Systemic Antibody Responses to the Immunodominant p23 Antigen and p23 Polymorphisms in Children with Cryptosporidiosis in Bangladesh


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Abstract. Cryptosporidium is a major cause of diarrhea in children in developing countries. However, there is no vaccine available and little is known about immune responses to protective antigens. We investigated antibody responses to p23, a putative vaccine candidate, in children in Bangladesh with cryptosporidiosis and diarrhea (cases) and uninfected children with diarrhea (controls), and p23 gene polymorphisms in infecting species. Serum IgM, IgG, and IgA responses to p23 were significantly greater in cases than controls after three weeks of follow-up. Cases with acute diarrhea had significantly greater serum IgA and IgM responses than those with persistent diarrhea, which suggested an association with protection from prolonged disease. The p23 sequences were relatively conserved among infecting species and subtype families. Although most children were infected with Cryptosporidium hominis, there was a cross-reactive antibody response to C. parvum antigen. These results support further development of p23 as a vaccine candidate.

INTRODUCTION

Cryptosporidium spp. of the phylum Apicomplexa are a significant cause of diarrheal illness worldwide, particularly in untreated patients with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and children in resource-poor countries. In these countries, cryptosporidiosis in early childhood is associated with subsequent malnutrition, impairment in growth, physical fitness, and cognitive development. The immune system of the host is critical in mediating protection from and resolution of cryptosporidiosis. In immunocompetent hosts, the infection is generally asymptomatic or self-limited. However, in immunocompromised hosts including patients with HIV/AIDS, the disease can be chronic, severe, and possibly fatal. Therapeutic options for cryptosporidiosis are limited. Nitazoxanide is effective in immunocompetent hosts and is the only drug that has been approved by the Food and Drug Administration for treatment of cryptosporidiosis in the United States. However, this drug is not effective in the immunocompromised host and has not been widely tested in children in developing countries. Children in these countries are considered an important target group for vaccine development. However, there is no vaccine available for cryptosporidiosis. Thus, identification of putative protective antigens and characterization of human immune responses to them is essential for development of effective vaccines for this disease in vulnerable populations.

One of these antigens is p23 (also known as Cp23, p27 or 27-kDa antigen), which is a surface-associated, immunodominant antigen present on invasive stages of the parasite and shed from their surface during gliding motility. Monoclonal and bovine colostral antibodies to p23 protect against Cryptosporidium parvum challenge in mice and calves, respectively. The p23 antigen induces serum, mucosal, humoral, and cell-mediated immune responses in experimentally infected or immunized animals and active immunization with DNA or peptide vaccines targeting p23 has been shown to confer varying degrees of protection in animal models.

The p23 antigen is one of the most immunodominant Cryptosporidium antigens and is consistently recognized by serum from actively infected or previously exposed humans. The presence of pre-existing antibodies to this antigen was associated with reduced oocyst shedding and protection from diarrhea in infected human volunteers. In addition, serum antibody responses to p23 were associated with a reduced risk of diarrheal illness in immunosuppressed persons. The p23 antigen also induced cell-mediated and antibody responses in persons previously exposed to Cryptosporidium spp. Taken together, these findings identify p23 as a putative vaccine candidate. This antigen is considered one of the most promising candidates for vaccine development. However, there have been few clinical studies of immune responses to this antigen in well-defined cohorts, particularly in children in resource-poor countries.

Most Cryptosporidium infections are caused by two species: C. hominis almost exclusively infects humans, whereas C. parvum infects humans in addition to animals. Most human infections, particularly in resource-poor countries, are caused by C. hominis. Genes encoding other surface-associated Cryptosporidium proteins including gp40 and Muc4 and are highly polymorphic among clinical isolates of different species and subtypes. If p23 is to be considered as a vaccine candidate, it is essential to investigate polymorphisms in the gene that encodes it among clinical isolates in different geographic areas. A previous investigation of Cryptosporidium isolates found ten nucleotide polymorphisms between C. hominis and C. parvum, three of which result in amino acid changes. However, polymorphisms in this gene have not been extensively investigated in samples from clinical studies in different geographic areas.

