Prevalence of Cryptosporidiosis in children with diarrhea and its correlation with mucosal immunity

Purbasha Bera[1], Shukla Das[1], Rumpa Saha[1], Vishnampettai G. Ramachandran[1], Dheeraj Shah[2]

[1]Department of Microbiology, [2]Department of Pediatrics,
University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi-110 095

ABSTRACT

Background and objectives: *Cryptosporidium* is a recognized cause of diarrhea, particularly among children, in developing countries. *Cryptosporidium* diarrheal episode impinges heavily on the quantitative effector mucosal responses of subsets of T cell population, especially within the gut cytokines. The current study aims to estimate the prevalence of cryptosporidiosis in children of age $\leq$ 5 years old and also compared IL-10 and IFN-γ levels in immunocompetent children responding to gut infection with *Cryptosporidium*. Materials and methods: Diarrheal stool specimens from 175 young children (≤ 5 years) were collected. Kinyoun's acid fast staining was performed for identification of *Cryptosporidium*. ELISA was performed for antigen detection of *Cryptosporidium* and cytokine (IFN-γ and IL-10) analysis. For comparison a total of 30 stool samples from age and sex matched healthy children were also tested for IFN-γ and IL-10. Results: *Cryptosporidium* oocysts were found in 7 (4.0%) out of 175 children whereas 48 (27.4%) children suffering from diarrhea had *Cryptosporidium* antigen. A marker of a proinflammatory immune response, IFN-γ and the counter-regulatory cytokine IL-10 was also exclusively elevated in the patient population (p <001). Interpretations and conclusion: *Cryptosporidium* is present in a significant portion of children suffering from diarrhea in our setting. Antigen detection has much higher isolation rate than Kinyoun's acid fast staining. Results suggest that the Th response adapted at controlling cryptosporidial infection may be a dynamic one in which there is a strong early Th-1 response which later shifts to Th-2 response to facilitate parasite removal and to limit the infection.

Keywords: *Cryptosporidium*, immunocompetent children, mucosal immunity

INTRODUCTION

Our knowledge of the protozoan parasite *Cryptosporidium parvum* has increased considerably since 1976 when it was first discovered as a cause of diarrhea in humans and animals, and in present times the organism is recognized as one of the important opportunistic parasites. *Cryptosporidium* is an obligate intracellular protozoan parasite and is a major cause of diarrheal illness worldwide. *Cryptosporidium* primarily infects the distal small intestine (distal jejunum and ileum). Immunocompetent hosts control and eliminate the infection, which typically manifest as acute, self-limited watery diarrhea lasting 5 to10 days. However, in patients with defects in cellular immune responses, *Cryptosporidium* frequently causes persistent or chronic diarrhea and may also involve the biliary tract.[1] In malnourished children, recurrent diarrheal episodes, can lead to death or chronic nutritional and cognitive sequelae.[2] Thus, the host immune response plays a critical role in the control of human cryptosporidiosis. The mechanism by which *C. parvum* causes a spectrum of clinical illnesses with different outcomes are poorly understood. To understand the pathophysiology of cryptosporidiosis, it is important to define the intestinal mucosal immune responses to *C. parvum*

Corresponding author: Dr. Purbasha Bera
E-mail: drpurbasha@gmail.com

Received: 06-02-2016 Accepted: 20-07-2016

How to cite this article: Bera P, Das S, Saha R, Ramachandran VG, Shah D. Prevalence of Cryptosporidiosis in children with diarrhea and its correlation with mucosal immunity. J Gastrointest Infect, 2016; 6: 39-44

infection and their potential impact on intestinal secretory responses. Although extensive studies with various animal models have provided important insight into the host immune response to *C. parvum*, the ability of these models to explain the human immune response is limited.[3] *C. hominis*, infects only humans and gnotobiotic pigs, thus limiting the data from animal models. Healthy human volunteers can be studied, but they typically experience a milder illness than malnourished children and AIDS patients. Human intestinal tissue samples can be obtained only by invasive procedures, limiting the number of subjects and samples available. Some data can be obtained from *in vitro* infections, but most of the target cells are immortalized cell-lines and may not be ideal for studying mechanisms involving apoptosis. Furthermore, the immune cells in the peripheral blood may exhibit properties different from the properties of cells found in the intestinal compartment. Thus, knowledge about the human immune response towards *Cryptosporidium* infection is far from complete. In the face of these limitations, important recent advances have been made.

