Antibody Responses to the Immunodominant *Cryptosporidium* gp15 Antigen and gp15 Polymorphisms in a Case–Control Study of Cryptosporidiosis in Children in Bangladesh

Genève M. Allison,† Kathleen A. Rogers,† Anoli Borad, Sabeena Ahmed, Mohammad Mahbubul Karim, Anne V. Kane, Patricia L. Hibberd, Elena N. Naumova, Stephen B. Calderwood, Edward T. Ryan, Wasif A. Khan, and Honorine D. Ward*

Division of Geographic Medicine and Infectious Diseases, Tufts Medical Center and Department of Public Health and Community Medicine, Tufts University School of Medicine, Boston, Massachusetts; Clinical Sciences Division, Centre for Health and Population Research, International Center for Diarrheal Disease Research, Dhaka, Bangladesh; Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts

Abstract. Although *Cryptosporidium hominis* is the dominant *Cryptosporidium* species infecting humans, immune responses to cognate antigens in *C. hominis*-infected persons have not been reported. We investigated antibody responses to the immunodominant gp15 antigen from *C. hominis* and *C. parvum*, in *C. hominis*-infected Bangladeshi children less than five years of age with diarrhea (cases) and uninfected children with diarrhea (controls). We also investigated polymorphisms in the *C. hominis* gp15 sequence from cases. Serum IgG responses to gp15 from both species were significantly greater in cases than controls. In spite of polymorphisms in the gp15 sequence, there was a significant correlation between antibody levels to gp15 from both species, indicating cross-reactivity to conserved epitopes. Cases with acute diarrhea had a significantly greater serum IgA response to gp15 compared with those with persistent diarrhea, suggesting that this response may be associated with protection from prolonged disease. These findings support further investigation of gp15 as a vaccine candidate.

INTRODUCTION

Cryptosporidium species are an important cause of diarrheal disease worldwide, particularly in immunocompromised hosts such as patients with acquired immunodeficiency syndrome and malnourished children in developing countries.^{1–4} Cryptosporidiosis is usually either asymptomatic or self-limiting in immunocompetent hosts. However, in immunocompromised patients, the disease can be severe and chronically debilitating. Additionally, *Cryptosporidium* infection in malnourished children in developing countries may result in serious long-term sequelae, including developmental delays and growth stunting.^{1,5–7} Nitazoxanide, the only drug approved by the United States Food and Drug Administration for treatment of patients with cryptosporidiosis, is not effective in immunocompromised hosts,⁸ and has not been extensively evaluated in children in developing countries. Although children in these countries are considered an essential group to target for vaccine development,⁹ there is currently no vaccine available for prevention of cryptosporidiosis. A major focus of research on cryptosporidiosis has been the identification and characterization of surface-associated parasite proteins that mediate attachment and invasion, with the goal of developing interventions such as vaccines to prevent these interactions.^{10,11} Understanding the immune response to these proteins is a key step in the identification of potential vaccine targets.¹¹

It is well known that cell-mediated immune responses are essential for protection from and clearance of *Cryptosporidium* infection.^{11,12} However, although antibody responses against *Cryptosporidium* associated with protection from diarrhea have been reported in infected humans, it is not known whether these responses are themselves protective or whether they are merely reflective of protective cell-mediated responses.¹²

Two *Cryptosporidium* spp. cause most infections in humans. *Cryptosporidium parvum* infects animals and humans, whereas *C. hominis* primarily infects humans.¹³ Although most human infections, particularly in developing countries, are caused by *C. hominis*,¹³ most studies have used *C. parvum* oocyst lysates or native or recombinant *C. parvum* proteins as antigens to evaluate immune responses. Previously, we investigated the systemic antibody response to *Cryptosporidium* in a matched case–control study of children less than five years of age who presented with diarrhea to the International Center for Diarrheal Disease Research, Bangladesh (ICDDR, B).¹⁴ Using *C. parvum* oocyst lysate as antigen for enzyme-linked immunosorbent assays (ELISAs), we found that serum IgM levels to *C. parvum* were higher at presentation in cases (children with diarrhea and *Cryptosporidium* detected by stool microscopy) compared with controls (age-matched children with diarrhea but no *Cryptosporidium* detected by microscopy), and that the IgG levels increased significantly in cases compared with the controls over a three week follow-up period.¹⁴ However, the nature of specific antigens recognized by serum samples from these children is not known. Subsequently, using polymerase chain reaction (PCR) restriction fragment length polymorphism analysis at the 18S ribosomal RNA (rRNA) locus, we determined that *Cryptosporidium* was detected in feces in 7 of the controls and that most children with PCR-confirmed infection (90%) were infected with *C. hominis*.¹⁵

A number of studies have reported dominant serum antibody responses to 15–17-kD and 23–27-kD groups of *C. parvum* antigens in *Cryptosporidium*-infected persons.^{16–21} One of the 15–17-kD group of antigens was subsequently identified to be gp15 (also called Cp17, 15/17-kD antigen or 17-kD antigen), an immunodominant surface antigen encoded by the *Cryptosporidium* gp40/15 (also known as gp60) gene, which we and others have cloned and characterized.^{22–25} The gp15 is the C-terminal proteolytic cleavage product of the precursor gp40/15 (also known as gp60) protein,^{22,23} and is linked to the surface membrane via a glycosylphosphatidylinositol anchor.^{26,27} The presence of pre-existing serum antibodies to gp15 was associated with protection from diarrhea and reduced oocyst shedding in naturally or experimentally infected humans

*Address correspondence to Honorine D. Ward, Division of Geographic Medicine and Infectious Diseases, Tufts Medical Center, Box 041, 800 Washington Street, Boston, MA 02111. E-mail: hward@tuftsmedicalcenter.org

†These authors contributed equally to this article.

(reviewed by Borad and Ward¹¹ and Riggs¹²). In addition, gp15 induced interferon- γ -mediated cellular responses in previously infected humans.²⁸ These findings indicate that gp15 induces humoral and cellular immune responses that may be protective in humans, and raise the possibility that gp15 may be a putative vaccine candidate.

The *gp40/15* (*gp60*) gene encoding gp15 is highly polymorphic among *C. hominis* and *C. parvum* isolates.^{23,29} Although most of the polymorphisms are clustered in the gp40 portion of the molecule, there are several single nucleotide (SNP) and single amino acid (SAAP) polymorphisms between *C. hominis* and *C. parvum* gp15.²⁹ Polymorphisms within the gp40 region of the *gp40/15* (*gp60*) gene form the basis for subtyping of *Cryptosporidium* spp. from humans and animals worldwide into at least 17 major subtype families.¹³ However, polymorphisms in the gp15 part of the molecule have not been extensively characterized in clinical samples.