The present study is part of a prospective case–control study to evaluate the clinical, epidemiologic, and immunologic features of cryptosporidiosis in children with diarrhea coming to

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the Dhaka Hospital of the International Center for Diarrheal Disease Research (ICDDR, B) in Dhaka, Bangladesh. We previously reported the clinical and epidemiologic features and immune responses to total antigens in oocyst lysate preparations from *C. parvum* and *C. hominis* and to gp15.43 another immunodominant *Cryptosporidium* antigen in the same cohort of children.

The aim of the current study was to investigate serum IgG, IgM, and IgA responses to p23 in serum samples from these children using recombinant *C. parvum* p23 (rp23) protein as antigen in enzyme-linked immunosorbent assays (ELISAs), to determine if there are any clinical or epidemiologic associations with antibody responses to p23 and to compare antibody responses to p23 with those to *C. parvum* lysate and gp15. An additional aim was to characterize polymorphisms in the p23 nucleotide and deduced amino acid sequences among the *Cryptosporidium* spp. infecting these children.

**METHODS**

**Patients.** Children 15 days to 5 years of age with diarrhea who came to the Dhaka Hospital of the ICDDR, B were enrolled in the original study.46 Details of the recruitment procedure have been described.46 Informed consent was obtained from the parents or guardians of all children recruited to the study according to the guidelines of the Ethical Review Committee of the ICDDR, B, which approved the study.46 Diarrhea was defined as ≥3 watery stools within a 24-hour period. A diarrheal episode was defined as diarrhea for ≥72 hours. The end of a diarrheal episode was defined as absence of diarrhea for 48 hours. Persistent diarrhea was defined as a diarrheal episode lasting <14 days. Acute diarrhea was defined as a diarrheal episode lasting ≥14 days.

In the original study, 46 children with diarrhea and stools positive for *Cryptosporidium* spp. by microscopy were enrolled as cases and an equal number of age-matched children with diarrhea but whose stools were negative for *Cryptosporidium* spp. by microscopy were enrolled as controls.46 Subsequently, *Cryptosporidium* was detected by polymerase chain reaction (PCR) at the 18s ribosomal RNA locus in all 46 case-patients and in 7 control subjects.47 Thus, for subsequent studies, the study design was unmatched to include 53 cases and 39 controls. For this study, serum was available for 49 cases and 32 controls at hospitalization and for 32 cases and 17 controls at follow-up (Figure 1).

**Recombinant p23.** The plasmid containing the p23 insert cloned into the pGEX 4T-2 vector was obtained from Dr. Jeffrey Priest (Centers for Disease Control and Prevention, Atlanta, GA) under a Materials Transfer Agreement with North Carolina State University (Raleigh, NC) and over-expressed in *Escherichia coli* BL21 cells as described.30 The recombinant p23 (rp23)—glutathione-S-transferase fusion protein was purified by affinity chromatography using a glutathione Sepharose 4B column (Amersham Biosciences, Piscataway, NJ). The glutathione-S-transferase tag was cleaved off with thrombin as described.40 Protein concentration was measured using the Micro BCA protein assay kit (Pierce, Rockford, IL).

**Gene sequencing of p23.** DNA was extracted from two stool samples representative of each of five *C. hominis* and one *C. parvum* subtype families identified from children in the study43 and from three children infected with the subtype Iic family in Vellore, India48 by using a QIAmp Stool Extraction Kit (QIAGEN, Valencia, CA). A 549-basepair fragment of the p23 gene was amplified by using primers 5’-ATTATTTTTACGTTTCTTCCACTTG-3’ and 5’-ACC TAAATAAAAACACTCTATTGG-3’ and the proofreading polymerases ProofStart (QIAGEN) or PFX-4 (Invitrogen, Carlsbad, CA).

When using the ProofStart enzyme, PCR conditions were 35 amplification cycles at 95°C for 5 minutes, 94°C for 1 minute, 50°C for 1 minute, followed by 72°C for 1 minute. For the PFX-4 enzyme, PCR conditions were 94°C for 5 minutes (initial activation), followed by 35 cycles at 94°C for 15 seconds, 50°C for 30 seconds, and 68°C for 1 minute. The PCR products were sequenced at Mclab (Molecular Cloning Laboratories, South San Francisco, CA) using an ABI 3730XL sequencer (Applied Biosystems, Foster City, CA).

