Delayed Time to Cryptosporidiosis in Bangladeshi Children is Associated with Greater Fecal IgA against Two Sporozoite-Expressed Antigens

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Abstract. Cryptosporidiosis is common in early childhood, and both diarrheal and subclinical infections are associated with adverse developmental outcomes. Improved therapeutic medications may help reduce the burden of cryptosporidial diarrhea; however, an effective vaccine would be better able to prevent the detrimental impact of both diarrheal and subclinical disease. A more complete understanding of naturally occurring immunity may further inform strategies to develop an effective vaccine. In this prospective cohort study of Bangladeshi children, greater fecal IgA at 12 months, but not plasma IgG, directed against two sporozoite-expressed, immunodominant and vaccine candidate antigens was associated with delayed time to subsequent cryptosporidiosis to 3 years of life. These findings extend prior work and further support the role of mucosal antibody responses in naturally developing protective immunity to Cryptosporidium.

Cryptosporidiosis, caused by intracellular protozoan parasites of the genus Cryptosporidium, is a globally common infection with long-term adverse developmental consequences.1–3 The parasite is a leading cause of childhood diarrheal morbidity and mortality in many low- and middle-income countries; however, there is increasing recognition that subclinical (non-diarrheal) cryptosporidiosis is common and associated with impaired growth.5–7 Although several new therapeutics are under development, nitazoxanide is currently the only U.S. Food and Drug Administration–approved treatment for cryptosporidial diarrhea, and its efficacy in young children and other vulnerable populations is limited.8 Addressing the burden of cryptosporidial diarrhea through better therapeutics is crucial; however, even if proven clinically effective, these therapies would rely on infected children presenting to medical care for diagnosis and treatment. In the absence of diarrhea, one may expect most subclinical infections to remain untreated with subsequent developmental consequences remaining unchecked. Development of an effective vaccine remains an important and appealing approach to better mitigate the full burden of the parasite. Greater understanding of naturally occurring immunity to the parasite is important to better inform vaccine development strategies. Cell-mediated immune responses, particularly CD4+ T-cell production of interferon gamma (IFN-γ), are critical to clearance of established Cryptosporidium infection.6,7 We previously showed that greater fecal IgA directed against a sporozoite-expressed antigen measured at 12 months of age was associated with delayed time to subsequent cryptosporidiosis over the subsequent year, suggesting that antibody-mediated immune responses may play a role in preventative immunity.8 Whether this protective association persisted beyond age 2 years and whether it was unique to the studied sporozoite-expressed antigen (Cp23) or whether responses to other sporozoite-expressed antigens may similarly be protective remained uncertain.

To address these questions, we again leveraged an ongoing, prospective birth cohort study located in Mirpur, Dhaka, Bangladesh, and in which active surveillance of children, for whom informed written consent was provided by a parent or guardian, was continued through 3 years of life, as previously described.3 The Ethics and Research Review Committee at the icddr,b approved this study, and a reliance agreement was granted by the Institutional Review Board of the University of Virginia. Diagnosis of cryptosporidiosis was based on detection by real-time PCR of stool samples which were collected monthly and during diarrheal episodes (≥3 loose stools in 24 hours).9 Plasma was obtained from children at 12 months of age. The sporozoite-expressed antigens Cp17 and Cp23 lacking the glutathione S-transferase expression tag were prepared, as previously described.9 These antigens were chosen because both are surface-expressed during the infective sporozoite parasite stage, have been previously shown to induce both antibody- and cell-mediated immune responses, and are considered potential vaccine candidates.9–12 Anti-Cp17 and anti-Cp23 plasma IgG and fecal IgA were measured using ELISA.8,9 Subjects were divided into the upper and lower 50th percentiles for each antigen–antibody pairing. Survival probabilities for time to first Cryptosporidium PCR-positive stool from 12–36 months of age were estimated with the Kaplan–Meier method for both plasma IgG and fecal IgA for Cp17 and Cp23, respectively. Univariate Cox regression of time to subsequent cryptosporidiosis was performed for demographic, socioeconomic, and anthropometric variables (Supplemental Table 1). Variables for which P < 0.1 were then included in multivariable Cox regression analysis. Analyses were performed using R version 4.0.0 with package “survival” version 3.1–12 (R Foundation for Statistical Computing, Vienna, Austria) with function “coxph.”

