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Recent Diarrhea is Associated with Elevated Salivary IgG Responses to *Cryptosporidium* in Residents of an Eastern Massachusetts Community

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Abstract

Background: Serological data suggest that *Cryptosporidium* infections are common but underreported. The invasiveness of blood sampling limits the application of serology in epidemiological surveillance. We pilot-tested a non-invasive salivary anti-*Cryptosporidium* antibody assay in a community survey involving children and adults.

Materials and Methods: Families with children were recruited in a Massachusetts community in July; symptoms data were collected at 3 monthly follow-up mail surveys. One saliva sample per person (n = 349) was collected via mail, with the last survey in October. Samples were analyzed for IgG and IgA responses to a recombinant *C. hominis* gp15 sporozoite protein using a time-resolved fluorometric immunoassay. Log-transformed assay results were regressed on age using penalized B-splines to account for the strong age-dependence of antibody reactions. Positive responses were defined as fluorescence values above the upper 99% prediction limit.

Results: Forty-seven (13.5%) individuals had diarrhea without concurrent respiratory symptoms during the 3-month-long follow-up; eight of them had these symptoms during the month prior to saliva sampling. Two individuals had positive IgG responses: an adult who had diarrhea during the prior month and a child who had episodes of diarrhea during each survey month (Fisher's exact test for an association between diarrhea and IgG response: p = 0.0005 for symptoms during the prior month and p = 0.02 for symptoms during the entire follow-up period). The child also had a positive IgA response, along with two asymptomatic individuals (an association between diarrhea and IgA was not significant).

Conclusion: These results suggest that the salivary IgG specific to *Cryptosporidium* antigens warrants further evaluation as a potential indicator of recent infections.

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Introduction

Cryptosporidium is an intestinal protozoan parasite with a simple life cycle, which is completed within one host [1]. Two species of *Cryptosporidium* cause most infections in humans: *C. hominis*, which almost solely infects humans, and *C. parvum*, which can infect cattle, humans, and many other mammals. Outbreaks of cryptosporidiosis have been linked to the contamination of drinking water [2] and swimming pools [3]. The most common symptom is diarrhea. Approximately half of *Cryptosporidium* infections may be completely asymptomatic [4, 5]. The incidence of reported cryptosporidiosis in the US and Massachusetts fluctuates from approximately one to three cases per 100,000 persons per year [6–8]. The incidence is highest in children under 10 years of age; another peak is observed in 30–34-year-old adults. The seasonal peak of infections typically occurs in Massachusetts in August–October [6].

Cryptosporidiosis is likely to be substantially underreported in all age groups. Most symptomatic infections result in mild self-resolving illness, which do not require medical care. Only a fraction of those who receive primary care have their diarrheal stool samples analyzed for this parasite [9]. The parasite detection rates in fecal samples can also vary widely, depending on the analytical method used [10]. An epidemiological study in Michigan

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demonstrated that *Cryptosporidium* could be responsible for 12% of acute gastroenteritis of ill-defined etiology in children [11]. Epidemiological survey data from other industrialized nations demonstrated that *Cryptosporidium* was responsible for 2.1% of physician visits due to acute gastroenteritis in The Netherlands [12], 0.4% of physician visits and 0.4% of community cases of gastroenteritis in England [13], and 0.7% of physician visits due to acute gastroenteritis in Austria [14].

Two major groups of *Cryptosporidium* proteins, 15–17 kDa and 23–27 kDa, induce strong antibody responses in humans [15]. Serum antibody responses to these proteins increase shortly after infection and then gradually decay to the pre-infection level [16]. The median half-life of serum IgG to these proteins can vary from 12 weeks [17] to almost 15 months [18]. Serum IgA response can decay to a non-detectable level within 10 weeks post-infection [16, 19]. High seroprevalence of anti-*Cryptosporidium* antibodies in the US population also suggests that infections are very common [20]. Blood sampling, however, is invasive and expensive, which is an impediment for its use in community studies, especially those involving children.