In addition, p23 PCR products from two samples belonging to the *C. parvum* Iim subtype family from this study and two samples from the *C. parvum* subtype Iic family from India, which were identified by PCR restriction fragment length polymorphism analysis or sequence analysis of the gp40/15 gene as described,47,49 were cloned into the pCR II-TOPO vector using the TOPO TA cloning Dual promoter kit (Invitrogen). Plasmid DNA was isolated from the bacterial cultures by using the QIAPrep Spin Miniprep Kit (QIAGEN) and sequenced.

Nucleotide and deduced amino acid sequences were aligned by using the Clustal W algorithm in the Align X program of Vector NTI Advance v 11.5 software (Invitrogen). The consensus p23 sequences from ≥2 clones from the Iim and Iic subtype families were identified by using the same program. Phylogenetic analysis was performed by using the maximum-likelihood method and the Phylogeny.fr server (http://www.phylogeny.fr). Predicted sites of N and O-glycosylation were identified by using the NetNGLyce 1.0 and NetOGlyc 3.1 servers respectively at the ExPASy proteomics server (http://expasy.org/). Nucleotide and deduced amino acid sequences from this study were deposited in GenBank (accession nos. JF927201-12).
ELISA. Serum samples were shipped to Boston on dry ice and stored at −80°C. Approval for use of de-identified serum samples from children in the study was obtained from the Tufts Medical Center Institutional Review Board. Serum IgG, IgA, and IgM responses to p23 were assessed by ELISA using rp23 as antigen as described. Briefly, 96-well microtiter plates (Nunc, Rochester, NY) were coated with 0.1 µg of rp23 per well, overnight at 4°C. The plates were washed three times with 0.05% Tween 20 in 20 mM sodium phosphate buffer, pH 7.0, 150 mM NaCl (phosphate-buffered saline [PBS]) by using an automated plate washer (BioTek-Elx505, Winooski, VT). Non-specific binding was blocked with 0.25% bovine serum albumin (BSA) in PBS for 2 hours at 37°C. Serum diluted 1:100 in 0.25% PBS-BSA was added and plates were incubated for 1 hour at 37°C.

After three washes with 0.05% Tween 20-PBS, alkaline phosphatase-conjugated IgG (γ chain specific), IgA (α chain specific), or IgM (μ chain specific) (Southern Biotech, Birmingham, AL) diluted in 0.25% PBS-BSA were added, and the plates were incubated at 37°C for 1 hour. After three washes, substrate solution containing 1 mg/mL of p-nitrophenyl phosphate (Sigma, St. Louis, MO) in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl2 was added, and the plates were incubated at room temperature for 30 minutes in the dark. Absorbance at 405 nm (A405 nm) was measured with a Bio-Rad Microplate Reader (Model 550; Bio-Rad Laboratories, Hercules, CA). The same Cryptosporidium-positive and -negative serum samples (positive or negative for reactivity with p23 by Western blotting of a C. parvum lysate) were run on each plate. All samples were run in triplicate, and the mean was determined.

To control for plate-to-plate variability, values were normalized by dividing the A405 nm of each sample on a plate by the A405 nm of the positive control for that plate and multiplying by 100. The effect of such normalization on inter-plate and intra-plate variability in ELISAs using the same serum samples was validated previously by using mixed effects regression models that indicated that the normalized values represent the original measures and are invariant to plate performance. Therefore, results presented are expressed in normalized ELISA units.

Statistical analysis. Statistical analysis was performed using S-plus version 8 (Insightful, Inc., Seattle, WA) statistical software and data plotted with Prism software version 5 (GraphPad Software Inc., San Diego, CA). Dichotomous demographic and clinical characteristics were compared by using Fisher’s exact test. Continuous variables were compared using the unpaired t-test with Welch’s correction for normally distributed variables, as defined by a standard test of normality, or the Mann-Whitney U test for non-normally distributed variables. To accommodate for the right skew in the distribution of antibody levels, the summary statistics are presented as the median and interquartile range (25th to 75th percentiles). Serum antibody levels to p23 at the initial and follow-up time points between cases and controls and between cases with acute or persistent diarrhea were compared by using the Mann-Whitney U test. Serum antibody levels at the initial and follow-up time points within cases and controls were compared by using the Wilcoxon matched pairs test. Changes in serum antibody levels from the initial to the follow-up time points in cases and controls were compared by using the unpaired t-test with Welch’s correction. Associations between antibody levels to p23 and gp15 or C. parvum lysate were measured by using Spearman’s correlation.