Knowledge of whether immune responses to gp15 are cross-reactive or species and/or subtype-specific is crucial if this antigen is to be considered as a vaccine candidate. The goals of this study were 1) to compare antibody responses to gp15 from *C. hominis* and *C. parvum* in *C. hominis*-infected children with diarrhea (cases) and in children with diarrhea but with no PCR-detected *Cryptosporidium* in feces (controls) by using recombinant proteins derived from both species as antigens in ELISAs; 2) to compare antibody responses to gp15 with those to antigens in oocyst lysates from the cognate species and 3) to characterize SNPs and SAAPs in the gp15 sequence from *Cryptosporidium* spp. identified in fecal samples of case children in the study.

MATERIALS AND METHODS

Patients. Patients recruited for the study were children 15 days to 60 months of age who came to the Dhaka Hospital of the International Center for Diarrheal Diseases Research,

Bangladesh (ICDDR,B), with diarrhea.¹⁴ Details of the recruitment procedure have been described.¹⁴ Informed consent was obtained from the parents or guardians of all children recruited into the study according to the guidelines of the Ethical Review Committee of the ICDDR, B which reviewed and approved the study.¹⁴ The original study was designed as a case-control study in which 46 children with diarrhea and *Cryptosporidium* spp. identified in feces by microscopy were enrolled as cases and 46 age-matched children with diarrhea but no *Cryptosporidium* spp. in feces by microscopy were enrolled as controls.¹⁴ Diarrhea was defined as ≥ 3 stools within a 24-hour period. A diarrheal episode was defined as diarrhea for at least 72 hours. The end of a diarrheal episode was defined as absence of diarrhea for 48 hours. Acute diarrhea was defined as a diarrheal episode lasting < 14 days. Persistent diarrhea was defined as a diarrheal episode lasting ≥ 14 days. In this study, children with persistent diarrhea included those who presented with diarrhea for ≥ 14 days and those who continued to have diarrhea for ≥ 14 days during the follow-up period and thus met the definition of persistent diarrhea.

In a subsequent study, fecal samples from seven controls that were negative for *Cryptosporidium* spp. by microscopy were found to be positive by PCR for the 18S rRNA locus.¹⁵ Therefore, for subsequent studies, the study design was unmatched to include 53 cases and 39 controls. In the previous study, the species and subtype of PCR-detected *Cryptosporidium* were determined by PCR-restriction fragment length polymorphism analysis and sequencing at the 18S rRNA and *gp40/15* loci respectively.¹⁵ Because the number of *C. parvum* (4) and *C. felis* (1)-infected cases were small, in the current study, we focused only on antibody responses in the 47 *C. hominis*-infected cases and 39 controls at the initial visit and in 33 cases and 17 controls that returned for follow up after 3 weeks (Figure 1). For each patient, clinical

FIGURE 1. Schematic showing numbers of cases and controls at the initial and follow-up time points enrolled in the current study, Bangladesh.

and epidemiologic factors such as age, sex, water source, and exposure to family members with diarrhea were obtained at enrollment.¹⁴ Nutritional status (measured by height-for-age Z score [HAZ] and weight-for-age Z score [WAZ]) was assessed using World Health Organization ANTHRO software (<http://www.who.int/childgrowth/software/en/>).

Recombinant *Cryptosporidium* gp15. Sequences encoding gp15 from *C. parvum* (GCH1 isolate) and *C. hominis* (TU502 isolate) and that of a control protein that contains the vector-encoded thioredoxin tag, S-tag, and His-tag (Novagen, Madison, WI) were cloned into the pET-32 Xa/LIC vector (Novagen) and overexpressed in *Escherichia coli* as described.^{21,27} Recombinant fusion proteins were purified by using Talon metal affinity chromatography (Clontech, Palo Alto, CA).

***Cryptosporidium hominis* oocyst lysate.** *Cryptosporidium hominis* oocysts (TU502 isolate) were obtained from Dr. Saul Tzipori (Tufts Cummings School of Veterinary Medicine, North Grafton, MA). Oocyst lysates were prepared as described and stored in aliquots at -80°C .¹⁴ The same batch of oocysts was used for all assays. Data using *C. parvum* oocyst lysate in ELISAs from our previous study¹⁴ were used for correlations between antibody responses to *C. parvum* gp15 and antigens in *C. parvum* oocyst lysate.

Enzyme-linked immunosorbent assay. Serum samples were shipped to Boston on dry ice and analyzed by ELISA at Tufts Medical Center. Approval for use of deidentified serum samples from children in the study was obtained from the Tufts Medical Center Institutional Review Board. Serum IgG, IgM, and IgA responses to gp15 proteins were assessed by ELISA as described.¹⁴ Briefly, microtiter plates (Nunc, Rochester, NY) were coated overnight with $0.4\text{ }\mu\text{g}$ of protein/well of recombinant (r) *C. hominis* or *C. parvum* gp15 or the control protein or *C. hominis* oocyst lysate equivalent to 5×10^5 oocysts at 4°C . Plates were washed three times with phosphate-buffered saline (PBS)–0.05% Tween 20, and non-specific binding was blocked with 0.25% bovine serum albumin in PBS for 2 hours at 37°C . Serum diluted 1:100 in 0.25% bovine serum albumin in PBS was added, and the plates incubated for 1 hour at 37°C . Plates were washed three times with PBS–0.05% Tween 20, alkaline phosphatase-conjugated goat anti-human IgG (γ -chain specific), IgA (α -chain specific) or IgM (μ -chain specific) (Southern Biotech, Birmingham, AL) was added, and the plates incubated for one hour at 37°C . The plates were washed three times with PBS–0.05% Tween 20, and substrate solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl_2) containing *p*-nitrophenyl phosphate (1 mg/mL) (Sigma, St. Louis, MO) was added.