Saliva has the potential to replace serum for the evaluation of immune responses to common infections [21, 22]. Antibodies present in saliva are relatively stable, even when stored without preservatives [23]. The collection of saliva using specially designed samplers, such as Oracol™, is non-invasive, simple, safe, and painless, making inexpensive population surveys by mail feasible [24, 25]. Previous studies demonstrated that anti-*Cryptosporidium* IgA and IgG in saliva are elevated in individuals who were recently infected with *Cryptosporidium* [26, 27]. The objectives of this study were to evaluate the feasibility of a salivary antibody survey by mail in a US community, and demonstrate an application of time-resolved fluorometry for the analysis of salivary antibody responses to *Cryptosporidium*.

Materials and Methods

Study Design

Families with at least one elementary school age child were recruited in an Eastern Massachusetts community at places frequented by parents, such as local elementary schools and public recreational centers (convenience sampling). Children younger than 1 year of age were excluded. Informed consent was obtained at recruitment. The study protocol and survey forms were approved by the Institutional Review Boards of Tufts University School of Medicine and Centers for Disease Control and Prevention. Demographic data were collected at enrollment in July. Follow-up illness symptom surveys were mailed to the study participants with pre-paid return envelopes in August, September, and October. Each illness survey asked about acute gastrointestinal, respiratory, and other symptoms since the previous survey or enrollment (approximately 1-month intervals). Each person could report up to four episodes of illness at each survey. Data on each episode included specific symptoms, such as diarrhea, runny nose, sore throat, cough, and fever. Diarrhea was defined as passing three or more unformed stools during 24 h or passing at least one liquid stool. Fecal samples were not collected.

The participants were asked to collect one saliva sample each with the last symptoms survey. Saliva samples were collected using Oracol™ sampling kits (Malvern Medical Developments, Ltd., Worcester, UK), which include a small cylindrical sponge with a handle and a container. Sampling kits and instructions were mailed to participants in insulated shipping containers with ice packs and FedEx labels for pre-paid overnight return shipping. Participants were instructed to rub their gums with the sampling sponge for approximately 1 min until the sponge became saturated with saliva and ship their saliva samples to the laboratory in containers with frozen ice packs. Upon delivery to the laboratory, saliva was separated from sampling sponges by centrifugation and stored at –80 °C until analysis.

Analysis of Salivary Antibody Responses

The sequence encoding the gp15 protein of *C. hominis* (TU502 isolate) was cloned into the pET32 Xa/LIC vector, the recombinant protein overexpressed in *Escherichia coli* and purified by metal affinity chromatography as described previously [28, 29]. The purified recombinant protein contained thioredoxin, His, and S tags. IgA and IgG antibody responses to gp15 were analyzed using time-resolved fluorometry (TRF) in the format of the Dissociation-Enhanced Lanthanide Fluorescence Immuno-Assay (DELFIA®, PerkinElmer, Inc.) using this recombinant protein as the antigen. Sterile Nunc® flat-bottom 96-well microplates were incubated overnight at 4 °C with a filter-sterilized solution of the recombinant gp15 protein (rgp15), 50 µl per well at 0.4 µg/ml in bicarbonate coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6). The plates were washed four times with 0.05% Tween20–phosphate-buffered saline, blocked with 100 µl of 0.25% bovine serum albumin buffer at 37 °C for 1 h, and stored at –80 °C until use. Affinity-purified monoclonal mouse anti-human IgA and IgG detection antibodies (KPL, Inc.) were labeled with Samarium or Europium, respectively, at PerkinElmer. Saliva samples were diluted 1:5 with the proprietary DELFIA® dilution buffer and assayed in triplicate at 100 µl per well. Microplates were incubated with samples for 1 h at 37 °C on a rotary shaker, washed four times with DELFIA® washing buffer using an automatic microplate washer, incubated on a rotary shaker for 1 h at 37 °C with 100 µl of 100 ng/ml lanthanide-labeled anti-human IgA and IgG detection antibodies, washed four times with DELFIA® washing buffer, incubated for 20 min with 150 µl of DELFIA® enhancement solution, and analyzed using a Wallac VICTOR^2 D time-resolved fluorometer (PerkinElmer, Inc.).

