cases with diarrhea was higher than cases without diarrhea but this difference was not significant (Table 10.3).

Among the 154 cases for whom a CD_4 count was available, there was no difference in proportion of seropositive cases with counts above or below 200 for both antigens tested.

Figure 10.6: Anti gp15 IgG Antibodies in Cases with and without Cryptosporidiosis

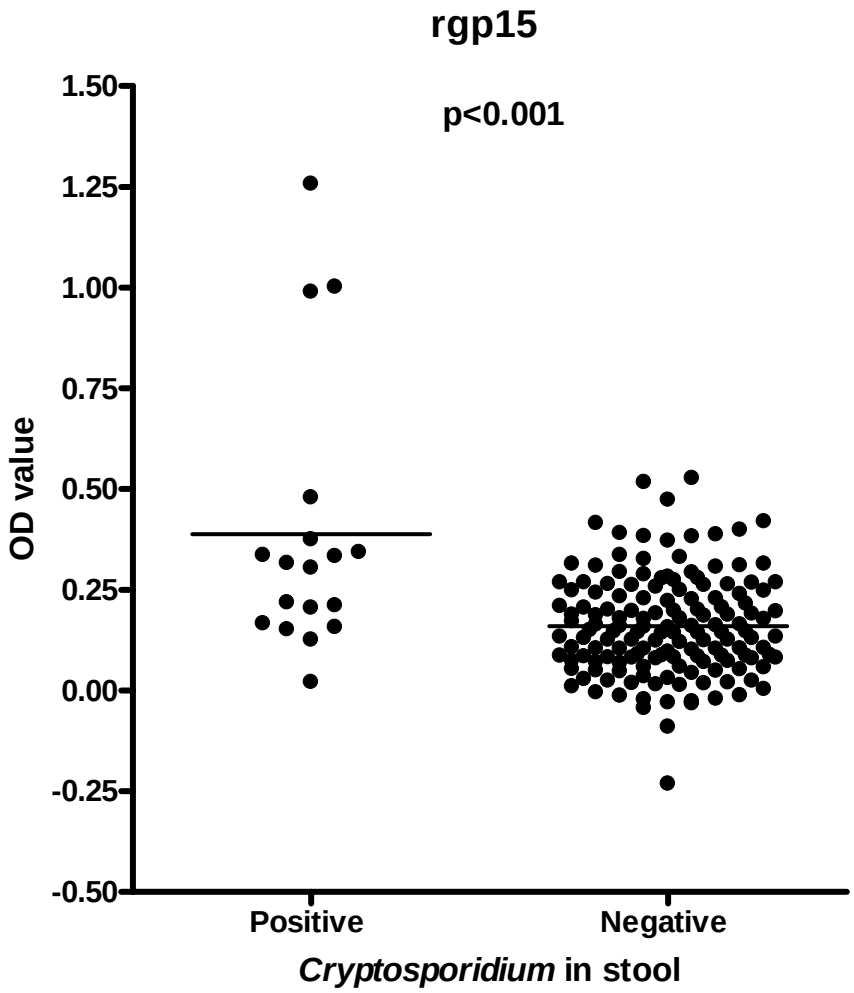


Figure 10.7: Anti Cp23 IgG Antibodies in Cases with and without Cryptosporidiosis

