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

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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 compared with 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. 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. 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, 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, 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. Understanding the immune response to these proteins is a key step in the identification of potential vaccine targets.

It is well known that cell-mediated immune responses are essential for protection from and clearance of Cryptosporidium infection. 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.

Two Cryptosporidium spp. cause most infections in humans. Cryptosporidium parvum infects animals and humans, whereas C. hominis primarily infects humans. Although most human infections, particularly in developing countries, are caused by C. hominis, 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). 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. 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.

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. One of the 15–17-kD group of antigens was subsequently identified to be gp15 (also called Cpl7, 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. The gp15 is the C-terminal proteolytic cleavage product of the precursor gp40/15 (also known as gp60) protein, and is linked to the surface membrane via a glycosphatidyl inositol anchor. 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.
gp15 induced interferon-γ–mediated cellular responses in previously infected humans. 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. 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. 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. 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. Details of the recruitment procedure have been described. 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. 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. 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. 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. 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

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**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. 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. 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.[C] The same batch of oocysts was used for all assays. Data using C. parvum oocyst lysate in ELISAs from our previous study 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. Briefly, microtiter plates (Nunc, Rochester, NY) were coated overnight with 0.4 μg of protein/well of recombinant (r) C. hominis or C. parvum gp15 or the control protein or C. hominis oocyst lysate equivalent to 5 × 10⁴ 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, and substrate solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂, containing p-nitrophenyl phosphate (1 mg/mL) (Sigma, St. Louis, MO) was added.

After 30 minutes, the absorbance at 405 nm (A₄₀₅nm) 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 A₄₀₅nm value obtained for the control protein was subtracted from the A₄₀₅nm value for gp15 protein. None of the A₄₀₅nm 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 A₄₀₅nm of the sample (after subtracting the A₄₀₅nm of the control protein) by the A₄₀₅nm 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 study 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. The demographics and clinical characteristics of the 47 C. hominis-infected cases and 39 uninfected controls in the present study after unmatching 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
Demographic and clinical characteristics of cases and controls at presentation, Bangladesh

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases (n = 47)</th>
<th>Controls (n = 39)</th>
<th>P</th>
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<tbody>
<tr>
<td>Age (months)</td>
<td>12.88 ± 5.80</td>
<td>12.59 ± 6.46</td>
<td>0.829</td>
</tr>
<tr>
<td>Sex</td>
<td>29 (59%)</td>
<td>27 (69%)</td>
<td>0.378†</td>
</tr>
<tr>
<td>Duration of diarrhea (days)</td>
<td>8 (4, 13.5)‡</td>
<td>3 (1, 4)‡</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Nutritional status</td>
<td>18 (37%)</td>
<td>0 (0%)</td>
<td>&lt;0.0001†</td>
</tr>
<tr>
<td>Weight-for-age Z score</td>
<td>−2.73 ± 1.13</td>
<td>−2.46 ± 1.18</td>
<td>0.291</td>
</tr>
<tr>
<td>Height-for-age Z score</td>
<td>−1.94 ± 1.28</td>
<td>−1.66 ± 1.75</td>
<td>0.408</td>
</tr>
<tr>
<td>Weight-for-height Z score</td>
<td>−3.37 ± 1.37</td>
<td>−2.09 ± 1.51</td>
<td>0.383</td>
</tr>
<tr>
<td>Exposure to family member</td>
<td>6 (12%)</td>
<td>5 (13%)</td>
<td>1.00†</td>
</tr>
<tr>
<td>Contact with animals</td>
<td>7 (14%)</td>
<td>7 (18%)</td>
<td>0.771†</td>
</tr>
<tr>
<td>Water supply</td>
<td>30 (61%)</td>
<td>23 (59%)</td>
<td>1.00‡</td>
</tr>
<tr>
<td>Municipal water supply</td>
<td>19 (39%)</td>
<td>16 (41%)</td>
<td>1.00‡</td>
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</table>

*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 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.

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) and 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 = 26–75, 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. 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>
<thead>
<tr>
<th>Table 2</th>
<th>Antibody levels to Cryptosporidium gp15 in cases and controls, Bangladesh*</th>
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<tbody>
<tr>
<td></td>
<td>C. parvum gp15</td>
</tr>
<tr>
<td>Group</td>
<td>Initial Follow-up</td>
</tr>
<tr>
<td>IgG controls</td>
<td>17 [10; 26]†</td>
</tr>
<tr>
<td>Cases</td>
<td>24 [11; 56]‡</td>
</tr>
<tr>
<td>IgM controls</td>
<td>16 [0; 47]</td>
</tr>
<tr>
<td>Cases</td>
<td>52 [28; 103]</td>
</tr>
<tr>
<td>IgA controls</td>
<td>7 [0; 35]</td>
</tr>
<tr>
<td>Cases</td>
<td>12 [0; 54]</td>
</tr>
</tbody>
</table>