Enrolled infants were born between July 2014 and April 2016 and were subsequently prospectively followed to age 3 years. Stool and plasma samples obtained at 12 months of age were available for 442 children. Details of gender, household size and income, duration of excluding breastfeeding, water source and treatment, and length-for-age z score (LAZ) at 12 months have previously been reported.9 As previously described, 126 children (28.5%) had detectable
cryptosporidiosis in the first year of life. By age 3, 340 children (81.9%) had a PCR-detected Cryptosporidium infection; 27 children were included in the analysis but censored (migrated out of the study area or a parent/guardian elected to withdraw from the study) before age 3 years without preceding Cryptosporidium infection. There was no difference observed in cryptosporidiosis-free survival through age 3 years of life between children in the upper or lower 50th percentiles for plasma IgG measured at 12 months of age and directed against either Cp23 or Cp17 (Figure 1A and C). However, children in the upper 50th percentile of fecal IgA measured at 12 months and directed against either Cp23 or Cp17 were more likely to be subsequently cryptosporidiosis-free through age 3 years of life than children in the lower 50th percentile (Figure 1B and D; \( P = 0.0034 \) and 0.031 for Cp23 and Cp17, respectively).

Univariate Cox regression analysis was performed for potentially important covariables including anti-Cp23 and anti-Cp17 antibody responses and demographic, socioeconomic, and anthropomorphic factors (Supplemental Table 1). As we have previously shown seasonal and yearly differences in the incidence of cryptosporidiosis in Bangladesh, the month and year of birth were also included (Supplemental Table 1). In addition to fecal IgA in the upper 50th percentile against Cp23 and Cp17, only monthly income and LAZ at 12 months had \( P < 0.1 \). Multivariable Cox regression analysis to time to subsequent cryptosporidiosis was performed using these variables with \( P < 0.1 \); because of correlation of fecal Cp23 and Cp17 IgA only fecal anti-Cp23 IgA was included. In multivariable analysis, only fecal Cp23 IgA in the upper 50th percentile remained statistically significant with a reduction in the hazard ratio of 21% (95% CI: 1–36%; \( P = 0.04 \); Table 1).

These findings extend our previous observations of association of greater anti-Cryptosporidium fecal IgA (but not plasma IgG) with delayed time to subsequent cryptosporidiosis in two important ways. First, we show that the association of greater fecal IgA directed against well-described and immunogenic sporozoite-expressed antigens at 12 months of age with subsequent protection from infection persists (though wanes) through 3 years of life. Importantly, this association of greater fecal anti-Cryptosporidium IgA with delayed subsequent cryptosporidiosis persisted and remained statistically significant in stepwise multivariable analysis; other demographic, socioeconomic, anthropometric, and month and year of birth were not associated with protection. Second, we show that this protective effect of
Multivariable Cox regression indicating a statistically significant decrease in the hazard ratio of subsequent Cryptosporidium infection through 3 years of life for children in the upper 50th percentile of fecal anti-Cp23 IgA

| Variable                                      | Hazard ratio (95% CI) | P-value |
|-----------------------------------------------|-----------------------|---------|
| Fecal anti-Cp23 IgA in the upper 50th percentile | 0.79 (0.64–0.99)      | 0.04    |
| Monthly income (in thousands)                 | 0.99 (0.99–1.00)      | 0.17    |
| Bangladesh Taka                                | 0.91 (0.81–1.01)      | 0.09    |

Variables included in multivariable analysis were selected if univariate P < 0.1 (see Supplemental Table 1). As fecal anti-Cp23 IgA in the upper 50th percentile was highly correlated with fecal anti-Cp17 IgA in the upper 50th percentile, only fecal anti-Cp23 IgA was included in multivariable analysis.

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Note: Supplemental table appears at www.aftmh.org.

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