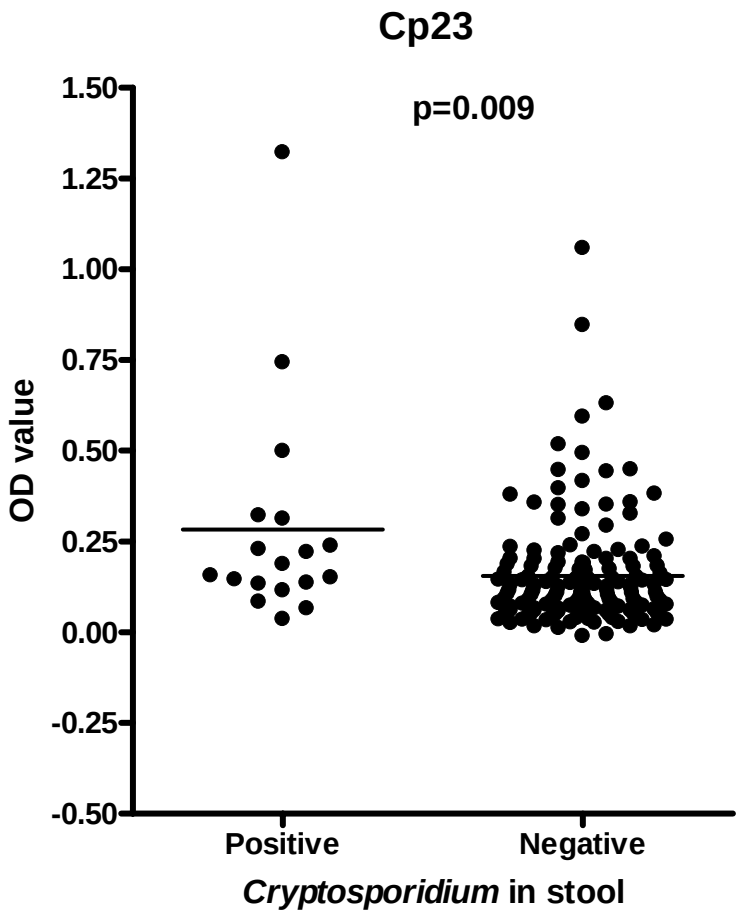


Table 10.3: Median OD levels of anti gp15 and Cp23 antibodies

All cases enrolled	**n**	**median OD for gp15**	**p(test)**	**median OD for Cp23**	**p(test)**
Cryptosporidium positive	18	0.310 (0.166 - 0.375)		0.171 (0.133 - 0.312)	
Cryptosporidium negative	150	0.145 (0.079 - 0.247)	<0.001	0.110 (0.058 - 0.189)	0.009
Cases with diarrhea					
Cryptosporidium positive	13	0.316 (0.205 - 0.343)		0.220 (0.115 - 0.312)	
Cryptosporidium negative	74	0.153 (0.070 - 0.257)	0.002	0.120 (0.062 - 0.192)	0.054
Cases with no diarrhea					
Cryptosporidium positive	5	0.218 (0.151 - 0.375)		0.151 (0.136 - 0.156)	
Cryptosporidium negative	76	0.138 (0.085 - 0.236)	0.095	0.109 (0.054 - 0.186)	0.095
Cases with cryptosporidiosis					
With diarrhea	13	0.316 (0.205 - 0.343)		0.220 (0.115 - 0.312)	
No diarrhea	5	0.218 (0.151 - 0.375)	0.657	0.151 (0.136 - 0.156)	0.805

Discussion - Cryptosporidiosis remains an important cause of diarrhea in the immunocompromised due to the lack of effective therapy. Although cryptosporidiosis in HIV infected patients has been widely reported in India (5, 11, 21, 124, 144, 155, 156, 199, 209), very little is known about circulation and transmission patterns of the infection in this part of the world. In this study, the prevalence of cryptosporidial diarrhea was 15% and asymptomatic infections occurred in 6% determined by a screening PCR. A previous study from the same center had reported a higher prevalence of 25% in diarrhea even though a less sensitive tool like microscopy was used. This decrease in prevalence could be attributed to several causes, including the increasing accessibility of HAART for Indian patients. Nearly 30% of cases enrolled in this study had some history of antiretroviral therapy. Among the other coccidian parasites, *Isospora belli* has also been reported frequently with prevalence rates ranging from 2.5 to 60% in patients with diarrhea. Most recent studies in India, including this study shows much lower prevalence rates than *Cryptosporidium* (probably due to prophylactic treatment with trimethoprim-suphamethoxazole)(17, 61, 116, 156, 191).

When CD_4 counts were examined, a greater number of patients with diarrhea and cryptosporidial infections had counts below 200 cells per cu. mm. More than 90% of cases identified to have cryptosporidial diarrhea had CD_4 counts below 200. Several studies in India have also documented mean CD_4 counts in these patients with most studies showing that symptomatic cases had CD_4 counts < 200 cell/mm^3 and asymptomatic cases had CD_4 counts > 300 cell/mm^3 reinforcing the importance of CD_4 T cells in mediating resistance to this pathogen (17, 61, 80, 156). A study from Delhi that stratified patients based on their CD_4 counts showed that *Cryptosporidium* was the commonest parasite seen in 46% patients with

counts < 200 cell/mm^3. The prevalence of cryptosporidial diarrhea (56%) was also significantly higher when CD_4 counts were below 200 compared to patients with higher CD_4 counts (40%) (199) in a previous study from this hospital.