Statistical Analysis of Data

Episodes of diarrhea were divided into two categories: (i) diarrhea with concurrent respiratory symptom(s); and (ii) diarrhea without any concurrent respiratory symptoms. Individual data were dichotomized as to the presence or absence of each type of diarrheal illness during 1 month, 2 months, or 3 months prior to saliva sampling.

Statistical analysis was performed using SAS 9.2 software (SAS Institute, Inc., Cary, NC, USA). The data were log-transformed for regression analysis. Associations between age and anti-gp15 antibody responses were modeled using penalized B-splines with automatic selection of the smoothing parameter to minimize the corrected Akaike Information Criterion (AICC). Positive IgG or IgA antibody responses were defined as

Table 1
Characteristics of the study population.

Parameter and category		Number of individuals	Percentage of individuals
All participants		349	100
Gender	Males	173	50
	Females	176	50
Age category (years)	0–5	67	19
	6–10	63	18
	11–20	38	11
	21–40	71	20
	41–60	110	32

responses above the upper limit of the corresponding 99% prediction band (the upper 0.5% of age-specific distribution), which was produced using the SAS procedure TRANSREG. This cut-off was selected in order to identify outlying antibody responses which are likely to be associated with very recent infections. Associations between diarrheal illness and anti-gp15 IgG and IgA response status were analyzed using Fisher's exact test.

Results

Reflecting the composition of the source Massachusetts community, the study population was predominantly (95%) white. It consisted of approximately equal groups of children and adults (Table 1). Reflecting a typical structure of families with elementary school age children, the study population did not include any individuals in the age interval from 18 to 26 years. After excluding one senior individual who was an outlier with respect to age, the maximum age of the remaining 349 study participants was 55 years.

Eight study participants had episodes of diarrhea without any concurrent respiratory symptoms during the month prior to saliva sampling (Table 2). None of them sought medical attention. In addition, 19 individuals had diarrhea with concurrent respiratory symptoms during the month prior to saliva sampling (Table 2). Six of these 19 individuals went to see a doctor and four of them were diagnosed with viral infections (not shown in Table 2). During the entire 3-month-long follow-up, 47 (13.5%) individuals had episodes of diarrhea without concurrent respiratory symptoms and an additional 29 (8.3%) individuals had episodes of concurrent diarrheal and respiratory symptoms.

Log-transformed age-specific IgA and IgG antibody responses had approximately normal distribution (Figure 1a, b). Log-transformed antibody responses were also homoscedastic (had approximately equal scatter) across the entire range of age values, demonstrating that using the upper 99% prediction band as a flexible age-dependent cut-off was statistically appropriate. B-spline fitted regression lines demonstrated monotonous increases in antibody responses with age in children, with subsequent plateaus in adults. Two individuals (0.57%), a swimming pool-attending adult and a diaper-wearing day care-attending child, had IgG responses above the upper limit of the 99% prediction band (defined as ''positive''). Both of them had diarrhea without concurrent respiratory symptoms: an adult had an episode of diarrhea, vomiting, and abdominal pain during the month prior to saliva sampling, and the child had episodes of diarrhea during each survey month (Figure 1a). Three individuals (0.86%) had positive IgA responses. These included the IgG-positive child and two asymptomatic individuals (Figure 1b). The IgG-positive adult had an IgA antibody response that was below the upper 99% prediction limit (negative).