After 30 minutes, the absorbance at 405 nm ($A_{405\text{ nm}}$) was measured by using a Microplate Reader (Model 550; Bio-Rad Laboratories, Hercules, CA). All samples were run in triplicate. All serum samples were tested using rgp15 from both species and the control protein containing the fusion tags alone. For each sample, rgp15 from both species and the control protein were run on the same plate. For each sample, the $A_{405\text{ nm}}$ value obtained for the control protein was subtracted from the $A_{405\text{ nm}}$ value for gp15 protein. None of the $A_{405\text{ nm}}$ values for gp15 or control proteins exceeded 2.0 optical density units. To minimize effects of plate-to-plate variation, initial and follow-up serum samples of the same patient were always run on the same plate and the same known *Cryptosporidium*-negative (by ELISA and immunoblot), and *Cryptosporidium*-positive serum samples were run on each plate. To adjust for

inter-plate variability, values were normalized by dividing the $A_{405\text{ nm}}$ of the sample (after subtracting the $A_{405\text{ nm}}$ of the control protein) by the $A_{405\text{ nm}}$ of the positive control for that plate and multiplying by 100. The effect of such standardization on inter- and intra-plate variability in the ELISAs were examined by using all values from triplicates in mixed effects regression models that indicated that the normalized values well represent the original measures and are invariant to plate performance. Therefore, results presented are expressed in normalized ELISA units (EU).

Comparison of gp15 sequences of *Cryptosporidium* species from case children. Nucleotide and deduced amino acid sequences of gp15 obtained by PCR amplification and sequencing of the *gp40/15* gene from DNA extracted from fecal samples of case children in the study¹⁵ and *C. parvum* (GCH1) and *C. hominis* (TU502) sequences used to generate recombinant gp15 proteins for this study were aligned by using the Align X program of Vector NTI Advance version 11.5 software (Invitrogen, Carlsbad, CA). Predicted sites of N and O-glycosylation were identified by using the NetNGlyc 1.0 and NetOGlyc 3.1 servers, respectively, at the Expasy proteomics server site (<http://expasy.org/>).

Statistical analysis. Statistical analysis was performed by using S-plus statistical software (Insightful, Inc., Seattle, WA) and data plotted with Prism version 5.0 software (Graphpad, San Diego, CA). Because the study design for the current study was changed from a matched pairs analysis (in the original study) to an unmatched analysis, Fisher's exact test was used to compare dichotomous demographic and clinical characteristics, and continuous variables were compared by using the unpaired *t*-test with Welch's correction for normally distributed variables or the Mann-Whitney U test for non-normally distributed variables. The ELISA data were compared by using the Mann-Whitney U test or Wilcoxon test. The associations between antibody levels to rgp15 or oocyst lysates of both species were measured by using Spearman's correlation. To accommodate for the left skew in the distribution of antibody levels, the summary statistics are presented as the median and interquartile range (25th–75th percentiles). To assess the temporal change in antibody levels with respect to potential contributing clinical and epidemiologic factors such as age (as a proxy to exposure and/or re-exposure), sex, HAZ score, WAZ score, water source, and exposure to family members with diarrhea multiple linear regression models were used.

RESULTS

Demographics, clinical features, and epidemiologic parameters. Comparison of demographics and clinical features of age-matched case and control children in the original matched study have been described.¹⁴ The demographics and clinical characteristics of the 47 *C. hominis*-infected cases and 39 uninfected controls in the present study after unmatched are shown in Table 1 and are similar to those in the original matched study. There were no significant differences in age, sex, and nutritional status (assessed by WAZ and HAZ scores) between the cases or controls. However, the duration of diarrhea and number of children with persistent diarrhea was significantly greater in cases than in controls. There was no significant difference in the duration of diarrhea or occurrence of persistent diarrhea at presentation in the

TABLE 1
Demographic and clinical characteristics of cases and controls at presentation, Bangladesh*

Characteristic	Cases (n = 47)	Controls (n = 39)	P
Age (months)	12.88 ± 5.80	12.59 ± 6.46	0.829
Male sex	29 (59%)	27 (69%)	0.378†
Duration of diarrhea (days)	8 (4, 13.5)‡	3 (1, 4)‡	< 0.0001
Persistent diarrhea§	18 (37%)	0 (0%)	< 0.0001†
Nutritional status			
Weight-for-age Z score	-2.73 ± 1.13	-2.46 ± 1.18	0.291
Height-for-age Z score	-1.94 ± 1.28	-1.66 ± 1.75	0.408
Weight-for-height Z score	-2.37 ± 1.37	-2.09 ± 1.51	0.383
Exposure to family member with diarrhea	6 (12%)	5 (13%)	1.00†
Contact with animals	7 (14%)	7 (18%)	0.771†
Water supply			
Tube well	30 (61%)	23 (59%)	1.00†
Municipal water supply	19 (39%)	16 (41%)	1.00†

*Values are mean ± SD unless otherwise indicated. All comparisons were made by using the unpaired *t*-test with Welch's correction unless otherwise specified.

† By Fisher's exact test.

‡ Data not normally distributed, represented as median (25th, 75th percentile), and comparisons were made by using the Mann-Whitney test.

§ Includes *Cryptosporidium*-infected persons who had persistent (≥ 14 days) diarrhea and *Cryptosporidium*-infected persons who had diarrhea for < 14 days at enrollment but continued to have diarrhea during the follow-up period for a total duration of diarrhea for ≥ 14 days, thus meeting the definition of persistent diarrhea.

patients who returned for follow-up compared with those who did not return for follow up. There were no differences in the frequencies of reported family members with diarrhea, animal contact, or type of water supply between cases and controls.

Serum antibody responses to *C. hominis* and *C. parvum* gp15. The serum IgG, IgA, and IgM levels to *C. hominis* and *C. parvum* gp15 at the initial (day 1) and follow-up (day 21) time points and the temporal change values (from initial to follow-up time points) are shown in Table 2 and summarized in Figure 2. At presentation, *C. hominis*-infected cases had significantly higher IgM levels to *C. hominis* gp15 and *C. parvum* gp15 than controls. At follow-up, IgG and IgA levels to *C. hominis* gp15 and *C. parvum* gp15 were significantly higher in the cases than in controls. However, although IgM levels to *C. hominis* gp15 were higher in cases than controls, IgM levels to *C. parvum* gp15 were not. Similarly, the change from the initial to the follow-up time points in IgG (Figure 3), IgA, and IgM levels to *C. hominis* gp15 was significantly greater in cases than in controls, but only for IgG and IgA to *C. parvum* gp15. In the controls, serum IgA levels to gp15 from *C. parvum* (but not *C. hominis*) were significantly higher at follow-up than at the initial time point.

