How Clean Must Our Drinking Water Be: The Importance of Protective Immunity

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Background. Cryptosporidium parvum is an important cause of epidemic diarrhea. Few studies have assessed whether serological evidence of prior infection in adults is related to a reduced occurrence of enteric illness.

Methods. Serum samples and enteric illness event data were obtained in 2000 and 2001 from 326 people served by 1 of 2 unfilted surface sources or 1 groundwater source. In 2001, filtration was initiated at 1 of the surface sources. Poisson regression related illness episodes with serological responses to the 15/17- and 27-kDa Cryptosporidium antigen groups.

Results. Subjects with moderately strong responses to the 15/17-kDa antigen had <65% of the risk of all 1–3-day episodes of diarrheal or gastrointestinal illness and <40% of the risk of all ≥4-day episodes, compared with subjects without a moderately strong response. Water source, change in water treatment, and very weak responses were unrelated to illness events.

Conclusions. Endemic Cryptosporidium infections are a common cause of diarrheal and gastrointestinal illness in persons without a moderately strong response to the 15/17-kDa antigen group. Users of surface-derived drinking water are more likely to have strong serological responses to this antigen group and may be at a lower risk of endemic gastrointestinal illness caused by Cryptosporidium infection.
parasite transmission [2–5, 10, 11]. The use of these assays avoids the potential underascertainment of a history of Cryptosporidium infection. Unfortunately, previous studies have not related serological responses to these antigens with the risk of illness from infection.

Protective immunity has been postulated to be a reason for the infrequent occurrence of illness caused by exposure to Cryptosporidium oocysts [12–14]. Protective immunity may develop from repeated low-dose exposure to the oocysts present in surface-derived drinking water [9]. However, the oocyst dose and frequency of exposure needed to initiate a serological response are unknown. If responses to the 15/17- and 27-kDa antigen groups are markers of protection from cryptosporidiosis and if Cryptosporidium infections commonly occur, then people with elevated serological markers may be at lower risk for Cryptosporidium-related illness. We prospectively collected illness and symptom data to evaluate the health benefit from adding the filtration of drinking water to a previously unfiltered surface-water supply. The relationship between the intensity of serological responses to Cryptosporidium antigens and rates of self-reported diarrhea, gastrointestinal illness, and other human illness was examined. Given the difficulty obtaining stool samples and detecting oocysts, differences in rates of enteric illness among people with and those without the markers may better estimate the magnitude of Cryptosporidium-related illness than do reported rates of cryptosporidiosis.

**SUBJECTS AND METHODS**

**Study sites and population.** The present prospective cohort study was conducted in 3 distinct geographic sites in 2 cities in the northwestern United States. Sites A and B were areas of the same city supplied by different unfiltered chlorinated surface-water sources. Both surface sources came from well-protected watersheds with no evidence of human sewage contamination. Site C, located 280 miles away from sites A and B, was served by several utilities using the same groundwater aquifer. Water in all 3 communities was chlorinated during the entire study period. In February 2001, the water-treatment plant for site A was upgraded to include ozonation, filtration and chlorination. No changes were made to the water-treatment processes at sites B and C.

Two separate institutional review boards approved the study before the recruitment of subjects. Illnesses were tracked in the 3 cohorts for a 6-month period before the initiation of filtration at site A (phase 1, June–November 2000) and for the same 6-month period the next year, after the implementation of filtration (phase 2, June–November 2001). Drinking water for all sites, before and after the treatment changes, met the current US Environmental Protection Agency Safe Drinking Water Standards for coliform bacteria.

Families were enrolled from the 3 geographic areas if they had either a child 2–10 years old or an adult at least 65 years old living in the home, drank municipal water and did not have a filtration system in their home, had lived in their residence for at least 6 months, planned on staying in the community for the next 2 years, and were in self-reported overall good mental and physical health. Only family members who were healthy and immunocompetent were enrolled.

During the initial home visit, the family’s main contact person was trained to complete the daily diaries and to record illnesses for each enrolled family member for each day. At the end of each week, the diaries were mailed to the study office. The majority of the data was submitted through the mail, with some data being collected by telephone. Serum samples were collected at local clinics once during phase 1 and again during phase 2 from healthy people ≥18 years old who were either a parent of a young child or an elderly person. No diagnostic stool specimens were collected during illness episodes.

**Illness outcomes.** Three categories of symptoms, reported by contact persons, were considered: (1) diarrheal, defined as at least 1 episode of soft or loose stools; (2) gastrointestinal, defined as nausea, any vomiting, or abdominal cramps; and (3) other symptoms, defined as fever, chills, headache, or cold. Illnesses could be classified in ≥1 illness category if symptoms in ≥1 category were reported. For example, an illness was classified as both gastrointestinal and diarrheal if the person reported nausea and an episode of soft stools.

Symptoms occurring within 5 days of each other were considered to be related and were counted as 1 episode. The duration of the episode was the number of days from the beginning of the episode until the last day of the episode. A new diarrheal or gastrointestinal episode could begin after ≥6 days without diarrheal or gastrointestinal symptoms. For example, the following would be classified as a single diarrheal episode of 6 days duration: a person with no diarrhea for 6 days experiences diarrhea on days 7 and 8, no diarrhea on days 9 and 10, diarrhea on days 11 and 12, and no diarrhea during the next 6 days.