Figure 1c, d show the exponentiated predicted response values (age-specific median responses), the upper limits of the 99% prediction bands, and non-transformed original fluorescence values for IgG and IgA responses. This figure highlights the greater variability of non-transformed antibody responses in adults than children and the corresponding increases in cut-off values for positivity in adults. It also shows that the positive responses are extreme values of the corresponding age-specific distributions. The markedly stronger IgG fluorescence values are mainly due to the stronger fluorescence of Europium, which was used with anti-IgG detection antibodies, than of Samarium, which was used with anti-IgA antibodies [30].

Fisher's exact tests demonstrated that diarrhea without concurrent respiratory symptoms was a significant predictor of positive anti-gp15 IgG responses (Table 3). The association was the strongest ($p = 0.0005$) for symptoms that occurred during the month prior to saliva sampling (two IgG-positives among eight symptomatic individuals vs. zero IgG-positives among 341 individuals without symptoms). It was also significant ($p = 0.02$) for the entire 3-month-long follow-up period (two IgG-positives among 47 symptomatic individuals vs. zero IgG-positives among 302 asymptomatic individuals).

Table 2
Illnesses in the study participants: number (percentage) of individuals who were ill at least one time.

Type of diarrheal illness	Previous month	Previous 2 months	Previous 3 months
Diarrhea without any concurrent respiratory symptoms	8 (2.3%)	26 (7.5%)	47 (13.5%)
Diarrhea with concurrent respiratory symptom(s)	19 (5.4%)	23 (6.6%)	29 (8.3%)