The significant increase in IgG response to gp15 of both species was maintained after controlling for age, sex, duration of diarrhea, type of diarrhea, nutritional status (assessed

by WAZ and HAZ scores), presence of family members with diarrhea, and the baseline IgG levels, as shown by multivariate analyses. Overall, the magnitude of this increase was 41 EU (95% confidence interval = 25–79, *P* < 0.001) for *C. parvum* and 52 EU (95% confidence interval = 26–57, *P* < 0.001) for *C. hominis*. However, there were no significant changes in IgM or IgA levels to gp15 from either species in cases or controls after controlling for covariates in multivariate analysis.

Because changing the analysis strategy from a matched pairs design to an unmatched study design based on the PCR results rather than microscopy could introduce bias, we also conducted a matched analysis of serum antibody levels to gp15 based on a smaller number of matched case–control pairs that were PCR and microscopy positive or negative. There were no differences in the results. For this reason, we report the unmatched analysis that includes a greater sample size.

Comparison of antibody responses to gp15 in patients with acute versus persistent diarrhea. Previously, using *C. parvum* oocyst lysate as antigen for the ELISA, we found that persistent diarrhea (≥ 14 days) was associated with a significant decrease in IgA and IgM levels from the initial to the follow-up time period in cases.¹⁴ To determine if this was the case with antibody levels to gp15 alone, we compared anti-gp15 responses in cases with acute diarrhea (*n* = 22) with those with persistent diarrhea (*n* = 11) who returned for follow up. Cases with acute diarrhea had significantly lower IgM levels to *C. parvum* and *C. hominis* gp15 compared with cases with persistent diarrhea at presentation (Table 3). In addition, cases with acute diarrhea had a significantly greater increase in IgA levels to *C. hominis* gp15 from the initial to the follow-up time points than cases with persistent diarrhea.

Correlation between antibody levels to *C. parvum* and *C. hominis* gp15. To determine if there was cross-reactivity in antibody responses to gp15 from *C. hominis* and *C. parvum*, we compared antibody levels by using Spearman's correlation. There were statistically significant correlations between serum IgG, IgA, and IgM levels to gp15 from *C. hominis* and *C. parvum*, in cases at the initial and follow-up time points and in the temporal change between them (except for IgM), indicating that there is cross-reactivity to peptide epitopes of gp15 from both species (Table 4). In addition there were some significant correlations between serum antibody levels to gp15 and a mixture of antigens in oocyst lysates from the cognate species at the initial and follow-up time points or in the temporal change between them (Table 4).

Identification of polymorphisms in the gp15 sequence of *Cryptosporidium* spp. from fecal samples of case children. We

TABLE 2
Antibody levels to *Cryptosporidium* gp15 in cases and controls, Bangladesh*

Group	<i>C. parvum</i> gp15			<i>C. hominis</i> gp15		
	Initial	Follow-up	Change	Initial	Follow-up	Change
IgG controls	17 [10; 26]	19 [10; 28]†	4 [0; 10]	21 [14; 43]	24 [13; 29]†	0 [–7; 7]
Cases	24 [11; 56]‡§	110 [67; 149]‡§¶	68 [28; 110]‡¶	30 [19; 43]‡	110 [77; 185]‡¶§¶	62 [19; 124]‡¶
IgM controls	16 [0; 47]	0 [0; 14]†	0 [–; 0]	23 [5; 38]	10 [4; 18]†	0 [–7; –8]
Cases	52 [28; 103]‡¶§	0 [0; 48]‡§	–29 [–97; 13]‡¶	45 [23; 74]‡	42 [21; 95]‡¶§	2 [–20; 20]‡
IgA controls	7 [0; 35]	47 [3; 66]†¶	40 [3; 61]	22 [8; 68]	13 [3; 29]†	4 [–1; 24]
Cases	12 [0; 54]‡¶	95 [41; 258]‡¶§¶	83 [19; 243]‡¶	32 [0; 81]‡	80 [31; 158]‡¶§¶	50 [10; 138]‡¶

* Data are median [25th percentile; 75th percentile].

† Controls compared for initial vs. follow-up.

‡ Cases vs. controls compared at the initial and follow-up time points and the difference between them (change).

§ Cases compared for initial vs. follow-up.

¶ *P* < 0.05.

FIGURE 2. Median change in serum IgG, IgM and IgA levels to *Cryptosporidium parvum* and *C. hominis* gp15 in *C. hominis*-infected cases and uninfected controls over the three-week follow up period using rgp15 from the respective species as antigens for an enzyme-linked immunosorbent assay, Bangladesh. Closed squares = *C. parvum* rgp15, cases; open squares = *C. hominis* rgp15, cases; closed circles = *C. parvum* rgp15, controls; open circles = *C. hominis* rgp15, controls. The 25th and 75th percentiles and *P* values are shown in Table 2.

examined the gp15 nucleotide and deduced amino sequences of gp15 from 45 *C. hominis*, 4 *C. parvum*, and 1 *C. felis* samples from case-children and those of the *C. parvum* GCH1 and *C. hominis* TU502 isolates²⁹ used to generate the recombinant gp15 proteins used as antigens for ELISA in this study. Previously, we identified 7 subtype families (Ia, Ib, Id, Ie, If, IIa, and IIm) based on polymorphisms in the gp40/15 gene¹³ in samples from case children in the study.¹⁵ Five subtype families (Ia, Ib, Id, Ie, and If) were identified among the 45 *C. hominis* samples. All 4 *C. parvum* samples were of the IIm subtype family and the *C. felis* sample was of the IIa subtype family. The *C. parvum* GCH1 and *C. hominis* TU502 isolates belong to the IIa and Ia subtype families respectively. Multiple alignment of all 52 nucleotide and deduced amino acid sequences showed several SNPs and SAAPs among them. Deduced amino acid sequences of gp15 representative of each of the subtype families identified in children in the study and those of the GCH1 and TU502 isolates are shown in Figure 4. There were 30 SAAPs among the different subtype families identified in this study. The IIa and Ia subtype family sequences were more similar to each other than to the other subtype families and showed 16 SAAPs between them (Figure 4). However, despite the polymorphisms among them, all gp15 sequences analyzed displayed several conserved regions (Figure 4). Examination

of the deduced amino acid sequences showed that the experimentally determined N-terminal glutamic acid (E) residue of the mature gp15 protein from the GCH1 isolate²² was conserved among the Ia, Ib, Id, If, and IIa subtype families, but was replaced with an aspartic acid (D) residue in subtype family Ie and a glycine (G) residue in subtype family IIm. The predicted NTVLLKDAGS glycoposphatidyl inositol anchor attachment site previously identified in subtype family IIa was conserved among all subtype families, except for replacement of the threonine (T) residue with a lysine (K) in the Ia, Id, and IIm and an asparagine (N) residue in the Ib, Ie, and If subtype families.²⁷ A single N terminal threonine residue was predicted to be O-glycosylated in subtype families IIa, Ib, and Id, as was a carboxyl-terminal threonine residue in subtype family IIm. No predicted N-glycosylation sites were identified in any of the sequences.