In our study, an attempt was made to estimate the prevalence of *Cryptosporidium* in immuno-competent children, under the age of 5 years, presenting with acute or persistent diarrhea. This will help us to measure not only the parasitic burden, but will also correlate the demographic, nutritional and immunological status of the child with the disease burden. Additionally since the immune response to *Cryptosporidium* is not clearly defined till now, an attempt was made to investigate the indicators of an inflammatory component at mucosal surface, in naturally acquired cryptosporidial diarrhea, by stool assay of interferon-γ (IFN-γ) and interleukin-10 (IL-10) which may help to understand the intestinal immune response of young children with cryptosporidiosis.

MATERIALS AND METHODS

i) Patient population

In the present prospective study from 2012 to 2013, a total of 175 stool specimens were collected from children under 05 years of age and suffering from diarrhea. The samples were collected only once from the patient. Diarrhea episodes were defined as acute (three or more loose stools per day over 72 hours) or persistent (persisted more than 14 days). Ethical clearance was obtained from Institutional Ethics Committee. The parents/guardians of the children were interviewed using a designed proforma for demographic, epidemiological data and clinical history. Children with known immune suppression, history of receiving antibiotics or antiparasitic drug for current episode of diarrhea, known allergy to lactose, gluten or any other food, history of recurrent hospitalization due to infections, history of prolonged steroids intake in last three months or history of infection with unusual organisms were excluded from our study.

(ii) Routine microscopy and culture

Fecal samples meeting the inclusion criteria were collected in a clean, dry, leak-proof plastic container. Routine microscopic examination inclusive of Kinyoun's acid-fast staining of the stool samples were performed as per standard laboratory protocols. Bacterial culture was done using MacConkey's media, xylose lysine deoxycholate agar and bile salt agar for primary plating of stool sample and selenite F broth and alkaline peptone water for enrichment of stool sample.

(iii) Antigen detection

A portion of each sample was preserved in 10% formalin for antigen detection till the time of assay by commercially available *Cryptosporidium* antigen detection ELISA kit (DRG international Inc, USA).

(iv) Cytokine analysis

A portion of stool sample was preserved at -70^{0}C for cytokine analysis till the time of assay. Stool samples which were positive for *Cryptosporidium* on microscopy or ELISA test for antigen detection were further tested for IFN-γ and IL-10 analysis in stool supernatant. Thirty samples from healthy age, sex matched children were also subjected to cytokine analysis and served as controls.

Before the assay the stool samples were removed from -70^{0}C freezer and thawed. Small aliquots were diluted in 1:2 (w/v) in PBS (phosphate buffered saline pH-7.2-7.4) containing protease inhibitor (AEBSF, E -64, pepstatin A.1, and 10-phenanthrrolin) (Sigma, St. Louis, Missouri). Stool samples were thoroughly homogenized and centrifuged for 10 minutes at 12,000 rpm. The supernatants were collected in fresh sterile eppendorf tubes. IFN-γ and IL-10[4] determinations were performed in stool supernatants using commercially available human IFN-γ and IL-10 ELISA kit (Gen-Probe Diaclone, France). Each test was performed according to the manufacturer's specification. Briefly, standards and samples were pipetted into wells pre-coated with highly specific antibodies to IFN-γ and IL-10. After binding of IFN-γ and IL-10 in samples and known standards to the capture antibodies and subsequent binding of the biotinylated anti IFN-γ and anti IL-10 secondary antibody to the analyte was completed during the same incubation period; excess unbound analyte and secondary antibody was removed. The HRP conjugate solution was then added to every well including the zero wells. Following incubation, excess conjugate is removed by careful washing. A chromogen substrate

was added to the wells resulting in the progressive development of colored complex with the conjugate. The absorbance of the color complex was then measured and the generated OD values for each standard were plotted against expected concentration forming a standard curve. Standard curve was used to accurately determine the concentration of IFN-γ and IL-10 in any sample tested.

RESULTS

Among the 175 patients of diarrhea, seven were positive by microscopy and 48 were positive by antigen detection kit. All microscopically positive samples were also positive for antigen by ELISA. According to the onset of illness (diarrhea) only one (2.08%) *Cryptosporidium* positive patient presented with persistent diarrhea whereas the remaining 47 (97.91%) presented with acute diarrhea. The demographic profile of these patients is represented in Table 1. Fever was present in nine (18.7%), vomiting in 19 (39.58%), abdominal pain in six (12.5%) and abdominal distention in two (4.16%) children with *Cryptosporidium* species in diarrheal stool. The epidemiological characteristic is presented in Table 2.

Hydration status was defined according to WHO classification as (No, some or severe dehydration). Twenty three (47.91%) children did not have dehydration; some dehydration was present in 21 (43.75%) whereas severe dehydration was present in four (8.33%) children positive for *Cryptosporidium* species. Breast-fed children with cryptosporidiosis were 64.58%.