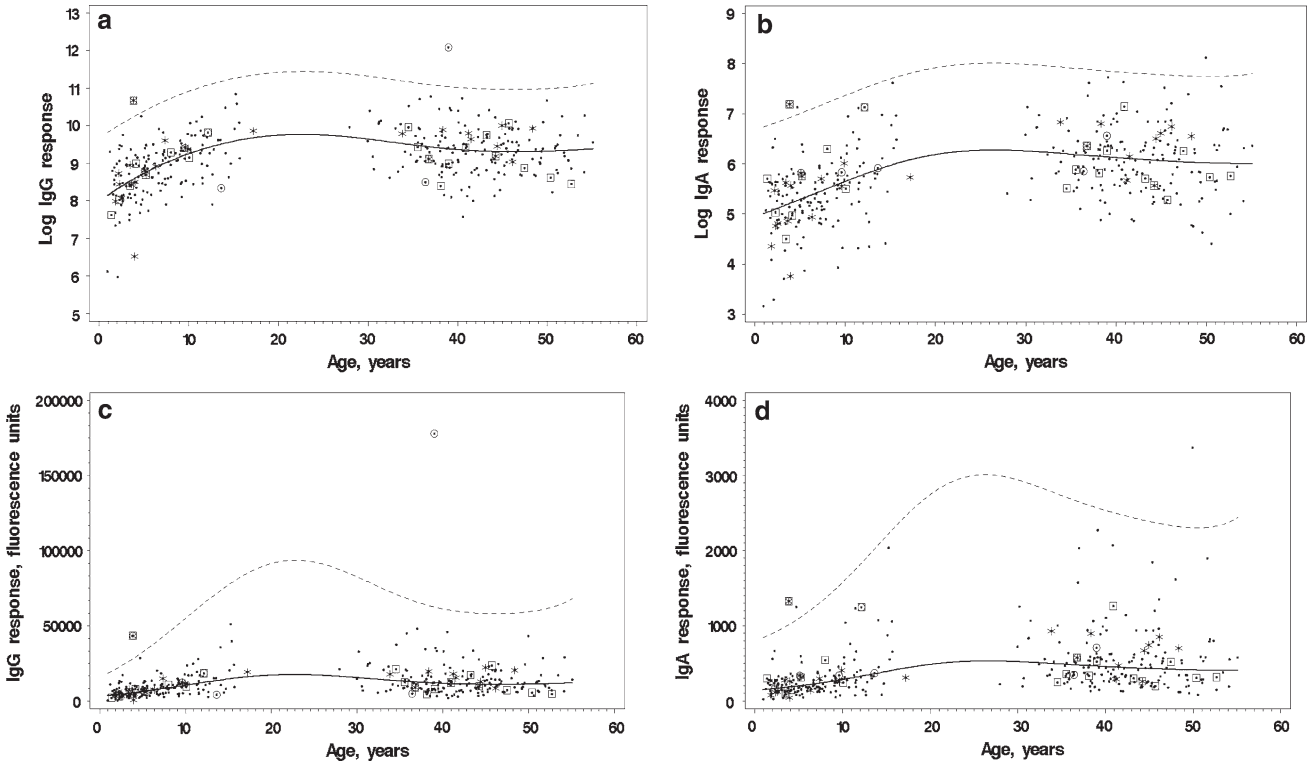

Figure 1. Associations between age and salivary anti-*Cryptosporidium* antibody responses with fitted penalized B-spline regression (*solid line*) and the upper limit of the 99% prediction band (*dashed line*). Episodes of diarrhea without concurrent respiratory symptoms are marked with: (i) *circles* for episodes reported at the last survey (month prior to saliva sampling); (ii) *squares* for episodes reported during the second survey; and (iii) *asterisks* for episodes reported during the first survey. **a)** Log-transformed IgG. **b)** Log-transformed IgA. **c)** Non-transformed IgG, Europium fluorescence units. **d)** Non-transformed IgA, Samarium fluorescence units.

Table 3
Associations between diarrhea without concurrent respiratory symptoms and anti-*Cryptosporidium* salivary antibody responses: counts of individuals by illness and antibody response category, and *p*-values for association from Fisher's exact tests.

Antibody isotype		Interval prior to saliva sampling					
		Previous month		Previous 2 months		Previous 3 months	
		Diarrhea+	Diarrhea–	Diarrhea+	Diarrhea–	Diarrhea+	Diarrhea–
IgG	Positive	2	0	2	0	2	0
	Negative	6	341	24	323	45	302
	p-value	0.0005		0.005		0.02	
IgA	Positive	1	2	1	2	1	2
	Negative	7	339	25	321	46	300
	p-value	0.07		0.2		0.3	

Associations between diarrhea without concurrent respiratory symptoms and IgA responses were not significant (Table 3).

None of the four individuals who had a positive IgG or IgA anti-gp15 response had episodes of diarrhea with concurrent respiratory symptom(s) during follow-up. Thus, a combination of gastrointestinal and respiratory symptoms was not associated with salivary anti-*Cryptosporidium* antibody in this data set.

Discussion

This pilot study demonstrated an association between recent episodes of diarrhea and elevated salivary IgG antibody responses to the recombinant gp15 protein of *Cryptosporidium* in residents of a Massachusetts community using the time-resolved fluorometric immunoassay. To account for the generally stronger antibody responses to this parasite in adults than children, this study used age-specific cut-offs for positive responses

based on the upper limit of the 99% prediction band from a non-parametric regression of antibody responses on age. Outlying antibody responses (the upper 0.5% of the age-specific distribution) are likely to be associated with very recent infections.

The strong age-related increase in anti-*Cryptosporidium* antibody responses in children observed in this study is consistent with previously published data [20, 31, 32]. It has also been demonstrated that the number of prior *Cryptosporidium* infections increases with age in children and that serum anti-*Cryptosporidium* IgG responses are stronger in children who have had more prior infections [32]. Using age-specific cut-off values may be important for the detection of recent infections in young children because of the relatively small amplitude of their peak antibody response.

The *Cryptosporidium* Cpgp40/15 gene encodes a precursor glycoprotein which undergoes proteolytic cleavage to produce gp40 and gp15 surface glycoproteins [28, 33]. The Cpgp40/15 gene is highly polymorphic and varies substantially between *C. parvum* and *C. hominis* [33–35]. However, most of this variability is contained within the region encoding the gp40 protein, while the gp15 protein is much less variable, despite being an immunogenic zoite surface antigen [33, 34]. Analysis of serum samples from children in Bangladesh and India using rgp15 proteins of *C. hominis* and *C. parvum* as antigens in enzyme-linked immunosorbent assay (ELISA) confirmed a lack of species specificity of antibody responses to the gp15 proteins (Honorine Ward, unpublished data). Therefore, strong antibody responses to the gp15 protein of *C. hominis* likely reflected recent infections with either *C. hominis* or *C. parvum*.