DISCUSSION

Although *C. hominis* is the dominant species infecting humans, particularly in developing countries,¹³ there have been no studies documenting immune responses to cognate (from the same species) antigens in persons infected with this species and none that have investigated immune responses

FIGURE 3. Comparison of the change in serum IgG levels to *C. hominis* gp15 in individual *C. hominis*-infected cases and uninfected controls over the three-week follow up period, Bangladesh, using the Mann Whitney U test. *P* = 0.0001.

TABLE 3
Antibody levels to *Cryptosporidium* gp15 in cases with acute vs. persistent diarrhea, Bangladesh*

Antibody	<i>C. parvum</i> gp15			<i>C. hominis</i> gp15		
	Initial	Follow-up	Change	Initial	Follow-up	Change
IgG AD†	23 [11; 55]	111 [69; 155]	74 [35; 124]	31 [20; 83]	110 [73; 230]	62 [18; 176]
IgG PD‡	36 [15; 59]§	107 [61; 147]§	61 [17; 97]§	36 [18; 108]§	103 [95; 143]§	62 [20; 90]§
IgM AD	50 [28; 74]	14 [0; 66]	0 [0; 17]	31 [24; 61]	38 [21; 81]	4 [-21; 36]
IgM PD	103 [41; 167]§¶	14 [0; 66]§	0 [0; 10]§	64 [46; 155]§¶	43 [25; 123]§	-12 [-24; 22]§
IgA AD	11 [0; 31]	140 [41; 253]	113 [42; 232]	32 [0; 63]	82 [30; 333]	67 [21; 287]
IgA PD	48 [0; 90]§	82 [41; 181]§	19 [0; 181]§	61 [0; 125]§	62 [33; 136]§	29 [-40; 110]§¶

* Data are median [25th percentile; 75th percentile].

† Acute diarrhea (< 14 days), n = 22.

‡ Persistent diarrhea (≥ 14 days), n = 11.

§ Acute vs. persistent diarrhea compared at the initial and follow-up time points and the difference between them (change).

¶ P < 0.05.

to any *C. hominis* antigen. In this study in Bangladesh where cryptosporidiosis is endemic³⁰⁻³⁴ we found that serum antibody responses to the immunodominant gp15 antigen developed in *C. hominis*-infected children with diarrhea less than five years of age, in contrast to control children in the same age group with diarrhea but no evidence of *Cryptosporidium* infection. For the first time, we showed antibody responses to the cognate *C. hominis* gp15 antigen in *C. hominis*-infected children. We also found that despite considerable variation in the gp15 sequence among different *C. hominis* subtype families from children in the study, there was a statistically significant correlation between antibody levels to *C. hominis* and *C. parvum* gp15, indicating that there is cross-reactivity to peptide epitopes of this antigen from both species.

TABLE 4

Correlation of antibody levels to *Cryptosporidium hominis* and *C. parvum* gp15 and oocyst lysate in *C. hominis*-infected cases, Bangladesh*

<i>C. hominis</i> gp15 vs. <i>C. parvum</i> gp15		Spearman's R	P
IgG	Initial	0.82	< 0.001
	Follow-up	0.81	< 0.001
	Change	0.88	< 0.001
IgM	Initial	0.86	< 0.001
	Follow-up	0.02	NS
	Change	0.01	NS
IgA	Initial	0.94	< 0.001
	Follow-up	0.90	< 0.001
	Change	0.91	< 0.001
<i>C. hominis</i> gp15 vs. <i>C. hominis</i> oocyst lysate			
IgG	Initial	0.51	< 0.001
	Follow-up	-0.04	NS
	Change	0.29	NS
IgM	Initial	0.55	< 0.0001
	Follow-up	0.70	< 0.0001
	Change	0.34	< 0.05
IgA	Initial	0.06	NS
	Follow-up	0.46	< 0.01
	Change	0.58	< 0.001
<i>C. parvum</i> gp15 vs. <i>C. parvum</i> oocyst lysate			
IgG	Initial	0.44	< 0.01
	Follow-up	0.37	< 0.05
	Change	0.41	< 0.05
IgM	Initial	0.47	< 0.001
	Follow-up	0.24	NS
	Change	0.34	< 0.05
IgA	Initial	0.16	NS
	Follow-up	0.54	< 0.01
	Change	0.57	< 0.001

* Initial (n = 47), follow-up and change are those who returned for follow-up (n = 33). NS = not significant.

Serum antibody responses to the gp15/15-17-kD antigen have been reported in children with cryptosporidiosis in developing countries²¹ in children and adults after outbreaks of cryptosporidiosis³⁵⁻³⁷ and in adults infected with human immunodeficiency virus in developed countries.³⁸ However, all of these studies used partially purified native *C. parvum* 17-kD antigen or *C. parvum* lysate as antigen in ELISA or immunoblots. Our study is the first to use recombinant gp15 from both major species as antigens in ELISAs. In addition, our study is the first to investigate immune responses to native *C. hominis* antigens in oocyst lysates from this species and to compare antibody responses to a single antigen to that of a mixture of antigens in oocyst lysates.

Serum antibody levels of all three isotypes to gp15 from both species were significantly greater in cases than in controls. However, after we controlled for covariates such as age, sex, nutritional status, water source, and exposure to family members with diarrhea in multivariate analysis, at follow up only serum IgG levels to gp15 from both species and the increase in IgG levels from the initial to the follow-up time points were significantly greater in cases than in controls, which is consistent with our earlier findings using *C. parvum* oocyst lysate as antigen, although multivariate analysis was not performed in the previous study.¹⁴ In contrast to the significant increase in IgG levels to gp15 at follow-up, IgM levels were significantly lower at follow-up. This finding could be explained by the fact that an IgM response occurs immediately after an acute infection and levels decrease within weeks, whereas IgG responses are slower to appear but persist for a longer period. However, the decrease in IgM levels was not significant after controlling for covariates in multivariate analysis, whereas the increase in IgG levels remained significant. In the controls, serum IgA levels to gp15 from *C. parvum* were significantly higher at follow-up than at the initial time point. The reason for this finding is not clear. However, there was no significant increase in serum IgA levels to gp15 from *C. hominis*, with which all the cases included in this study were infected. At presentation, some cases and two controls had relatively high levels (> 100 ELISA units) of serum IgG to gp15 of both species (Figure 3 for *C. hominis* gp15 and data not shown for *C. parvum* gp15). This finding may reflect previous infection with *Cryptosporidium*, perhaps with a different species or subtype family.