*Data are median [25th percentile; 75th percentile].
†Controls compared vs. 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.
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 isolates 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, Iiia, and Iiim) based on polymorphisms in the gp40/15 gene in samples from case children in the study. 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 Iiim subtype family and the C. felis sample was of the Iiia subtype family. The C. parvum GCH1 and C. hominis TU502 isolates belong to the Iiia 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 Iiia and Ia subtype family sequences were more similar to each other than to 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 isolate was conserved among the Ia, Ib, Id, Ie, and Iiia subtype families, but was replaced with an aspartic acid (D) residue in subtype family Ie and a glycine (G) residue in subtype family Iiim. The predicted NTVLLKDGAGS glycosphatidyl inositol anchor attachment site previously identified in subtype family Iiia was conserved among all subtype families, except for replacement of the threonine (T) residue with a lysine (K) in the Ia, Id, and Iiim and an asparagine (N) residue in the Ib, Ie, and If subtype families. A single N terminal threonine residue was predicted to be O-glycosylated in subtype families Iiia, Ib, and Id, as was a carboxyl-terminal threonine residue in subtype family Iiim. 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, 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...
to any C. hominis antigen. In this study in Bangladesh where cryptosporidiosis is endemic,\textsuperscript{10-38} 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.

Serum antibody responses to the gp15/15–17-kD antigen have been reported in children with cryptosporidiosis in developing countries\textsuperscript{39} in children and adults after outbreaks of cryptosporidiosis\textsuperscript{35-37} and in adults infected with human immunodeficiency virus in developed countries.\textsuperscript{40} 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.\textsuperscript{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 relative 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.\textsuperscript{14} This finding raises the possibility that increased serum

\begin{table}[h]
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\caption{Correlation of antibody levels to Cryptosporidium hominis and Cryptosporidium parvum gp15 and oocyst lysate in C. hominis-infected cases, Bangladesh\textsuperscript{8}}
\begin{tabular}{lccc}
\hline
\textbf{C. hominis gp15 vs. C. parvum gp15} & \textbf{Spearman’s R} & \textbf{P} \\
\hline
\textbf{IgG} & Initial & 0.82 & < 0.001 \\
& Follow-up & 0.81 & < 0.001 \\
& Change & 0.88 & < 0.001 \\
\textbf{IgM} & Initial & 0.86 & < 0.001 \\
& Follow-up & 0.02 & NS \\
& Change & 0.01 & NS \\
\textbf{IgA} & Initial & 0.94 & < 0.001 \\
& Follow-up & 0.90 & < 0.001 \\
& Change & 0.91 & < 0.001 \\
\hline
\textbf{C. hominis gp15 vs. C. hominis oocyst lysate} & & & \\
\begin{tabular}{lccc}
\hline
\textbf{IgG} & Initial & 0.51 & < 0.001 \\
& Follow-up & -0.04 & NS \\
& Change & 0.29 & NS \\
\textbf{IgM} & Initial & 0.55 & < 0.0001 \\
& Follow-up & 0.70 & < 0.0001 \\
& Change & 0.34 & < 0.05 \\
\textbf{IgA} & Initial & 0.06 & NS \\
& Follow-up & 0.46 & < 0.001 \\
& Change & 0.58 & < 0.001 \\
\hline
\textbf{C. parvum gp15 vs. C. parvum oocyst lysate} & & & \\
\begin{tabular}{lccc}
\hline
\textbf{IgG} & Initial & 0.44 & < 0.01 \\
& Follow-up & 0.37 & < 0.05 \\
& Change & 0.41 & < 0.05 \\
\textbf{IgM} & Initial & 0.47 & < 0.001 \\
& Follow-up & 0.24 & NS \\
& Change & 0.34 & < 0.05 \\
\textbf{IgA} & Initial & 0.16 & NS \\
& Follow-up & 0.54 & < 0.01 \\
& Change & 0.57 & < 0.001 \\
\hline
\end{tabular}
\end{tabular}
\end{table}
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 tgp15 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.\(^{22}\) 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.\(^{23}\) 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.

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**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, JF27172, A700389, KY770350, KY770393, KY770398 for samples 7, 10, 13, 30, 35, 76 and 82 respectively). **C. parvum** GCH1 Ila (Genbank accession no. AAFL2349) and **C. hominis**Tu502 la (Genbank accession no. AAL07533) isolates used to generate recombinant gp15 proteins used as antigens in the study. Conserved residues are indicated in dark gray. The experimentally determined N-terminus (glutamic acid, E) of the **C. parvum** GCH1 Ila isolate is double underlined. The predicted glycosphophatidyl inositol anchor attachment site is underlined. Predicted mucin type O-glycosylation sites are in italics.
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