Molecular typing data from this study indicated that although *C. hominis* was the most common species identified, around 40% of cases with diarrhea were infected with other zoonotic species, raising the question about the sources of infection. Several studies indicate that immunocompromised individuals are susceptible to a wider range of species and genotypes and that host factors must play a role in controlling susceptibility to these divergent parasites(74, 75). Immunodeficiency, therefore, alters host susceptibility to *Cryptosporidium spp*. that are not normally infectious in humans (98). Additionally, variation in virulence among different species has also been demonstrated in volunteer studies with respect to attack rate and duration of diarrhea (230). Considerable circumstantial evidence from previous studies have shown a zoonotic exposure in these patients (65). A previous report analyzing isolates from Kenya, Switzerland and the United States identified animal contact in patients (using a questionnaire) infected with zoonotic isolates(151). Animal human mixing patterns, access to potable drinking water and antiretroviral therapy are all factors that play a major role in the risk of acquiring cryptosporidiosis and are very different from region to region. A wide diversity in *Cpgp40/15* subgenotypes was also seen and was similar in distribution to the previous study (156).

This is the first study to quantitate cryptosporidiosis in HIV infected patients. Real time PCR has been used extensively in previous studies to detection and quantitation of cryptosporidial

oocysts extensively in sewage and water samples (90), as well as in animal and cell culture studies (82, 136). Recently 2 studies have been carried out on stool samples, including a multiplex PCR (92, 177). The limit of detection in the real time PCR was much lower (10 -100 copies) than the other studies on stool samples (1000 copies) respectively. However, there was no difference in median Ct values between cases with and without diarrhea.

Immune response to 2 immunodominant antigens, gp15 and Cp23 was characterized in this HIV infected population. This is the first study on humoral immune response in HIV infected patients in India. Previous studies on humoral immune response in cryptosporidial infections in HIV have been in developed countries like the United States and Australia (63, 70, 202). In this study, more than 90% of patients were found to be seropositive for both antigens, a much greater prevalence rate than that seen in studies from the West where rates of 44-59% have been reported (36, 202). A previous study that followed up HIV infected individuals longitudinally has estimated a decay rate of around 300 days for Cp23 antibodies suggesting that most patients in this study probably have repeated exposure to *Cryptosporidium* and therefore have higher seropositivity levels. However, similar to a study in San Francisco, cases with cryptosporidiosis had higher median levels of antibody than patients without cryptosporidiosis (63). Due to the high prevalence of antibodies, serological studies in this setting may not be able to identify recent episodes of cryptosporidial diarrhea as in the West (202). The cut off used in this study was based on the mean+2SD of the negative control which may be too conservative an approach. Other workers have used a receiver operating curve analysis to determine optimal cut off rates while others used a predetermined cut off rate based on the positive control. Most workers also found Cp23 a better indicator of exposure than

gp15. When antibody levels in patients with and without cryptosporidial diarrhea were analysed, no protective effect could be determined for either antigen tested in this study. A study from Australia has documented protection from diarrhea without weight loss in patients with higher Cp23 antibodies. In this study, CD_4 counts did not have any impact on the seropositive status of the patient. This lack of association between antibody response and CD_4 count has also been documented in these previous studies (36, 63, 202). This could possibly be due to the fact that CD_4 T cells probably play a more important role in immunity from this parasite while the antibody response may act as a marker of infection or prior exposure.

This study has used multiple tools to characterize cryptosporidiosis in HIV infected adults in India. These data will help in understanding the transmission and natural history of cryptosporidiosis in India. It is necessary to document the full public health significance of this infection in immunocompromised hosts in order to formulate more rational approaches to the control of this disease, especially in the context of developing countries.

- **CHAPTER 11**

SUMMARY AND CONCLUSIONS

Cryptosporidiosis affects young children and HIV infected adults in the developing world. Among children it is a common cause of diarrhea in under-fives, especially in the malnourished. Infections with *Cryptosporidium* spp., along with *Giardia* and enteroaggregative *E. coli,* also result in long term effects on growth causing stunting and may also result in deficits of cognitive function. Among the HIV infected, *Cryptosporidium* spp is the most common cause of opportunistic diarrhea in less-developed countries with a clear correlation with worsening cell mediated immunity. Although the pathogen is endemic in these countries, its ability to survive in the environment for a prolonged duration can lead to diarrheal disease epidemics affecting children in the entire community. Prior studies in India identified this parasite in children and in the HIV infected in various parts of the country but little information was available on the circulating species and subgenotypes or immunity to this parasite.