Interestingly, there was a significantly greater increase in IgA levels to *C. hominis* gp15 from the initial to the follow-up time points in cases with acute diarrhea than in those with persistent diarrhea, which is similar to results from our previous study, in which there was a significant increase in IgA and IgM levels.¹⁴ This finding raises the possibility that increased serum

AAF82349 I Ia ETSEAAATVDLFAFTLDGGKRIEAVPNVEDASKRDKYSLVADDDKPFYTGANS GTTNGVYRLNENGDLVDKDNTVLLKDAGSSAFGLRYIVPSVFAALFVL
 76 I Ia ETSEAAATVDLFAFTLDGGKRIEAVPNVEDASKRDKYSLVADDDKPFYTGANS GTTNGVYRLNENGDLVDKDNTVLLKDAGSSAFGLRYIVPSVFAALFVL
 7 I Im GSE-TPATVDLFAFTLDGGKRIEAVPNVEDASKRDKYSLVADDDKPFYTGANS GTTNGVYRLNENGDLVDKDNTVLLKDAGSSAFWTSSDTSFLV
 AAL07533 I a ENGETVATVDLFAFTLDGGRRIEVAVPKDENADKRSEYSLVADDDKPFYTGANS GTTNGVYRLNENGDLVDKDNTVLLKDAGSSAFGRYIVPSVFAALFVL
 35 I a ENGETVATVDLFAFTLDGGRRIEVAVPKDENADKRSEYSLVADDDKPFYTGANS GTTNGVYRLNENGDLVDKDNTVLLKDAGSSAFGRYIVPSVFAALFVL
 13 I b ETS-ETATVDLFAFTLDGGKRIEAVPSDEDVSKRNKYSLVAGDKTFYTGANS GNTDGIYRLNDNGDLVDKNNVLLKDAGSSALGFR
 82 I d ETS-ETATVDLFAFTLDGGKRIEAVPNAEETSKRDKYSLVADDDKPFYTGANS GSTDGVYRLNENGDLVDKDNTVLLKDAGSSAFGRYIVLPF
 30 I e DGTETAATVDLIAFTLDGGKRIEAVPSDEDVSKRNKYSLVAGDKTFYTGANS GNTDGIYRLNDNGDLVDKNNVLLKDAGSSALGFR
 10 I f ESD-EVATVDLFAFTLDGGKRIEAVPSDDVSKRDKYSLVADDDKPFYTGANS GAENG IYKLDSDGNLVDKDNVLLKDAGSSALD

FIGURE 4. Multiple alignment of deduced amino acid sequences of gp15 from representative *Cryptosporidium hominis* and *C. parvum* subtype families identified in this study (Genbank accession nos. JF27170, JF927172, A700389, AY7700390, AY700393, AY700398 for samples 7, 10, 13, 30, 35, 76 and 82 respectively), *C. parvum* GCH1 Ia (Genbank accession no. AAF82349) and *C. hominis* TU502 Ia (Genbank accession no. AAL07533) isolates used to generate recombinant gp15 proteins used as antigens in the study. Conserved residues are outlined in dark gray. The experimentally determined N-terminus (glutamic acid, E) of the *C. parvum* GCH1 Ia isolate is double underlined. The predicted glycoposphatidyl inositol anchor attachment site is underlined. Predicted mucin type O-glycosylation sites are in italics.

IgA (which mainly reflects mucosal IgA) levels to gp15 may be associated with protection from persistent diarrhea.

The finding that there were significant correlations between antibody responses to gp15 and a mixture of antigens in oocyst lysates from the cognate species is consistent with gp15 being one of, if not the most, immunodominant *Cryptosporidium* antigens. It also suggests that antibody responses to gp15 could be used as a surrogate marker of antibody responses to *Cryptosporidium* in seroepidemiologic studies.

A previous study investigated longitudinal serum IgG responses to the 17-kD antigen (same as gp15) in children in a birth cohort in Peru by using partially purified 17-kD antigen from *C. parvum* (Iowa strain) as antigen in ELISAs.²¹ Although *C. hominis* was the predominant species identified, serum IgG responses to the 17-kD antigen were detected in children infected with other species, including *C. parvum*, *C. meleagridis*, and *C. felis* and with different subtype families, suggesting that IgG responses to this antigen are cross reactive. In the present study of *C. hominis*-infected children, despite significant variation in the deduced amino acid sequence of gp15 among five subtype families identified in these children, we found a statistically significant correlation between serum antibody levels to gp15 from both major species, directly demonstrating cross-reactivity to peptide epitopes of this antigen. Although we found a cross-reactive antibody response to gp15 from both species in *C. hominis*-infected children by using the same preparation of rgp15 from both species as antigen, our collaborators found that *C. hominis* but not *C. parvum* gp15 induced interferon- γ -mediated cellular immune responses in adult volunteers with serologic evidence of prior *Cryptosporidium* infection.²⁸ The infecting *Cryptosporidium* species in their study was not known. However, it is possible that in *C. hominis*-infected persons, cell-mediated immune responses may be directed at polymorphic peptide epitopes present in *C. hominis* but not in *C. parvum*.

Although the dominant antibody response appears to be targeted to conserved peptide epitopes of gp15, antibody responses to polymorphic, species- or subtype specific epitopes may also occur. In the study in Peru in which *C. parvum* 17-kD antigen was used in ELISAs, the authors noted that there was a lower sensitivity of detection of *C. hominis* infections and raised the possibility that this may reflect the greater variation observed in the 17-kD antigen sequence within this species.²¹ Similarly, in our study, the increase in IgA responses to *C. hominis* but not *C. parvum* gp15 was significantly greater in patients with acute diarrhea than in those with persistent diarrhea, raising the possibility that antibodies to specific epitopes on *C. hominis* gp15 may be involved. In addition, as noted above,

interferon- γ -mediated cellular responses were detected to *C. hominis* but not *C. parvum* gp15 in humans with serologic evidence of prior *Cryptosporidium* infection.²⁸

The findings of our study support further investigation of gp15 as a putative component of a subunit vaccine for cryptosporidiosis. However, there are a number of limitations to our study. In addition to the small numbers of cases and controls and the significant loss to follow-up, we were limited to investigation of serum antibody responses to peptide epitopes of gp15 in children from an urban setting presenting to hospital with diarrhea. In addition, we were not able to definitively determine whether cases or controls in the study were previously infected with *Cryptosporidium*. Also, cases (particularly those with persistent diarrhea) presented at varying times after the onset of diarrhea.¹⁴ Furthermore, we were not able to assess mucosal secretory IgA responses or cell-mediated responses to gp15, or to investigate immune responses in asymptomatic infection.

