Serum Antibody Responses to Polymorphic *Cryptosporidium* Mucin Antigen in Bangladeshi Children with Cryptosporidiosis

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Abstract. *Cryptosporidium* is a significant cause of diarrheal disease in children in developing countries. The sporozoite antigen Muc4 is important for infection of host cells, and could be a candidate vaccine antigen. However, this antigen is polymorphic between *Cryptosporidium hominis* and *C. parvum*. We investigated antibody responses to *C. hominis* Muc4 and *C. parvum* Muc4 antigen in children in Bangladesh infected with *C. hominis*. Antibody responses were compared between children with cryptosporidial diarrhea (cases) and uninfected children with diarrhea (controls). There was a significant IgM response to Muc4 from both species in cases compared with controls, which increased over time, and was higher in children with persistent diarrhea. Despite sequence polymorphisms, antibody responses to *C. hominis* Muc4 and *C. parvum* Muc4 were significantly correlated. These results suggest that the human antibody response to Muc4 is cross-reactive between species, but in young children does not mature to an IgG response within the period observed in this study.

INTRODUCTION

Cryptosporidium sp. are waterborne gastrointestinal protozoa that are ubiquitous in industrialized and developing countries.¹ In immunocompromised patients, *Cryptosporidium* can cause a severe and chronic diarrheal disease, whereas in immunocompetent patients, the disease follows a milder course and infection is often asymptomatic.^{2–5} In developing countries where access to clean water sources is limited, *Cryptosporidium* is one of the commonest causes of parasitic diarrheal disease in children less than five years of age.⁶ These children often experience repeated infections with this parasite,^{7,8} leading to significant growth and developmental delays,^{9–11} even in children with asymptomatic infection.¹⁰ Cryptosporidiosis is also associated with malnutrition,^{12–16} although cause and effect remain unclear.¹⁷ Currently, treatment and prevention options are severely limited: there is no vaccine, and nitazoxanide, the only drug approved for treatment of cryptosporidiosis,¹⁸ is ineffective in immunocompromised patients.¹⁹

To develop better prevention and treatment strategies, it is important to characterize the immune responses to the parasite. Although the CD4⁺ immune response is essential for control of infection,²⁰ the serum antibody response to immunodominant antigens is correlated with protection from symptoms.²¹ The humoral immune response has not been shown to directly participate in clearance of the parasite, and may well simply reflect development of protective cellular responses. However, several studies have found that patients with acquired immunodeficiency syndrome living in areas where cryptosporidiosis is endemic often have asymptomatic cryptosporidiosis even when severely immunosuppressed.^{22,23} This observation suggests that in the absence of a robust CD4⁺ response, other immune responses such as antibodies or cytokines, may make a more significant contribution to protection from symptomatic infection.

There are two *Cryptosporidium* species (*C. hominis* and *C. parvum*) that cause most human infections.²⁴ Some anti-

gens, such as the immunodominant Cp23 and gp15, are relatively conserved between the two species.^{25,26} A previous study from our group in this cohort of children using recombinant gp15 from both species as antigens in an enzyme-linked immunosorbent assay (ELISA) showed that both antigens induced serum antibody responses, and that there was cross-reactivity between gp15 from *C. hominis* and *C. parvum*.²⁷ Similarly, the immunodominant p23 antigen induced significant serum antibody responses in case children compared with controls in this study (Borad AJ and others, unpublished data). However, it is now recognized that *Cryptosporidium* also expresses antigens that are polymorphic between species and among isolates, most notably gp40,^{24,28,29} and the more recently described Muc4 and Muc5.³⁰ These antigens have also been implicated in the processes of parasite attachment and invasion, suggesting that an immune response directed against these antigens might be critically important for protection. Gp40 is highly polymorphic among *C. hominis* and *C. parvum* isolates; to date, 20 alleles of this gene have been identified.^{31,32}

A previous study from our group that investigated antibody responses to gp40 in children in southern India with cryptosporidiosis found that serum IgG responses to this antigen, although cross-reactive, are in part subtype specific.³³ Although there is only 58% identity between the *C. hominis* and *C. parvum* Muc4 alleles, there is little polymorphism at the Muc4 locus among *C. hominis* isolates, except for those isolates identified as human-adapted or anthroponotic *C. parvum*.³⁰ However, the nature and specificity of the immune response to Muc4 remain uncharacterized. To initiate studies on the immune response to Muc4 and the level of cross reactivity to the two protein isoforms, we expressed the *C. hominis* and *C. parvum* Muc4 proteins in *Escherichia coli* and examined antibody responses to both proteins in a cohort of children in Bangladesh infected with *C. hominis*.