The weight-for-age, height-for-age, and weight-for-height Z-scores were calculated by using World Health Organization Anthro software (http://www.who.int/childgrowth/software/en). Multivariate analysis of antibody responses was performed by using a generalized linear model to control for potential contributing clinical and epidemiologic factors such as age, sex, duration of diarrhea, nutritional status (expressed by the weight-for-age scores), water source, and exposure to family members with diarrhea.

RESULTS

Clinical and epidemiologic features. The demographic and clinical features of age-matched case and control children in the original age-matched study have been described. In the current unmatched case–control study, there were no significant differences in demographic and clinical characteristics such as age, sex, and nutritional status between cases or controls (Table 1). However, as in the original study, the duration of diarrhea at hospitalization was significantly greater in cases than in controls. In addition, 18 cases had persistent diarrhea (>14 days) compared with none in the controls (Table 1). Of note, there was no significant difference in the duration of

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**Table 1**

Demographic, clinical, and epidemiologic characteristics of cases of cryptosporidiosis in children and controls at presentation, Bangladesh

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases (n = 49)</th>
<th>Controls (n = 39)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in months, median (25th, 75th percentile)</td>
<td>12 (9, 15)</td>
<td>11 (7, 15)</td>
<td>0.68†</td>
</tr>
<tr>
<td>Male sex, no. (%)</td>
<td>29 (59)</td>
<td>27 (69)</td>
<td>0.38‡</td>
</tr>
<tr>
<td>Duration of diarrhea in days, median (25th, 75th percentile)</td>
<td>8 (4, 13.5)</td>
<td>3 (1, 4)</td>
<td>&lt;0.0001‡</td>
</tr>
<tr>
<td>Persistent diarrhea (&gt;14 days), no. (%)</td>
<td>18 (36.7)</td>
<td>0 (0)</td>
<td>&lt;0.0001‡</td>
</tr>
<tr>
<td>Nutritional status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight-for-age Z score, mean (SD)</td>
<td>−2.73 (1.13)</td>
<td>−2.46 (1.18)</td>
<td>0.29§</td>
</tr>
<tr>
<td>Height-for-age Z score, mean (SD)</td>
<td>−1.94 (1.28)</td>
<td>−1.66 (1.75)</td>
<td>0.41§</td>
</tr>
<tr>
<td>Weight-for-height Z score, mean (SD)</td>
<td>−2.37 (1.37)</td>
<td>−2.09 (1.51)</td>
<td>0.38§</td>
</tr>
<tr>
<td>Exposure to family member with diarrhea, no. (%)</td>
<td>6 (12)</td>
<td>5 (13)</td>
<td>1.00‡</td>
</tr>
<tr>
<td>Contact with animals, no. (%)</td>
<td>7 (14)</td>
<td>7 (18)</td>
<td>0.77‡</td>
</tr>
<tr>
<td>Water source</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tube well, no. (%)</td>
<td>30 (61)</td>
<td>23 (59)</td>
<td>1.00‡</td>
</tr>
<tr>
<td>Municipal water supply, no. (%)</td>
<td>19 (39)</td>
<td>16 (41)</td>
<td>1.00‡</td>
</tr>
</tbody>
</table>

*P values < 0.05 are indicated in bold.
†By Mann-Whitney test.
‡By Fisher’s exact test.
§By unpaired t-test with Welch’s correction.
diarrhea or occurrence of persistent diarrhea in the patients that returned for follow-up compared than in those who did not return for follow-up. Frequencies of reported family members with diarrhea or animal contact or type of water supply were similar in cases and controls.

**Serum antibody responses to C. parvum p23.** Serum IgA and IgM (but not IgG) levels to p23 were significantly greater in cases than in controls at hospitalization (Table 2). However, when antibody levels were compared by multivariate analysis using a generalized linear regression model to control for covariates such as age, sex, nutritional status, water source, and exposure to family members with diarrhea, there were no significant differences in these levels between cases and controls. In addition, there were no significant differences in serum anti-p23 IgG, IgM, or IgA levels between the initial and follow-up time points in controls. Of note, there were no significant differences between the initial antibody levels of cases or controls that returned for follow-up versus those who did not return for follow-up. Serum p23 IgG, IgA, and IgM levels to p23 at the three-week follow-up time point were significantly higher in cases than in controls (Table 2). These differences remained significant after multivariate analysis. In addition, there was a significantly greater increase in serum anti-p23 IgG, IgA, and IgM levels from the initial to the follow-up time points in cases compared with controls (Table 2 and Figure 2). These differences also remained significant in multivariate analysis.