Despite the limitations of the study, our findings have important implications for development of gp15 as a putative vaccine candidate. Additional, community-based, longitudinal studies with larger numbers of persons with symptomatic and asymptomatic infections with different species and subtype families and regular and frequent investigation of mucosal and systemic cellular and humoral immune responses to defined conserved and polymorphic peptide epitopes of gp15 from different species and subtypes are required to evaluate the potential of this antigen as a putative component for subunit vaccine development.

Received January 20, 2011. Accepted for publication April 19, 2011.

Acknowledgments: We thank all the study participants and field and laboratory staff who participated in this study.

Financial support: This study was supported by an opportunity pool grant and in part by grants UO1 AI45508, U01 AI058935, and RO1 AI52786, all from the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH) and K24 AT003683 from the National Center for Complementary and Alternative Medicine, NIH. Genève M. Allison and Kathleen Rogers were supported by T32 AI07389, and Anoli Borad was supported by T32 AI007438 from the National Institute of Allergy and Infectious Diseases, NIH.

Authors' addresses: Genève M. Allison, Anne V. Kane, and Honorine D. Ward, Division of Geographic Medicine and Infectious Diseases, Department of Medicine, Tufts Medical Center, Boston, MA, E-mails: gallison@tuftsmedicalcenter.org, akane@tuftsmedicalcenter.org, and hward@tuftsmedicalcenter.org. Kathleen A. Rogers, University of North Carolina Center for AIDS Research, Chapel Hill, NC, E-mail: rogerska@med.unc.edu. Anoli Borad, Division of Infectious Diseases, Department of Medicine, Yale School of Medicine, New Haven, CT, E-mail: anoli.borad@yale.edu. Sabeena Ahmed, Mohammad

Mahbubul Karim, and Wasif A. Khan, Clinical Sciences Division, Centre for Health and Population Research, International Center for Diarrheal Disease Research, Dhaka, Bangladesh, E-mails: mahbubul@icddr.org, sabeena@hotmail.com, and wakhan@icddr.org. Patricia L. Hibberd, Division of Global Health, Department of Pediatrics, Massachusetts General Hospital, Boston, MA, E-mail: phibberd@partners.org. Elena N. Naumova, Tufts Initiative for the Forecasting and Modeling of Infectious Diseases, Tufts University School of Engineering, Medford, MA, E-mail: elena.naumova@tufts.edu. Stephen B. Calderwood and Edward T. Ryan, Division of Infectious Diseases, Massachusetts General Hospital, Boston, MA, E-mails: scalderwood@partners.org and etryan@partners.org.