MATERIALS AND METHODS

Cohort. The study cohort has been described in detail.^{27,34} The study was conducted at the Dhaka Hospital of the International Center for Diarrheal Disease Research Center,

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Bangladesh in Dhaka, Bangladesh during May 2001–August 2002 and was designed as an age-matched case-control study. Children less than five years of age who had diarrhea when they came to the hospital were recruited. Forty-six children in whom microscopic examination identified *Cryptosporidium* oocysts in feces by modified acid-fast staining were enrolled as cases. An equal number of age-matched children with diarrhea, but with a negative stool examination results, were enrolled as controls. Subsequent evaluation by polymerase chain reaction (PCR) showed that seven of the controls had *Cryptosporidium* in feces.³⁵ Therefore, the study design was unmatched and the PCR-positive controls were reclassified as cases. Diarrhea was defined as three or more stools within a 24-hour period. A diarrheal episode was defined as diarrhea lasting 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. Children with persistent diarrhea included those who had diarrhea for ≥ 14 days and those who continued to have diarrhea for ≥ 14 days during the follow-up period.

Serum samples were obtained at the time of enrollment and at follow-up three weeks later. Of the 53 cases, 34 returned for follow-up three weeks later, and of the 39 controls, 19 returned for follow-up. Informed consent was obtained from the parents of the children, and the study was approved by the Institutional Review Board of the International Center for Diarrheal Disease Research Center, Bangladesh. Serum samples were shipped on dry ice to Boston, Massachusetts 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. For this study, we measured antibody levels in patients for whom we had initial and follow-up samples. Serum samples were available for all 34 cases, but for only 17 of the 19 controls.

We had previously sequenced the Muc4 gene from *C. hominis* and *C. parvum* isolates from this cohort and from *C. parvum* isolates from a cohort of children from India with cryptosporidiosis.³⁰ All *C. hominis* isolates had the published *C. hominis* Muc4 (*ChMuc4*) gene, whereas the *C. parvum* isolates had a unique Muc4 allele. All children whose serum samples were tested in this study were infected with *C. hominis* isolates,³⁵ and are presumed to carry the *ChMuc4* allele.

Expression of recombinant *CpMuc4* and *ChMuc4*. The coding sequences of *ChMuc4* and *C. parvum* Muc4 (*CpMuc4*), without the 19 amino acid N-terminal signal sequence, were PCR amplified from *C. hominis* genomic DNA purified from the TU502 isolate (obtained from Dr. Saul Tzipori, Tufts Cummings School of Veterinary Medicine, North Grafton, MA) by using a gNOME DNA extraction kit (Qbiogene, Carlsbad, CA) and from plasmids containing the *CpMuc4* gene,³⁰ respectively. The primers incorporated the overhang sequences necessary for cloning into the pET46EkLIC vector (Invitrogen, Carlsbad, CA). Expression from this vector produces a fusion protein with an N-terminal 6X His tag that can be cleaved with enterokinase. After cloning and sequencing to verify correct insertion of the Muc4 sequences, the plasmids were used to transform *E. coli* BL21, and expression was induced with isopropyl β-D-1-thiogalactopyranoside. The fusion proteins were purified by using nickel affinity chromatography (NTA agarose; Qiagen, Valencia, CA) following the manufacturer's directions, and purification evaluated by sodium dodecyl sulfate–polyacrylamide

gel electrophoresis (SDS-PAGE) and Coomassie staining. The identity of the fusion proteins was confirmed by Western blotting with anti-*CpMuc4* rabbit serum samples as described.³⁰ The 6X His-tags were cleaved and separated from the recombinant (r)Muc4 proteins by using the enterokinase cleavage kit (Invitrogen), and identity of the rMuc4 proteins was confirmed by N-terminal sequencing by automated Edman degradation at the Tufts University Core Facility.