Forty eight *Cryptosporidium* positive stool samples and 30 stool specimens from healthy controls were evaluated for IFN-γ and IL-10 assay. IFN-γ was measurable in 26 (54.16%) of 48 patients to a maximum of 159.2 pg/ml and in control groups it was measurable in four (13.3%) samples. IL-10 was measurable in 30 (62.5%) of 48 patients to a maximum of 320 pg/mL, while in the control groups it was detected in four (13.3 %) samples.

DISCUSSION

Cryptosporidiosis is a common cause of gastrointestinal disease, and it has been recognized worldwide as a cause of diarrhea in otherwise healthy children. The disease is widespread in many developed and developing countries. The present prospective case control study of cryptosporidiosis in urban North Indian children with diarrhea has revealed similarities as well as differences in clinical and epidemiological features when compared with previous studies from this country, and from other developing

Table 1

Clinical and demographic profile of children with and without cryptosporidiosis

Variable	Cryptosporidium positive cases (n=48)	Cryptosporidium negative cases (n=127)	Odds ratio	95% confidence interval	P value
Diarrheal type					
Acute	47	121	0.429	0.050 -3.660	0.426
persistent	1	6			
Hydration status					
No	23	76			0.304
Some	21	40	–	–	
Severe	4	11			
Still breast feeding	31	67	1.633	0.822 -3.244	0.160
Fever	9	25	0.942	0.404 -2.195	0.899
Vomiting	19	40	1.425	0.715 -2.838	0.313
Abdominal pain	6	9	1.873	0.629 -5.579	0.254
Abdominal distention	2	10	0.509	0.107 -2.411	0.387
WHZ Mean(SD)	0.80 (1.70)	-1.17 (1.90)	–	–	0.244
WAZ Mean(SD)	-1.80 (1.54)	-2.14 (1.48)	–	–	0.179
HAZ Mean(SD)	-1.88 (1.93)	2.32 (1.54)	–	–	0.117

Table 2

Epidemiological characteristics of *Cryptosporidium* positive cases with diarrhea

Characteristics	*Cryptosporidium* positive cases (n=48)	*Cryptosporidium* negative cases (n=127)	P value
Contact with animals	5	15	0.796
Tank supply	32	84	0.993
Well water	5	9	
Bottled water	1	3	
Boiled water	2	7	
Tube well water	4	11	
Boring water	3	10	
Exclusively breastfed	1	3	

Table 3

Cytokine data for children with cryptosporidiosis, compared with healthy control

Cytokine level pg/ml	***Cryptosporidium*** *positive diarrhea (cases) (n=48)*	*Non-diarrheal* ***Cryptosporidium*** *negative (control) (n=30)*	*P value (Mann - Whitney Test)*
IFN - γ			
No of detectable level	26	4	
Mean (SD)	25.50 (38.19)	4.75 (14.63)	<0.001
Median (range)	5.68 (0-159.20)	0 (0-57.85)	
IL -10			
No of detectable level	30	4	
Mean (SD)	47.34 (65.29)	12.88 (33.49)	<0.001
Median (range)	21.95 (0-320)	0 (0-103)	

countries. In addition, to our knowledge the present study describes for the first time mucosal immune responses to *Cryptosporidium* species in Indian immunocompetent children.

Our study demonstrated clearly a high prevalence rate (27.4%) of *Cryptosporidium* infection in children less than five years. Similar high prevalence of cryptosporidiosis has been reported from India; (18.9 %),[5] Bolivia (31.4%)[6] and Mexico (29.6%).[7] High prevalence of *Cryptosporidium* infection found among 1-2 years (32%) followed by 4-5 years age group (31.8%) is similar to studies conducted in the tropical countries [8,9] showing highest *C. parvum* infection in less than two years of age group children.

Regarding gender variation male:female ratio of 29% versus 24% was not statistically significant. However the contact with domestic animals may act as a risk factor in zoonotic infection and previous studies have reported an association of this disease with the domestic animals in the household.[10] In our study, however there was no statistically significant association between *Cryptosporidium* infection and animal contact similar to studies in Mexico and Brazil.[11,12] It is well known that, water borne transmission is a major route of infection with *Cryptosporidium* species and because of small size of *Cryptosporidium* oocyst and its ability to survive chlorination it was of interest to determine whether there was any difference in the type of water supply associated with the disease. In our study, consumption of untreated overhead tank water supply from municipality could have been a major source of contamination leading to infection and disease. *Cryptosporidium* species infected patients presenting with watery diarrhea (100%), vomiting (39.50%) and abdominal

pain (12.5%) is consistent with the previous studies [13,14] from developing countries. Cryptosporidial infection usually leads to chronic watery diarrhea with varying degree of dehydration as observed in our study.