**Serum antibody responses to p23 in cases with acute versus persistent diarrhea.** The only significant differences in clinical and epidemiologic features between cases and controls were the duration of diarrhea and occurrence of persistent diarrhea. We compared the antibody responses to p23 in 22 cases with acute (< 14 days) diarrhea with 10 cases with persistent (≥ 14 days) diarrhea. There were no significant differences in IgG levels (at the initial or follow-up time points or in the change from initial to follow-up time points) among cases with acute or persistent diarrhea. However, the initial IgA and IgM levels were significantly lower in those with acute diarrhea than in those with persistent diarrhea (P = 0.05 and P = 0.01 for IgA and IgM, respectively). In addition, the change in IgA and IgM levels from the initial to follow-up time points was significantly greater in those with acute diarrhea than in those with persistent diarrhea (Figure 3).

**Correlation of serum antibody responses to p23 with those to C. parvum gp15 and C. parvum lysate.** To determine if there was any association between serum antibody levels to p23 and

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases</th>
<th>Controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>62.4 (23.9, 190.3)</td>
<td>53.14 (23.71, 154.5)</td>
<td>0.98</td>
</tr>
<tr>
<td>Follow-up</td>
<td>307 (185.4, 478.7)</td>
<td>32.79 (10.5, 73.75)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Change¶</td>
<td>207.4 ± 187.6</td>
<td>−35.59 ± 97.05</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IgA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>47.18 (0, 304.2)</td>
<td>0 (0, 55)</td>
<td>0.03</td>
</tr>
<tr>
<td>Follow-up</td>
<td>305.7 (140.9, 541.5)</td>
<td>20 (20, 38.82)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Change¶</td>
<td>315 ± 671.7</td>
<td>6.45 ± 21.43</td>
<td>0.01</td>
</tr>
<tr>
<td>IgM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>113.5 (62.92, 221)</td>
<td>53.2 (31.3, 100.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Follow-up</td>
<td>170.6 (133.9, 291.2)</td>
<td>36.92 (21.45, 50.62)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Change¶</td>
<td>84.57 ± 169.7</td>
<td>−4.24 ± 15.48</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*Values are medians (25th, 75th percentiles) and comparisons were made by using the Mann-Whitney test unless otherwise indicated.*

*Cases: initial, n = 49; follow-up, n = 32.

1 Controls: initial, n = 49; follow-up, n = 17.

2 P values < 0.05 are indicated in bold.

3 Change in antibody levels from initial to follow-up time points are represented as mean ± SD and compared by using the unpaired t-test with Welch’s correction.

**Figure 2.** Median serum IgG, IgM, and IgA levels to *Cryptosporidium* (C) p23 in cases and controls at the initial and follow-up time points determined by enzyme-linked immunosorbent assay with recombinant p23 as antigen in children with cryptosporidiosis in Bangladesh. Changes in antibody levels over the three-week follow-up period were compared by using the unpaired t-test with Welch’s correction.

**Figure 3.** Median serum IgM and IgA levels to *Cryptosporidium* (C) p23 in cases with acute or persistent diarrhea at the initial and follow-up time points determined by enzyme-linked immunosorbent assay with recombinant p23 as antigen in children with cryptosporidiosis in Bangladesh. Changes in antibody levels over the three-week follow-up period were compared by using the unpaired t-test with Welch’s correction.
those to the immunodominant gp15 antigen, or to total antigens in C. parvum lysate, we compared antibody levels to p23 with each of these antigen preparations from previous studies in the same children. There were significant correlations in the initial, follow-up, and change between the IgG, IgA, and IgM (except for IgM follow-up) antibody levels to p23 with those to gp15 in cases over the three-week follow-up period (Table 3). In addition, there were significant correlations in antibody levels (except for IgG follow-up and change) to p23 with those to C. parvum lysate at the initial, follow-up, and change between them (Table 3).