ELISA. The ELISA for detection of antibodies to r*CpMuc4* and r*ChMuc4* was conducted according to protocols used in our laboratory.³⁴ The optimal coating concentration of *CpMuc4* and *ChMuc4* was determined empirically using *Cryptosporidium*-negative (by gp15 ELISA and immunoblot), and -positive serum samples. Maxisorp 96-well plates (Nunc, Rochester, NY) were coated with 10 µg/mL of the recombinant proteins overnight at 4°C. Plates were washed in phosphate-buffered saline (PBS), 0.05% Tween 20 and blocked in 0.25% bovine serum albumin–PBS for either 2 hours at 37°C or overnight at 4°C, and used immediately. Serum samples were added to the wells at a 1:100 dilution in 0.25% bovine serum albumin–PBS and incubated for 2 hours at 37°C. All samples were tested in triplicate. The plates were washed three times and antibodies against Muc4 were detected with alkaline phosphatase-conjugated goat anti-human IgG, IgM, or IgA and *p*-nitrophenyl phosphate substrate. After 30 minutes, the absorbance at 405 nm ($A_{405\text{ nm}}$) was measured with a Bio-Rad Microplate Reader (Model 550; Bio-Rad Laboratories, Hercules, CA). The $A_{405\text{ nm}}$ never exceeded 2.0 optical density units for any sample.

Statistical analysis. Samples were tested in triplicate. In those samples in which the triplicate values deviated from the other two samples by greater than 40%, the OD_{405} was calculated as the mean of the two values that were in close agreement. 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 and -positive serum samples were tested on each plate. To adjust for inter-plate variability, normalized ELISA units were calculated by dividing the $A_{405\text{ nm}}$ of the sample by the $A_{405\text{ nm}}$ of the positive control for that plate and multiplying by 100. This approach to standardization of ELISA values has been validated by using these same patient samples.²⁷

Statistical analysis was conducted by using PRISM version 4.03 (Graphpad, La Jolla, CA). Data was evaluated for normality by using the D'Agostino and Pearson omnibus normality test. Normally distributed data was compared by using the unpaired *t*-test to evaluate differences between cases and controls and by paired *t*-test to evaluate changes in antibody levels in initial and follow-up samples. For non-normally distributed data, the Mann-Whitney test and Wilcoxon matched-pairs test were used. Differences were considered significant at $P < 0.05$. Spearman correlations were used to test if there were significant correlations between antibody responses to the Muc4 proteins, and between the Muc4 proteins and total oocyst lysates.

RESULTS

Demographics. The demographics of the subgroup of the cohort we have examined in this study are described in Table 1. There were no statistically significant differences

TABLE 1

Demographics and clinical characteristics of patients with cryptosporidiosis and controls at presentation, Bangladesh*

Characteristic	Cases (n = 34)	Controls (n = 17)	P
Age (months)	12 (8.5–18)	13 (8–14.5)	0.98†
Female sex	12 (35.3)	2 (11.8)	0.10‡
Weight-for-age Z score	73 (64–80.5)	75 (69.5–82)	0.42†
Weight-for-height Z score	81 (76–86)	85 (80.5–89.5)	0.14†
Duration of diarrhea (days)	7 (4–12)	3 (1–4)	0.0001†
Persistent diarrhea	12 (35.3)	0 (0)	0.0044‡

*Values are median (interquartile range) or percentage.

† By Mann-Whitney test.

‡ By Fisher's exact test.

between cases and controls in age or nutritional status. The duration of diarrhea at presentation was significantly greater in cases compared with controls, and there were 12 cases with persistent diarrhea compared with none in the controls. Among cases, children with persistent diarrhea presented significantly later after the onset of diarrhea than those children with acute diarrhea. Children with acute diarrhea presented a median (interquartile range [IQR]) of 5 (3–7) days after onset of symptoms, whereas children with persistent diarrhea presented 14 (11.5–15.5) days after start of diarrhea ($P = 0.0001$, by Mann-Whitney test).