Lack of breast-feeding has been reported to be associated with cryptosporidiosis in many studies in developing countries.[15,16] However, some prospective studies have not shown a protective effect of breast-feeding, and one study found that *Cryptosporidium* species infection was actually more common in breast-fed children.[17] Although in the present study too, infection was seen more common among breast-fed (64%) as compared to non-breastfed (35%) patients, there was no statistically significant association of disease with breast-feeding.

This study demonstrated that 54% of *Cryptosporidium* patients had detectable levels of IFN-γ compared to control group and this difference was statistically significant. The mechanism by which the increased expression of IFN-γ controls intestinal cryptosporidiosis is unknown. Studies in a variety of systems have implicated IFN-γ in several aspects of the cellular immune system. Intestinal epithelial cells express IFN-γ receptors and IFN-γ directly activate intestinal epithelial cells to kill intracellular parasites. IFN-γ could potentially activate the epithelial cells directly or activate cytotoxic lymphocytes, which in turn might destroy infected cells to limit infection. Most importantly, comparison of animal and human data has shown that the immune response towards *Cryptosporidium* in humans differ significantly from that in animals; for example, in mice gamma interferon (IFN-?) production seems to be associated with the innate and primary immune responses, whereas in humans it is most probably associated with the memory response towards the parasite. Designing studies to elucidate human mucosal immune responses is difficult.

In a study in Haitan children,[15] IFN-γ was not detected in the stool samples with cryptosporidiosis, possibly due to degradation of the cytokine in the luminal contents of the gut, or the insensitivity of the ELISA method used, and /or collection of the samples at too frequent time-points such that secretion of this cytokine was missed.

In addition to IFN-γ, our study population also revealed evidence of a counter regulatory (anti-inflammatory) mucosal response, as demonstrated by IL-10 elevation in stool samples of 62.5% of positive subjects. IL-10 is produced by a broad range of cells in the gastrointestinal tract (including intestinal epithelial cells, B cells, and macrophages) and plays a central role in the immune balance between pathology and protection through its broad immune regulatory and immunosuppressive functions. The production of IL-10 can be considered as an attempt to "restrain" the immune system, shown to have an important role in regulation of mucosal immunity. Thus IL-10 may be key factor for repairing the intestinal epithelium after cryptosporidiosis mediating parasite clearance.

Our study showed parallel increase in both IFN-γ (inflammatory cytokine) and IL-10, (anti-inflammatory cytokine). It suggests that there exists a balance between these two cytokines determining the shift of Th1 to Th2 response, when there is a deficiency in IFN-γ production or a condition involving high levels of IL-10. Alternatively it can be said that the Th responses to cryptosporidiosis is a dynamic one in which there is an early Th1 response but later shifts to Th2 response to facilitate parasite removal and to limit the infection. The important observation of our study is that there is a predominant Th2 (IL-10) response and alternative activation of IL-10 may minimize tissue injury, or help in the escape of the parasite from the host. The quick appearance of IL-10 in the present study favors acute episode of diarrhea in Indian immunocompetent children which is in contrast with previous studies in Pakistan,[18] Haitan[15] and Uganda[19] where most of the *Cryptosporidium* affected patients presented with persistent diarrhea.

Cryptosporidium species is prevalent in a significant proportion (27%) in immunocompetent children presenting with acute diarrhea in North Indian tertiary care hospital. Outcome of a diarrheal episode impinges heavily on the quantitative effector responses of subsets of T cell population, especially within the gut. The presence of gut microbiota and proteases in the stool specimens can destroy secreted cytokines and can result in false low levels. Influence of other enteric pathogens in modulating the cytokine regulation is a probability. The inflammatory process observed in cryptosporidiosis requires additional investigation to comprehend the complex interrelationship between the pathogens, host-factors, malabsorption and malnutrition, especially in developing countries. Therefore such a study should be followed up sequentially to understand the role of cytokines in either remission or persistence of diarrhea with this agent.

ACKNOWLEDGEMENT

We thank the Principal UCMS & GTB Hospital, Delhi for Intramural Research Grant of the College as financial support.