**Analysis of p23 polymorphisms among infecting Cryptosporidium spp. subtypes.** Molecular characterization of the Cryptosporidium spp. infecting case children in the study identified seven subtype families from C. parvum (IIm), C. hominis (Ia, Ib, Id, Ie, and If), and C. felis (IIa). The C. parvum IIm subtype family has only recently been identified in Bangladesh, India, and Nigeria, and is considered an anthropo tonic subtype because it has not been identified in animals. The other anthropo tonic subtype family, IIc, was not identified in this study. We therefore amplified the p23 sequence from DNA from stool samples of three children infected with this subtype family in a previous study on cryptosporidiosis in children with diarrhea in Vellore, India.

To determine if there were differences in the p23 sequences from subtype families of C. parvum and C. hominis spp. infecting children in this study with those in the published C. parvum and C. hominis genomes, as well as other p23 sequences deposited in GenBank, we performed phylogenetic analysis. This analysis showed that the p23 nucleotide and deduced amino acid sequences from the major species clustered separately for the most part (Figure 4).

**TABLE 3**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Spearman correlation values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp23 vs. gp15</td>
<td></td>
</tr>
<tr>
<td>IgG Initial</td>
<td>0.53†</td>
</tr>
<tr>
<td>Follow-up</td>
<td>0.39‡</td>
</tr>
<tr>
<td>Change</td>
<td>0.51†</td>
</tr>
<tr>
<td>IgA Initial</td>
<td>0.36‡</td>
</tr>
<tr>
<td>Follow-up</td>
<td>0.47‡</td>
</tr>
<tr>
<td>Change</td>
<td>0.64‡</td>
</tr>
<tr>
<td>IgM Initial</td>
<td>0.70†</td>
</tr>
<tr>
<td>Follow-up</td>
<td>0.12</td>
</tr>
<tr>
<td>Change</td>
<td>0.57‡</td>
</tr>
<tr>
<td>Cp23 vs. C. parvum lysate</td>
<td></td>
</tr>
<tr>
<td>IgG Initial</td>
<td>0.50†</td>
</tr>
<tr>
<td>Follow-up</td>
<td>0.14</td>
</tr>
<tr>
<td>Change</td>
<td>0.10</td>
</tr>
<tr>
<td>IgA Initial</td>
<td>0.65†</td>
</tr>
<tr>
<td>Follow-up</td>
<td>0.48‡</td>
</tr>
<tr>
<td>Change</td>
<td>0.71†</td>
</tr>
<tr>
<td>IgM Initial</td>
<td>0.46†</td>
</tr>
<tr>
<td>Follow-up</td>
<td>0.53‡</td>
</tr>
<tr>
<td>Change</td>
<td>0.57‡</td>
</tr>
</tbody>
</table>

*Cases: initial, n = 49; follow-up, n = 32; change, n = 32.† P < 0.01.‡ P < 0.05.

The most divergent p23 sequence was from a C. parvum cervine genotype human isolate (GenBank accession no. DQ389174), which was reported to have low identity to C. parvum and C. hominis sequences and a multiple repeat region (Figure 4). Three other sequences from the mouse, rabbit, and pig II genotypes were more similar to each other than to the other sequences (Figure 4). All C. parvum sequences (except Iic sequences and one of the IIm sequences [B 7 from this study] clustered together as did the Iic and the second IIm (B 7) sequences (Figure 4). Finally all C. hominis p23 sequences including those from this study (Ia, Ib, Id, Ie, and If) and that of Sturbaum and others (Ia, Ib, Id, and Ie) clustered together. The deduced amino acid sequences of all C. parvum p23 sequences (except the Iic and the IIm B 7 sequence) were identical with each other and with that of the p23 sequence (which belongs to the Iia subtype family) from the C. parvum genome (Figure 4).

Similarly, all C. hominis sequences were identical with each other and with that of the published sequence (which belongs to the Ia subtype family) from the C. hominis genome (Figure 4). As reported, there were 10 nucleotide differences, which translated into three amino acid changes, P to S, A to S, and D to E (as indicated in Figure 4), between most C. parvum and C. hominis sequences. However, all three C. parvum Iic sequences and one C. parvum IIm (B 21) sequence (Figure 4) were identical with each other, but differed from other C. parvum and C. hominis sequences in that they shared the same P, A, and D residues as the other C. parvum sequences but had an A to S change in the C-terminal most residues compared with the rest of the C. parvum and all the C. hominis sequences.