Expression of *ChMuc4* and *CpMuc4* in *E. coli*. Both rCpMuc4 and rChMuc4 expressed in *E. coli* from the pET46EkLIC vector were soluble and easily purified by nickel affinity chromatography. The predicted sizes of the His-tagged recombinant *ChMuc4* and *CpMuc4* are 20.2 kD and 21.6 kD, respectively. However, Coomassie blue staining of the purified proteins showed multiple bands; for rChMuc4 there was a prominent band of 30 kD and two minor bands of 27 kD and 11 kD (Figure 1, left panel, lane 1); for rCpMuc4, there were two prominent bands of 25 kD and 18 kD (Figure 1, left panel, lane 2). Anti-CpMuc4 serum predominantly recognized the 30-kD *ChMuc4* band (Figure 1, right panel, lane 1) and 25-kD *CpMuc4* band (Figure 1, left panel, lane 2). The lower molecular weight bands are likely degradation products because we have found rMuc4 proteins to be unstable.

To confirm the identity of the bands, the His tags were cleaved from the recombinant proteins with enterokinase. When the tags were removed, rChMuc4 appeared as a 25-kD protein and rCpMuc4 appeared as 20-kD by SDS-PAGE gels. The N-terminal sequence obtained on both tagless recombinant proteins was the expected sequence of I²⁰NQLG²⁴ (the isoleucine is encoded from vector sequences). The reactivity of negative and positive control serum samples were evaluated in the ELISA on 6X His-tagged *ChMuc4* and *CpMuc4* and on *ChMuc4* and *CpMuc4* from which the tags had been removed. Because no significant difference was observed in the signal obtained, the ELISAs were conducted with 6X His-tagged recombinant proteins.

Antibody responses to homologous rChMuc4 antigen.

Levels of anti-*ChMuc4* IgM were significantly increased in cases as compared with controls at follow-up (median [IQR] = 173 [119.8–251.2] versus 102 [79.9–145.6], respectively; $P = 0.004$). There was also a significant increase from the initial to follow-up time points in cases compared with controls (a change of 20 [–7.3 to 74.1] for cases versus –11.2 [–44.5 to 0.53] for controls, $P = 0.005$). Cases had significantly greater anti-*ChMuc4* IgM levels at follow-up compared with the initial time points (Figure 2, upper left panel), whereas controls

FIGURE 1. Expression of recombinant *Cryptosporidium hominis* Muc4 (rChMuc4) and recombinant *C. parvum* Muc4 (rCpMuc4). Lane 1, affinity-purified 6XHis tagged rChMuc4; lane 2, 6XHis tagged rCpMuc4. The blot on the right was probed with rabbit antiserum against an rCpMuc4 fusion protein (lane 2). Arrowheads indicate protein bands that stain prominently with antibody against *CpMuc4*.

had significantly lower anti-*ChMuc4* IgM levels at this time point, although this difference was primarily caused by one person (Figure 2, upper right panel), and may be reflective of a previous *Cryptosporidium* infection. Fifteen of 30 cases had a $\geq 20\%$ increase in IgM response over time, and 3 of the 30 cases had a $\geq 20\%$ decrease in IgM. In contrast, only 1 of the 21 controls had an increase of $\geq 20\%$ in IgM, and 5 of the 21 had a $\geq 20\%$ decrease ($P = 0.0027$, by chi-square test). There were no differences between cases and controls, and no change over time in either group in anti-*ChMuc4* IgG or IgA.

Antibody responses to heterologous rCpMuc4 antigen.

Levels of anti-CpMuc4 IgG were significantly higher in cases compared with controls at follow-up (median [IQR] = 67.7 [45.9–117.7] versus 42.5 [46.7–57]; $P = 0.004$), and there was a significant increase from initial to follow-up time points in cases compared with controls (3.7 [–1.94 to 12.1] versus –4.2 [–15.3 to 0.18]; $P = 0.002$). Nine of the 30 cases had a $\geq 20\%$ increase in IgG levels, and 6 of the 30 had $\geq 20\%$ decrease. Among the controls, only one child had an increase in the anti-CpMuc4 IgG level, and six children had a decrease in IgG levels ($P = 0.0585$, by chi-square test). There was a trend for cases to have higher anti-CpMuc4 IgG levels at the initial time point (median [IQR] = 64.3 [41.7–113.2] for cases versus 46.9 [34.3–65.3] for controls; $P = 0.099$), and the change over time in the cases was small enough (3.7 ELISA units), although statistically significant, to be of questionable clinical significance.