The gp15 protein used in this study is identical to the Cp17 protein, which has been cloned by *Priest* et al. [36] using a different protein expression system. Salivary antibody responses to Cp17 have been shown to decay in individuals recently infected with *Cryptosporidium* [27]. The same protein has been used successfully as a marker of infection in serological studies [17, 32].

A main limitation of this pilot study is that it did not involve fecal sampling to microbiologically diagnose episodes of diarrheal illness. It is unknown who of the study participants actually had *Cryptosporidium* infections during the 3-month-long follow-up. Therefore, the specificity and sensitivity of this salivary antibody test cannot be evaluated using these data. The fact that both individuals who were IgG-positive also had recent symptoms consistent with cryptosporidiosis generates cautious optimism. A future larger size study with concurrent collection of fecal samples would allow the specificity and sensitivity of the salivary antibody method to be addressed.

IgA antibody response to the 17-kDa *Cryptosporidium* antigen group (represented by the recombinant gp15 protein in this study) can decay to a non-detectable level within 10 weeks after an episode of infection [16, 19]. Immunoblot tests have demonstrated that IgG responses to the 17-kDa antigen group tend to be stronger than IgA responses to the same antigens [18]. As IgG response is known to be a better indicator of *Cryptosporidium* infection than IgA or IgM, many recent serological or salivary antibody studies measured IgG responses only [17, 20, 27, 32]. Therefore, it is not surprising that only IgG response to the gp15 protein was significantly associated with recent diarrhea symptoms in this study, and that an individual with exceptionally strong IgG response to the gp15 protein had a relatively weak IgA response to the same protein.

In this study, 349 individuals were followed for approximately three months each. The total amount of follow-up time in this cohort was 94 person-years. The incidence rate of diarrhea without concurrent respiratory symptoms was 0.63 episodes per person-year (59 episodes in 47 individuals). This incidence is roughly consistent with the results of a larger study in the US [37].

The follow-up interval included the fall seasonal peak of *Cryptosporidium* infections [6, 38], but excluded the winter peak of viral gastrointestinal infections. It was expected that this parasite would account for several percent of all diarrheal illnesses in the study cohort. If two IgG-positive symptomatic individuals, indeed, had had *Cryptosporidium* infections and no one else had had it, this parasite would account for 3.4% of all cases of diarrhea in the cohort.

Acceptance of saliva sampling by individuals of all ages, including children, is an important advantage of the salivary antibody method over blood sampling. Saliva samples can be collected by individuals without special training and mailed to the laboratory, which can greatly reduce the study cost. Further research is warranted to evaluate the sensitivity and specificity of the salivary antibody test for the detection of recent *Cryptosporidium* infections in settings where fecal sampling can be used to confirm clinical cryptosporidiosis.

It has been shown that seroconversion can be used as a bioindicator of incident infections in children [32]. Prospective serological studies, however, are rare and difficult to conduct because of the parental reluctance to consent for their children to undergo potentially painful phlebotomy. The advantages of a non-invasive salivary antibody detection method would be even more important in prospective studies involving repetitive sampling to detect incident infections, which warrants evaluation of this method in the prospective study settings.

Conclusion

The results of this study suggest that salivary IgG antibodies can be used to identify *Cryptosporidium* infections among community-acquired diarrheal cases. This study also demonstrated a successful application of a low-cost saliva sampling method by mail in a US community. Further development and validation of the non-invasive

salivary antibody method for the surveillance of *Cryptosporidium* infections is warranted.

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