CONFLICT OF INTEREST: None

REFERENCES

1. Hunter PR, Nichols G. Epidemiology and clinical features of *Cryptosporidium* infection in immunocompromised patients. Clin Microbiol Rev. 2002;15:145-154.
2. Agnew DG, Lima AA, Newman RD, Wuhib T, Moore RD, Guerrant RL *et al.* Cryptosporidiosis in northeastern Brazilian children: association with increased diarrhea morbidity. J Infect Dis. 1998;177:754-60.
3. White AC, Robinson P, Okhuysen PC, Lewis DE, Shahab I, Lahoti S *et al.* Interferon-gamma expression in jejunal biopsies in

experimental human cryptosporidiosis correlates with prior sensitization and control of oocyst excretion. J Infect Dis. 2000;181:701-09.

4. Alcantara CS, Yang CH, Steiner TS, Barrett LJ, Lima AAM, Chappell CL *et al.* Interleukin-8, tumor necrosis factor–alpha, and lactoferrin in immunocompetent hosts with experimental and Brazilian children with acquired cryptosporidiosis. Am J Trop Med Hyg. 2003;68:325-28.
5. Kaur R, Rawat D, Kakkar M, Uppal B and Sharma VK. Intestinal parasites in children with diarrhoea in Delhi India. Southeast Asian J Top Med Public Health. 2002;33:725-9.
6. Esteban JG, Aguirre C, Flores A, Strauss W, Angles R, Mascoma S. High *Cryptosporidium* prevalence in healthy Aymara children from the Northern Bolivian Altiplano. Am J Trop Med Hyg. 1998;50:50-1.
7. Miller K, Duran-Pinales C, Lopez A, Lechuga LM, Teren D, Enriquez FJ. *Cryptosporidium parvum* in children with diarrhea in Mexico. Am J of Trop Med Hyg. 1994;5:47.
8. Perch M, Sodemann M, Jakobsen MS, Valentiner-Branth P, Steinsland H, Fischer TK *et al.* Seven years' experience with *Cryptosporidium parvum* in Guinea-Bissau, West Africa. Ann Trop Paediatr. 2001;21:313-8.
9. Pereira MD, Atwill ER, Barbosa AP, Silva SA, Garcia-Zapata MT. Intra-familial and extra-familial risk factors associated with *Cryptosporidium parvum* infection among children hospitalized for diarrhea in Goiania, Goias, Brazil. Am J Trop Med Hyg. 2002;66:787-93.
10. Rahman AS, Sanyal SC, Al-Mahmud KA, Sobhan A. *Cryptosporidium* diarrhoea in calves and their handlers in Bangladesh. Indian J Med Res. 1985;82:510-6.
11. Javier Enriquez F, Avila CR, Ignacio Santos J, Tanaka-Kido J, Vallejo O, Sterling CR. *Cryptosporidium* infections in Mexican children: clinical, nutritional, enteropathogenic, and diagnostic evaluations. Am J Trop Med Hyg. 1997;56:254-7.
12. Pereira MD, Atwill ER, Barbosa AP, Silva SA, Garcia-Zapata MT. Intra-familial and extra-familial risk factors associated with *Cryptosporidium parvum* infection among children hospitalized for diarrhea in Goiania, Goias, Brazil. Am J Trop Med Hyg. 2002;66:787-93.
13. Bhattacharya MK, Teka T, Faruque AS, Fuchs GJ. *Cryptosporidium* infection in children in urban Bangladesh. J Trop Pediatr. 1997;43:282-6.
14. Shahid NS, Rahman AS, Sanyal SC. *Cryptosporidium* as a pathogen for diarrhoea in Bangladesh. Trop Geogr Med. 1987;39:265-70.
15. Kirkpatrick BD, Daniels MM, Jean SS, Pape JW, Karp C, Littenberg B. Cryptosporidiosis stimulates an inflammatory intestinal response in malnourished Haitian children. J Infect Dis. 2002;186:94-01.
16. Molbak K, Aaby P, Hojlyng N, da Silva AP. Risk factors for *Cryptosporidium* diarrhea in early childhood: a case-control study from Guinea-Bissau, West Africa. Am J Epidemiol. 1994;139:734-40.
17. Nchito M, Kelly P, Sianongo S, Luo NP, Feldman R, Farthing M. Cryptosporidiosis in urban Zambian children: an analysis of risk factors. Am J Trop Med Hyg.1998;59:435-7.
18. Mumtaz S, Ahmed J, Liaqat A. Frequency of *Cryptosporidium* infection in children under five years of age having diarrhea in the North West of Pakistan. Afr J.Biotech. 2010;9:1230-5.
19. Tumwine JK, Kekitiinwa A, Nabukeera N, Akiyoshi DE, Rich SM, Widmer G *et al. Cryptosporidium parvum* in children with diarrhea in Mulago Hospital, Kampala, Uganda. Am J Trop Med Hyg. 2003;68:710-5.