When a paired analysis was conducted to determine if there was a difference in IgG levels at the initial and follow-up time

FIGURE 2. Serum IgM responses to recombinant *Cryptosporidium hominis* Muc4 (rChMuc4) (top) and recombinant *C. parvum* Muc4 (rCpMuc4) (bottom). IgM levels at the initial and follow-up time points were compared by using the Wilcoxon matched-pairs test.

points, there was a trend for IgG levels in cases to be higher at the follow-up than the initial time (median [IQR] = 64.3 [41.7–113.2] versus 67.7 [45.9–117.7]; $P = 0.062$, by Wilcoxon matched-pairs test). Controls had significantly less anti-CpMuc4 IgG at the follow-up time point compared with the initial time point (median [IQR] = 46.9 [34.3–65.3] versus 42.5 [36.7–67]; $P = 0.015$, by Wilcoxon matched-pairs test).

There were no significant differences in anti-CpMuc4 IgA or IgM between cases and controls except that anti-CpMuc4 IgA was significantly higher in cases at presentation (median [IQR] = 90.7 [58.4–111.8] versus 63.7 [51.5–74.7]; $P = 0.004$). Anti-CpMuc4 IgM was significantly higher at the follow-up compared with the initial time points in cases (Figure 2, lower right panel).

Antibody responses in children with acute diarrhea versus those with persistent diarrhea. The children in the cohort were divided into those with diarrhea that resolved within 14 days (acute), and those with diarrhea that persisted for ≥ 14 days (persistent).³⁴ All children in the control group had acute diarrhea. We compared the antibody response against Muc4 in the 22 cases with acute diarrhea and the 12 cases with persistent diarrhea. There were no significant differences in IgG or IgA responses to either antigen between children with acute diarrhea and children with persistent diarrhea. Children in both groups exhibited a strong trend to increased IgM levels to both antigens over time, but this increase did not achieve statistical significance (P values: for children with acute diarrhea: anti-ChMuc4 IgM $P = 0.051$, anti-CpMuc4 IgM $P = 0.073$; for children with persistent diarrhea: anti-ChMuc4 IgM $P = 0.064$, anti-CpMuc4 IgM $P = 0.052$, by Wilcoxon matched-pairs test). Children with persistent diarrhea had significantly higher anti-ChMuc4 IgM levels at presentation (Figure 3, upper panel) and continued to show higher levels at follow-up (Figure 3, lower panel). Higher IgM values in the persistent diarrhea group may reflect a more progressed immune response. There were no significant differences in anti-CpMuc4 IgM levels between children with acute diarrhea and those with persistent diarrhea.

Correlation of antibody response to ChMuc4 and CpMuc4 with each other and with antibody responses to Ch and Cp oocyst lysates. Antibody responses to ChMuc4 and CpMuc4 were highly correlated with each other at initial and follow-up time points (Table 2). This finding is likely attributable to epitopes shared between the two antigen isoforms. In previous studies in this cohort, antibody responses to *C. parvum*³⁴ and *C. hominis* oocyst lysates²⁷ were determined by using the same ELISA protocol. The antibody responses to CpMuc4 were tested for a correlation with antibody responses to *C. parvum* oocyst lysate, and likewise antibodies to ChMuc4 compared with the response to *C. hominis* oocyst lysate. There was a significant correlation between IgM responses to the Muc4 antigens and the respective oocyst lysates from each species at follow-up, and between CpMuc4 and *C. parvum* oocysts at the initial time point (Table 2). There were no correlations between IgA or IgG responses to oocyst lysate and Muc4 antigens.

DISCUSSION

Muc4 is a sporozoite surface antigen that localizes in the apical region of sporozoites and is implicated in the process of sporozoite invasion of host cells because of the ability of antibodies against CpMuc4 to inhibit *in vitro* infection and because crude³⁰ and purified (O'Connor RM, unpublished data) native CpMuc4 antigen binds to intestinal epithelial cells *in vitro*.³⁰ Additionally, the antigen is highly polymorphic between *C. hominis* and *C. parvum*, suggesting that Muc4 is subject to pressure from immune responses. In a previous study, we sequenced the Muc4 locus in a subset of isolates infecting the children in this cohort and found that all the isolates that typed as *C. hominis* carried the published ChMuc4 allele, except for a few anthroponotic *C. parvum* IIm isolates that carried a unique Muc4 allele.³⁰ All the children included in this study were infected with *C. hominis*. This cohort provided an opportunity to investigate the antibody response to a homologous and heterologous antigen in children in whom the infecting isolate had been identified.