REFERENCES

- Dillingham RA, Lima AA, Guerrant RL, 2002. Cryptosporidiosis: epidemiology and impact. *Microbes Infect* 4: 1059–1066.
- Collinet-Adler S, Ward HD, 2010. Cryptosporidiosis: environmental, therapeutic, and preventive challenges. *Eur J Clin Microbiol Infect Dis* 29: 927–935.
- Leav BA, Mackay M, Ward HD, 2003. *Cryptosporidium* species: new insights and old challenges. *Clin Infect Dis* 36: 903–908.
- Tzipori S, Ward H, 2002. Cryptosporidiosis: biology, pathogenesis and disease. *Microbes Infect* 4: 1047–1058.
- Checkley W, Epstein LD, Gilman RH, Black RE, Cabrera L, Sterling CR, 1998. Effects of *Cryptosporidium parvum* infection in Peruvian children: growth faltering and subsequent catch-up growth. *Am J Epidemiol* 148: 497–506.
- Guerrant DL, Moore SR, Lima AA, Patrick PD, Schorling JB, Guerrant RL, 1999. Association of early childhood diarrhea and cryptosporidiosis with impaired physical fitness and cognitive function four-seven years later in a poor urban community in northeast Brazil. *Am J Trop Med Hyg* 61: 707–713.
- Molbak K, Andersen M, Aaby P, Hojlyng N, Jakobsen M, Sodemann M, da Silva AP, 1997. *Cryptosporidium* infection in infancy as a cause of malnutrition: a community study from Guinea-Bissau, west Africa. *Am J Clin Nutr* 65: 149–152.
- Abubakar I, Aliyu SH, Arumugam C, Hunter PR, Usman NK, 2007. Prevention and treatment of cryptosporidiosis in immunocompromised patients. *Cochrane Database Syst Rev* CD004932.
- de Graaf DC, Spano F, Petry F, Sagodira S, Bonnin A, 1999. Speculation on whether a vaccine against cryptosporidiosis is a reality or fantasy. *Int J Parasitol* 29: 1289–1306.
- Wanyiri J, Ward H, 2006. Molecular basis of cryptosporidium-host cell interactions: recent advances and future prospects. *Future Microbiol* 1: 201–208.
- Borad A, Ward H, 2010. Human immune responses in cryptosporidiosis. *Future Microbiol* 5: 507–519.
- Riggs MW, 2002. Recent advances in cryptosporidiosis: the immune response. *Microbes Infect* 4: 1067–1080.
- Xiao L, 2010. Molecular epidemiology of cryptosporidiosis: an update. *Exp Parasitol* 124: 80–89.
- Khan WA, Rogers KA, Karim MM, Ahmed S, Hibberd PL, Calderwood SB, Ryan ET, Ward HD, 2004. Cryptosporidiosis among Bangladeshi children with diarrhea: a prospective, matched, case-control study of clinical features, epidemiology and systemic antibody responses. *Am J Trop Med Hyg* 71: 412–419.
- Hira KG, Mackay MR, Hempstead AD, Ahmed S, Karim MM, O'Connor RM, Hibberd PL, Calderwood SB, Ryan ET, Khan WA, Ward HD, 2011. Genetic diversity of *Cryptosporidium* spp. from Bangladeshi children. *J Clin Microbiol* Apr 6 [Epub ahead of print].
- Moss DM, Bennett SN, Arrowood MJ, Hurd MR, Lammie PJ, Wahlquist SP, Addiss DG, 1994. Kinetic and isotypic analysis of specific immunoglobulins from crew members with cryptosporidiosis on a U.S. Coast Guard cutter. *J Eukaryot Microbiol* 41: 52S–55S.
- Moss DM, Bennett SN, Arrowood MJ, Wahlquist SP, Lammie PJ, 1998. Enzyme-linked immunoelectrotransfer blot analysis of a cryptosporidiosis outbreak on a United States Coast Guard cutter. *Am J Trop Med Hyg* 58: 110–118.
- Mead JR, Arrowood MJ, Sterling CR, 1988. Antigens of *Cryptosporidium* sporozoites recognized by immune sera of infected animals and humans. *J Parasitol* 74: 135–143.
- Reperant JM, Naciri M, Chardes T, Bout DT, 1992. Immunological characterization of a 17-kDa antigen from *Cryptosporidium parvum* recognized early by mucosal IgA antibodies. *FEMS Microbiol Lett* 78: 7–14.
- Reperant JM, Naciri M, Iochmann S, Tilley M, Bout DT, 1994. Major antigens of *Cryptosporidium parvum* recognised by serum antibodies from different infected animal species and man. *Vet Parasitol* 55: 1–13.
- Priest JW, Bern C, Xiao L, Roberts JM, Kwon JP, Lescano AG, Checkley W, Cabrera L, Moss DM, Arrowood MJ, Sterling CR, Gilman RH, Lammie PJ, 2006. Longitudinal analysis of cryptosporidium species-specific immunoglobulin G antibody responses in Peruvian children. *Clin Vaccine Immunol* 13: 123–131.
- Cevallos AM, Zhang X, Waldor MK, Jaisson S, Zhou X, Tzipori S, Neutra MR, Ward HD, 2000. Molecular cloning and expression of a gene encoding *Cryptosporidium parvum* glycoproteins gp40 and gp15. *Infect Immun* 68: 4108–4116.
- Strong WB, Gut J, Nelson RG, 2000. Cloning and sequence analysis of a highly polymorphic *Cryptosporidium parvum* gene encoding a 60-kilodalton glycoprotein and characterization of its 15- and 45-kilodalton zoite surface antigen products. *Infect Immun* 68: 4117–4134.
- Priest JW, Kwon JP, Arrowood MJ, Lammie PJ, 2000. Cloning of the immunodominant 17-kDa antigen from *Cryptosporidium parvum*. *Mol Biochem Parasitol* 106: 261–271.
- Winter G, Gooley AA, Williams KL, Slade MB, 2000. Characterization of a major sporozoite surface glycoprotein of *Cryptosporidium parvum*. *Funct Integr Genomics* 1: 207–217.
- Priest JW, Mehlert A, Moss DM, Arrowood MJ, Ferguson MA, 2006. Characterization of the glycosylphosphatidylinositol anchor of the immunodominant *Cryptosporidium parvum* 17-kDa antigen. *Mol Biochem Parasitol* 149: 108–112.
- Priest JW, Xie LT, Arrowood MJ, Lammie PJ, 2001. The immunodominant 17-kDa antigen from *Cryptosporidium parvum* is glycosylphosphatidylinositol-anchored. *Mol Biochem Parasitol* 113: 117–126.
- Preidis GA, Wang HC, Lewis DE, Castellanos-Gonzalez A, Rogers KA, Graviss EA, Ward HD, White AC Jr, 2007. Seropositive human subjects produce interferon gamma after stimulation with recombinant *Cryptosporidium hominis* gp15. *Am J Trop Med Hyg* 77: 583–585.
- O'Connor RM, Thorpe CM, Cevallos AM, Ward HD, 2002. Expression of the highly polymorphic *Cryptosporidium parvum* Cpgp40/15 gene in genotype I and II isolates. *Mol Biochem Parasitol* 119: 203–215.
- Albert MJ, Faruque AS, Faruque SM, Sack RB, Mahalanabis D, 1999. Case-control study of enteropathogens associated with childhood diarrhea in Dhaka, Bangladesh. *J Clin Microbiol* 37: 3458–3464.
- Bhattacharya MK, Teka T, Faruque AS, Fuchs GJ, 1997. *Cryptosporidium* infection in children in urban Bangladesh. *J Trop Pediatr* 43: 282–286.
- Rahman M, Shahid NS, Rahman H, Sack DA, Rahman N, Hossain S, 1990. Cryptosporidiosis: a cause of diarrhea in Bangladesh. *Am J Trop Med Hyg* 42: 127–130.
- Shahid NS, Rahman AS, Sanyal SC, 1987. *Cryptosporidium* as a pathogen for diarrhoea in Bangladesh. *Trop Geogr Med* 39: 265–270.
- Haque R, Mondal D, Karim A, Molla IH, Rahim A, Faruque AS, Ahmad N, Kirkpatrick BD, Houpt E, Snider C, Petri WA Jr, 2009. Prospective case-control study of the association between common enteric protozoal parasites and diarrhea in Bangladesh. *Clin Infect Dis* 48: 1191–1197.
- McDonald AC, MacKenzie WR, Addiss DG, Gradus MS, Linke G, Zembrowski E, Hurd MR, Arrowood MJ, Lammie PJ, Priest JW, 2001. *Cryptosporidium parvum*-specific antibody responses among children residing in Milwaukee during the 1993 waterborne outbreak. *J Infect Dis* 183: 1373–1379.
- Frost FJ, Calderon RL, Muller TB, Curry M, Rodman JS, Moss DM, de la Cruz AA, 1998. A two-year follow-up survey of antibody to *Cryptosporidium* in Jackson County, Oregon following an outbreak of waterborne disease. *Epidemiol Infect* 121: 213–217.
- Frost FJ, Muller T, Craun GF, Fraser D, Thompson D, Notenboom R, Calderon RL, 2000. Serological analysis of a cryptosporidiosis epidemic. *Int J Epidemiol* 29: 376–379.
- Sandhu SK, Priest JW, Lammie PJ, Hubbard A, Colford JM Jr, Eisenberg JN, 2006. The natural history of antibody responses to *Cryptosporidium* parasites in men at high risk of HIV infection. *J Infect Dis* 194: 1428–1437.