In this study, we have documented the prevalent species among children with diarrhea in the community in Vellore and in the hospital setting in Delhi, Trichy and Vellore. In children both in the community and among those coming to the hospital, *C. hominis* was the most common species with a few infections due to zoonotic species like *C. parvum*, *C. felis* and *C. meleagridis*. Most of the children with cryptosporidial diarrhea both in the community and in the hospital setting were under the age of 2 years. In the community, *C. hominis*-infected children had a significantly greater severity of diarrhea. Our findings indicate that hospitalization due to cryptosporidial diarrhea is mainly due to *C. hominis*, probably attributable to greater severity of disease with anthroponotic than zoonotic species.

Predominance of a single subgenotype *Cpgp40/15* Ia was seen in the community indicating a common source of infection, probably water borne. A spatial analysis of children with cryptosporidial diarrhea was carried out to study the transmission dynamics of cryptosporidiosis in this community using GIS technology and two space time clusters were revealed. All of the *C. parvum* isolates in the community were the anthroponotic subgenotype Ic/IIc. Among children in the hospital setting, a more diverse set of subgenotypes was seen. Subgenotypes Ie, Ia, Ib and Id were common in all centers and 2 novel subgenotypes of *C. parvum* were seen in the south Indian centers of Trichy and Vellore.

Using real time PCR, when six children from the Vellore hospital based study were followed up for approximately 3 weeks, oocyst shedding decreased to low or undetectable levels within 12 days of admission in 3 cases, while in 3 cases, even at 16, 18 and 24 days post diarrhea, oocyst levels remained high long after the disappearance of any symptoms. This evidence that oocysts were shed for greater than 3 weeks following an episode of diarrhea may contribute to the prolonged effects of cryptosporidiosis, including stunting and growth retardation.

In order to identify the prevalence and impact of repeated infections, 20 children with cryptosporidial diarrhea in the community were screened. Multiple episodes of cryptosporidiosis occurred in 8/20 of the children. A total of 36 episodes were seen and most were symptomatic. In most cases, there was a decrease in duration and severity of diarrhea or occurrence of asymptomatic infections after the initial episode. When children with a single and multiple episodes were compared, significant differences in WAZ (weight for age) and HAZ (height for age) Z scores between the 2 groups at 24 months was seen and the difference

in WAZ persisted at 36 months. This study illustrates the importance of using molecular tools to estimate the true frequency of cryptosporidial infections and the extended duration of oocyst shedding during early childhood cryptosporidial diarrhea.

We also investigated maternal, longitudinal and post-diarrheal antibody responses to specific cryptosporidial antigens, gp15 and gp40 in the children from the community in Vellore. Mothers of children who had no cryptosporidial diarrhea were seropositive more often than the mothers of children with subsequent cryptosporidial diarrhea. The commonest transitional pathway was for the children of seropositive mothers who were seronegative at 3.5 months then seroconverted at 9 months and stayed seropositive at 24 months. Children of seropositive women seroconverted earlier (N/P ratio, 0.58 at 9 months) than children of seronegative women (1.0). In serum collected before and after an episode of cryptosporidial diarrhea, there was a statistically significant rise in titer to both antigens (gp15 and gp40) tested. Pre existing antibodies to gp15 were associated with a shorter duration of diarrhea. These findings on the immune response to gp40 and gp15 antigens has important implications in evaluating these glycoprotein antigens as potential vaccine candidates for cryptosporidiosis.

Cryptosporidiosis in HIV infected adults was also characterized in a hospital based study at Vellore. The prevalence of cryptosporidial diarrhea was 15% which was lower than a previous study that documented 25%. This decrease in prevalence could be attributed to several causes, including the increasing accessibility of HAART for Indian patients. Nearly 30% of cases enrolled in this study had some history of antiretroviral therapy. When CD4 counts were examined, a greater number of patients with diarrhea and cryptosporidial infections had counts

below 200 cells per cu. mm and more than 90% of cases identified to have cryptosporidial diarrhea had CD4 counts below 200.

Molecular typing data from this study indicated that although *C. hominis* was the most common species identified, around 40% of cases with diarrhea were infected with other zoonotic species. Several studies indicate that immunocompromised individuals are susceptible to a wider range of species and genotypes. A wide diversity in *Cpgp40/15* subgenotypes was also seen. This is the first study to quantitate cryptosporidiosis in HIV infected patients. However, there was no difference in median Ct values between cases with and without diarrhea.