FIGURE 3. IgM responses to *Cryptosporidium hominis* Muc4 (*ChMuc4*) in children in Bangladesh with acute and persistent diarrhea at presentation and follow-up. IgM levels to *ChMuc4* in children with acute versus persistent diarrhea were compared by using the Mann-Whitney test.

The predominant response to the Muc4 antigens was of the IgM isotype. IgM to *ChMuc4* was significantly higher in cases than controls at follow-up, the change in anti-*ChMuc4* IgM was significantly greater in cases than in controls, and IgM to heterologous and homologous antigens was significantly greater at the follow-up than the initial time points in the cases (Figure 2). The IgM response to Muc4 also showed a correlation with the IgM response to oocyst lysate (Table 2). An increasing IgM response is consistent with an initial infection, especially in the absence of an IgG response.^{36,37} Similarly, studies in human volunteers have documented a lack of IgG against *Cryptosporidium* after the primary infection, with IgG seroconversion occurring after secondary exposure.³⁶ However, previous studies on antibody responses to total

C. parvum antigens and the immunodominant gp15 antigen in the same cohort of children showed a significant increase in serum IgG levels over the three-week follow-up period in cases compared with controls^{27,33} and there was a significant increase in serum antibodies of all three isotypes to the p23 antigen in these children (Borad AJ and others, unpublished data). In Bedouin children, seroconversion increased with age, and IgG seroconversion occurred later than IgM and IgA.³⁷ In a longitudinal study of children in Peru with cryptosporidiosis, the magnitude of the antibody response increased with the number of infections,³⁸ and in general, the seropositivity of populations in disease-endemic areas increases with age.^{39,40} However, these studies examined the immune response to either oocyst lysates or immunodominant antigens, and Muc4 is not derived from an oocyst lysate and is not an immunodominant antigen. In this cohort, there was an IgG response to oocysts lysate over time,³⁴ and in human volunteers infected with *C. hominis*, an IgG response does develop after primary exposure,⁴¹ unlike volunteers infected with *C. parvum*.³⁶

The development of a mature immune response to a less abundant, non-immunodominant antigen such as Muc4 may require multiple exposures. There also may be a poor cellular immune response to Muc4, especially after primary infection, so that IgG class switching is not supported. Many of the cases did not have a significant serum antibody response to either Muc4 antigen. The only study that investigated the immune response to a non-immunodominant *Cryptosporidium* antigen, thrombospondin-related adhesive protein, showed that a significant antibody response to this protein that occurred only in volunteers who developed diarrhea and were shedding oocysts.⁴² Unfortunately, the responding antibody class was not identified. Similar observations have been made for the development of antibodies to the 27-kD (same as p23), 17-kD, (same as gp15) and 15-kD antigens, where the greatest antibody response was noted in those volunteers with symptomatic cryptosporidiosis.⁴³ Interestingly, both of these studies noted that persons with pre-existing antibodies to these antigens were more likely to be asymptomatic after challenge with *C. parvum* oocysts.

We also observed that the anti-*ChMuc4* IgM response was significantly higher in children with persistent cryptosporidial diarrhea at the initial time point, presumably from a longer exposure to the antigen because children with persistent diarrhea were enrolled in the study considerably later during the course of diarrhea than children with acute diarrhea. There was no difference in the change in anti-*ChMuc4* IgM between cases with acute diarrhea versus cases with persistent diarrhea. This finding is in contrast to antibody responses to total oocyst lysate, gp15, and p23 in the same children in whom the increase in serum IgA and/or IgM responses over the three-week follow-up period were higher in children with acute diarrhea compared with those with persistent diarrhea²⁷ (Borad AJ and others, unpublished data). Because the children were recruited on the basis of symptoms,³⁴ we cannot determine if there is a protective effect of pre-existing antibodies against anti-*ChMuc4*.