The predicted N-linked glycosylation site NKS (indicated in bold in Figure 4) is conserved among all p23 sequences as are 4 predicted O-linked glycosylation sites (indicated in bold and italics in Figure 4). An additional predicted O-glycosylated S residue is conserved among all C. parvum and C. hominis sequences. All C. hominis sequences share another putative O-glycosylated S residue, and the C-terminal-most S residue in all Iic and B 7 IIm sequences is predicted to be O-glycosylated (Figure 4). The C-terminal QDKPAD peptide against which the neutralizing 7A10 monoclonal antibody is directed is conserved among all (except the C. parvum cervine genotype) sequences, and the second QDKPAD peptide is conserved among all C. parvum sequences analyzed in this study (Figure 4). However, the C terminal D residue is replaced with an E in all C. hominis sequences (Figure 4).

**DISCUSSION**

Although p23 is considered one of the most promising vaccine candidates for cryptosporidiosis, there have been few clinical studies in well-defined cohorts that have characterized immune responses to this antigen and none that have analyzed polymorphisms in the gene encoding it from Cryptosporidium spp. and subtype families infecting patients in the study. In this case–control study of children less than five years of age with diarrhea in Bangladesh, we found that Cryptosporidium-infected case children, but not uninfected controls, showed development of statistically significant serum IgG, IgA, and IgM responses to this antigen over a three-week follow-up period. Serum IgA and IgM responses were significantly lower in children with persistent diarrhea than in those with acute
diarrhea over the three-week follow-up period, which sug-
gested that these responses may be associated with protection
from prolonged diarrhea.

The p23 nucleotide and deduced amino acid sequences from Cryptosporidium spp. infecting these children were relatively
conserved among different C. parvum and C. hominis subtype
families. Although most children were infected with different
subtype families of C. hominis, overall, there were significant
antibody responses to the C. parvum antigen in cases com-
pared with controls, which suggested that these cross-reactive
responses are directed at conserved epitopes. These results
support further development of p23 as a component of a sub-
unit vaccine for cryptosporidiosis.

A number of previous studies have reported serum anti-
body responses to p23 in immunocompetent and immuno-
compromised hosts. However, most studies were serosurveys
to estimate prevalence and investigate outbreaks1,23,56–60 to
demonstrate the utility of p23 as an antigen for ELISAs.30,35
Priest and others investigated serum antibody responses to a
27-kDa antigen (same as p23) in a birth cohort of children in
Peru by using the same recombinant C. parvum protein we
used as antigen for the ELISA. As in our study, most children
in their study were infected with C. hominis. However, serum
antibody responses to the C. parvum p23 antigen occurred
in children infected with a number of different species and
subtype families. Serum IgG responses to p23 increased with
age and with repeated infections.31 In a study of HIV-infected
persons from Australia, Frost and others reported that a strong
serologic response to p23 was associated with a reduced risk of
diarrhea without weight loss, but not in those who had weight
loss in addition to diarrhea.38

In this study, there were no significant differences in serum
IgG, IgM, or IgA levels to p23 between cases and controls after
controlling for covariates in multivariate analysis. However,
after three weeks of follow-up, levels of all three isotypes were
significantly higher in cases than controls, as was the change
in antibody levels from the initial to the follow-up time points.
These differences remained significant after controlling for
covariates. This finding is in contrast to antibody responses to
gp15 in the same children in whom only IgG levels at follow-
up and in the change from the initial to follow-up time points
were significantly greater in cases than controls, as was the change
in antibody levels from the initial to the follow-up time points.
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gp15 in the same children in whom only IgG levels at follow-
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were significantly greater in cases than controls, as was the change
in antibody levels from the initial to the follow-up time points.

Persistent diarrhea is a common consequence of cryp-
tosporidiosis in children in developing countries, particularly
in those who are malnourished.3,4 Approximately one-third
of the cases, but none of the controls in this study, had per-
sistent diarrhea. In these children, IgA and IgM levels were
significantly higher in those with persistent diarrhea than in those with acute diarrhea. The higher IgA and IgM levels in those with persistent diarrhea may be related to the longer duration of diarrhea in these children. However, there was a significantly greater increase in IgA and IgM levels from the initial to follow-up time points in those with acute diarrhea than in those with persistent diarrhea. This finding suggests that IgA and IgM responses (possibly mucosal) that persist for a longer time may protect against development of persistent diarrhea. These results are similar to those obtained in our studies of antibody responses to total Cryptosporidium antigens in oocyst lysates and to the immunodominant gp15 antigen in the same children.