Immune response to 2 immunodominant antigens, gp15 and Cp23 was characterized in this HIV infected population. More than 90% of patients were found to be seropositive for both antigens, a much greater prevalence rate than that seen in studies from the West. Cases with cryptosporidiosis had higher median levels of antibody than patients without cryptosporidiosis. When antibody levels in patients with and without cryptosporidial diarrhea were analyzed, no protective effect could be determined for either antigen tested in this study. In this study, CD4 counts did not have any impact on the seropositive status of the patient. This lack of association between antibody response and CD4 count has also been documented in previous studies and could possibly be due to the fact that CD4 counts or T cells probably play a more important role in immunity from this parasite while the antibody response may act as a marker of infection or prior exposure.

This study has used multiple tools to characterize cryptosporidiosis in children HIV infected adults in India. These data will help in understanding the transmission and natural history of cryptosporidial diarrhea in India and will help formulate more rational approaches to the control of this disease, especially in the context of developing countries.

• RECOMMENDATIONS

This study has characterized cryptosporidiosis in two populations in the country, young children and HIV infected adults. The recommendations of this study are the following:

- A more in depth longitudinal study of the transmission dynamics of *Cryptosporidium* in the community to identify the sources of infection and time to earliest infection in children is required.
- It is also important to identify potential methods of treatment and prevention of cryptosporidiosis as this study has clearly illustrated long term effects of infection. The effect of probiotics and nitazoxanide on cryptosporidial diarrhea has not been studied in Indian children.
- In this study, cryptosporidiosis was studied in the hospital setting from 3 centers; more detailed studies from additional centers in the country are required using sensitive PCR based tools to identify the true prevalence of disease. These data also need to be analyzed with environmental parameters like temperature, humidity and rainfall in order to be able to predict and peaks in occurrence so that appropriate prevention measures can be taken.
- In the HIV population as well, a multicenter study using sensitive PCR based tools is needed to identify the true prevalence of disease in India. The effect of HAART on the prevalence of disease also needs to be studied in long term follow up of a cohort.
- In both children and HIV infected patients, the role of cell mediated response to cryptosporidial antigens needs to be studied.

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- **APPENDICES**

Appendix -1

Genbank accession numbers of *Cpgp40/15* subgenotypes used in phylogenetic analysis

Subgenotype	Accession number	source
Ia	AF164502	human
Ia	AF440634	human
Ib	AY262031	human
Ib	AF440626	human
Ib	AF440628	human
Ic/ II c	AF440622	human
Ic/ II c	AF440621	human
Ic/ IIc	AF164491	human
Id	DQ665692	human
Id	AF440625	human
Ie	AY738184	human
Ie	AF440629 (Leav)	human
Ie	AF440630 (Leav)	human
Ie	AF440633 (Leav)	human
If	AF440638	human
Ig	EF208067	human
II	AF155624	
II	AF114166	cattle
IIa	AY262034	human and cattle
IIa	DQ192501	animal
IIb	AF402285	human and cattle
IIc	AF440636	human and cattle
IId	AY738194	human and cattle
IIe	AY382675	human and cattle
IIf	AY738188	human and cattle
IIg	AY873780	human and cattle
IIh	AY873781	human and cattle
IIi	AY873782	human and cattle
IIj	DQ648547	cattle
IIk	AB237137	cattle
IIl	AB242225	human and cattle
IIm	AY700401	human
C. meleagridis	AF401498	

- **PUBLICATIONS**

1) **Ajjampur, S. S.**, P. Sankaran, and G. Kang. 2008. *Cryptosporidium* species in HIV-infected individuals in India: an overview. Natl Med J India 21:178-84.

2) **Ajjampur, S. S.**, P. Sankaran, A. Kannan, K. Sathyakumar, R. Sarkar, B. P. Gladstone, and G. Kang. 2009. *Giardia duodenalis* assemblages associated with diarrhea in children in South India identified by PCR-RFLP. Am J Trop Med Hyg 80:16-9.

3) **Ajjampur, S. S.**, P. Rajendran, S. Ramani, I. Banerjee, B. Monica, P. Sankaran, V. Rosario, R. Arumugam, R. Sarkar, H. Ward, and G. Kang. 2008. Closing the diarrhoea diagnostic gap in Indian children by the application of molecular techniques. J Med Microbiol 57:1364-8.

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5) **Ajjampur, S. S.**, B. P. Gladstone, D. Selvapandian, J. P. Muliyil, H. Ward, and G. Kang. 2007. Molecular and spatial epidemiology of cryptosporidiosis in children in a semiurban community in South India. J Clin Microbiol 45:915-20.

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