For all antibody isotypes, antibody response to *ChMuc4* was significantly correlated with that to *CpMuc4*, indicating that at least some portion of the antibody response was directed against conserved epitopes. Because there is no data on the children's previous exposure to *Cryptosporidium*, an alternative possibility is that antibody to *CpMuc4* arose from previous

TABLE 2

Correlations between antibody responses to Muc4 and oocyst lysates in children with cryptosporidiosis, Bangladesh*

Antibody isotype	<i>ChMuc4/CpMuc4</i>		<i>ChMuc4/Ch</i> oocysts		<i>CpMuc4/Cp</i> oocysts	
	Initial	Follow-up	Initial	Follow-up	Initial	Follow-up
IgG	0.7†	0.56†	-0.09	-0.02	0.06	0.19
IgA	0.53†	0.63†	0.08	0.02	0.09	0.23
IgM	0.75†	0.65†	0.25	0.59†	0.3‡	0.54†

* Correlations were analyzed for all values for cases and controls. Values are Spearman r values. *ChMuc4* = *Cryptosporidium hominis* Muc4; *CpMuc4* = *C. parvum* Muc4.

† $P < 0.0001$.

‡ $P = 0.034$.

infections, although this seems unlikely because zoonotic *C. parvum* infections were not observed in this cohort.³⁵ In terms of vaccine development, it is encouraging that there appears to be cross-reactivity between these antigens because Muc4 is highly divergent between species.³⁰ Studies using monoclonal antibodies to p23 and gp900 domains 1 and 5 have found antigenic differences between *C. hominis* and *C. parvum* antigens, even though these sequences are relatively conserved.⁴⁴ However, the identity of immunologically protective epitopes on any of these antigens is unknown.

Although there was no IgG response to homologous antigen, IgG to heterologous antigen was significantly higher in cases than controls at follow-up, and there was a trend toward an increase in IgG against *CpMuc4* over time in the cases ($P = 0.062$). Similarly, levels of IgA against *CpMuc4* were significantly higher in cases than in controls at presentation, but there was no difference between groups in IgA levels to *ChMuc4*. It is difficult to account for this observation. Because there was no difference in levels of IgG or IgA against *ChMuc4* between groups, these observations cannot be attributed to cross-reactive antibodies. BLAST searches of other apicomplexan genomes have not identified significant homology between *CpMuc4* and any other protein sequence,³⁰ making it unlikely that this is caused by cross-reactivity with antibodies to another pathogen. It is possible that the children with cryptosporidiosis had previous infections with *C. parvum*, although the demographic data did not identify any differences between cases and controls in contact with animals or water sources.^{27,33} Characterization of antibody responses to Muc4 in children with cryptosporidiosis using a birth cohort study design might clarify these results.

There are several limitations in the study that must be taken into account when interpreting the data. Because children were recruited upon arriving at the hospital with diarrhea, there are no data on any previous *Cryptosporidium* infections, nor is there any information on the immune response in children with asymptomatic infections. Additionally, the initial sample was taken when the children came to the hospital at various times after the onset of diarrhea, resulting in the samples being taken at different points in the development of the immune response. The most important caveat is that the antigens used in this study are *E. coli* recombinant proteins that lack any post-translational modifications. Muc4 is predicted to be O- and N-glycosylated,³⁰ and although we have not found empirical evidence of glycosylation, the native antigen is 10 kD larger than the *E. coli* recombinant on SDS-PAGE gels (O'Connor R, unpublished data), which suggests significant post-translational modification. Thus, the immune response to the native antigens may be different than that observed in these studies, in which we only captured the response to protein epitopes. Future studies are planned using *Toxoplasma gondii*-expressed antigens, which carry similar glycotopes to native *Cryptosporidium* antigens.⁴⁵

We have shown in these studies that there is a humoral immune response to the Muc4 antigen in young children with cryptosporidiosis that consists primarily of IgM, and that some of this response is directed against conserved epitopes. Future studies using more appropriate antigens, and in other demographic groups such as adults positive for infection with human immunodeficiency virus, will provide more complete characterization of the immune response to this important antigen.

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