Although there were significant correlations in antibody levels to p23 with those to C. parvum lysate, serum IgG levels to antigens in the C. parvum lysate did not correlate well with the p23 response at the follow-up time point. The reason for this finding is not clear, but may reflect differences in the dynamics of the IgG response to different antigens present in the C. parvum lysate compared with that to p23.

In this study, PCR was used to re-classify seven microscopy-negative controls as cases. Because PCR is currently the most sensitive method for detection of Cryptosporidium spp., it is unlikely although possible that additional controls may have been misclassified. Three of the controls had relatively high levels of serum IgG (but not IgM) to p23, which decreased at the follow-up time point. This finding may represent previous symptomatic or asymptomatic infection with Cryptosporidium spp.

Cell-mediated immunity is critical for protection from and resolution of cryptosporidiosis. Although antibody responses to specific antigens such as gp15 and p23 have been associated with protection from symptoms of cryptosporidiosis, it is not known whether these responses are themselves protective or whether they merely reflect protective cellular responses. Smith and others examined serum antibody and T cell proliferative responses to p23 in residents of Haiti previously exposed to Cryptosporidium spp., as suggested by high levels of seropositivity. They found that antibody responses to p23 were greater in persons who displayed proliferative T cell responses to this antigen, suggesting that antibody responses may correlate with cellular responses. In our study, it was not possible to assess cell-mediated responses for logistical reasons.

The finding that there were relatively few single amino acid polymorphisms in the p23 deduced amino acid sequences from different C. parvum and C. hominis subtype families in this study and in others from different geographic locations indicates that this antigen is relatively conserved and supports its consideration as a vaccine candidate. This is the first report of p23 sequences from the anthropoontic C. parvum IIC and IIIm subtype families. The finding that these sequences clustered separately from the other C. parvum sequences is interesting and consistent with those of previous studies, which showed that they are divergent at other loci including that of the gp40/15 (also called gp60) and Muc4 genes.

Although most children in our study were infected with different C. hominis subtype families, the finding that they developed significant antibody responses to the C. parvum antigen is consistent with that reported by Priest and others. The results from both studies suggest that these responses are directed at epitopes conserved among species and different subtype families. The neutralizing monoclonal antibodies 7D10 and C6B6, which were first used to identify p23, are directed at linear (QDKPAD) and conformational epitopes, respectively, in the C-terminal region of p23. Sturbaum and others reported that 9 of 10 monoclonal antibodies generated against C. parvum p23 were reactive with the p23 antigen from three C. hominis isolates, confirming that for the most part p23 is antigenically conserved among isolates from both species. However, the epitopes recognized by human serum antibodies to p23 remain to be determined.

Because E. coli-expressed recombinant p23 was used as antigen for ELISAs, the findings of this study indicate that antibody responses were directed at peptide epitopes. However, as shown in the present study, p23, like many other surface-associated Cryptosporidium spp. antigens, is predicted to contain conserved N- and O-glycosylation sites, and we have shown that this antigen binds to Helix pomatia agglutinin, suggesting that it displays mucin-type O glycosylation (Ward H. D., unpublished data). However, it is not known whether immune responses to this antigen are directed against glycosylated as well as peptide epitopes.

As noted, there were a number of limitations with the parent study, including a small sample size and significant loss to follow-up in an urban hospital-based setting. In addition, we were not able to determine whether cases or controls experienced symptomatic or asymptomatic cryptosporidiosis previously. Furthermore, case children came to the hospital at various times after the onset of diarrhea. In the current study, assessment of immune responses was limited to measurement of systemic serum antibody levels to p23. For logistical reasons, we were not able to assess cell-mediated or mucosal responses to this antigen. Nonetheless, the findings of this study provide useful data that support further development of p23 as a vaccine candidate and will inform larger, community-based, longitudinal, studies on systemic and mucosal cell-mediated and humoral responses to protective peptide and glycosylated epitopes on this and other putative vaccine candidates.

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