**Western blot procedures.** Serum samples were collected, labeled with a study identification number, and stored at −70°C at local clinics within the same 1-month time frame during each phase. Samples were then shipped on dry ice to Lovelace Clinic Foundation in Albuquerque and again stored at −70°C. The samples were analyzed in batches by phase and, subsequently, by pairs. Serum samples were analyzed by immunoblot, to measure the IgG serological response to the 15/17- and 27-kDa Cryptosporidium antigen groups. No other Cryptosporidium antigen proteins or whole antigens were tested. These methods have been described elsewhere [2–4]. The laboratory technician was blind to residential, demographic, risk factor, or illness information for the individuals tested. The intensity of the serological responses to each antigen group was digitally
analyzed by an IS-2000 digital imaging system (Alpha Innotech) that calculates the pixel density of a manually selected band of the immunoblot. The intensity of each band is standardized by comparing the ratio of the response intensity of the unknown sample to the response intensity of a positive Cryptosporidium control serum sample contained on that blot. All positive control serum samples have an intensity that approximates the response of index serum samples obtained from people with laboratory-confirmed infection. Having comparable positive control-sample intensity for all our studies allowed the comparison of finding between studies. For the purposes of analysis, we categorized the imaged serological responses as non-detectable, detectable with a response of <20% of the positive control (very weak), and detectable with a response of ≥20% of the positive control (moderately strong).

**Poisson regression.** Analyses were performed by use of SAS statistical software (version 8.2; SAS Institute). Illness events were discrete counts and rare events, characteristic of a Poisson distribution of counts. Individual diaries encompassed varying lengths of time each year; adjustments for these varying lengths were made in the analysis models. Illness events per year were modeled by use of a Poisson regression that included variables for site location (i.e., surface site A, groundwater site C, and reference site B), study phase (reference, phase 1), sex (reference, female), age (45–69 and ≥70 years; reference age, <45 years). Separate analyses were performed for diarrhea, gastrointestinal illness, and other illness episodes that lasted 1, 2–3, and ≥4 days, to determine whether the protective effects could be observed for short- and long-term illnesses.

To account for correlated data (>1 observation per person), the generalized estimating equation approach was incorporated into the regression models [15]. Exponentiation of the main effect coefficient in the Poisson model estimates the regression-adjusted incidence density ratio (IDR) for the effect. The incidence density is the number of new illness events divided by the fraction of the year that the person contributed daily diary records. The IDR is the incidence density of illnesses in the exposed population divided by the incidence density of illnesses in the unexposed or reference population. A statistically significant ratio (i.e., <1.0) is consistent with a protective effect from the exposure. In the first analysis, models estimated the possibility of a significant relationship between illness events and a very weak serological response (<20% of the positive control) or a moderately strong serological response (≥20% of the positive control) to the 15/17- and the 27-kDa Cryptosporidium antigen groups, compared with no detectable response. In a second analysis, models estimated the relationship between illness-event rates and moderately strong serological responses to each antigen group.

In a previous study to determine whether a moderately strong serological response was protective for illness [5], we related questionnaire illness data collected at the time of the blood draw and serological response data. The questionnaire asked, “In the past 2 months have you had diarrhea (≥3 loose bowel movements a day) lasting 4 or more days?” Serological responses were determined by use of the same techniques as those used in the present study and were coded according to whether the intensity of response was moderately strong (i.e., ≥20% of the positive control).

**RESULTS**

Over the 2-year period, 522 serum samples were obtained from 326 individuals. Sixty-four subjects participated only in phase 1, 66 only in phase 2, and 196 in both phases. These 196 individuals were distributed, by water source, as follows: 95 from the intervention site A, 37 from site B, and 64 from site C.

The distribution of serum samples, by study area and phase, is given in table 1. Almost half of the samples were collected in site A. During phase 2, there was a decline in the number of samples from site B and an increase in the number from site C. The distribution of the intensity of responses is shown in table 2. The percentage of samples with a strong response remained almost unchanged between phases. Among the 196 people who participated in both phases, 40.3% had strong responses to the 15/17-kDa antigen in phase 1 and 46.3% in phase 2 (data not shown).

Of the 326 people, 44.4% reported an enteric disease event (e.g., diarrhea or gastrointestinal) during the study. In a Poisson regression that related illness events with very weak Cryptosporidium serological responses, by phase of study.

| Table 1. No. of serum samples collected, by site and phase of study. |
|-----------------|-----|-----|-----|-----|
| Phase | A   | B   | C   | Total |
| 1    | 124 | 65  | 73  | 262  |
| 2    | 125 | 39  | 96  | 260  |

| Table 2. Cryptosporidium serological responses, by phase of study. |
|-----------------|-----|-----|-----|
| Antigen group, phase | Undetectable | Very weak | Moderately strong |
| 27 kDa           | 29  | 28  | 43  |
| 15/17 kDa        | 46  | 18  | 36  |

a Responses with an intensity <20% of the positive control.

b Responses with an intensity ≥20% of the positive control.
tosporidium serological responses, IDRs for either the 15/17- or 27-kDa antigen group were not statistically different from 1.0 (P > .05, results not shown). This suggests that the ratio of illness events per unit of enrollment was, for those with a weak serological response, similar to the ratio for those with a nondetectable response. For further analyses, we combined the nondetectable responses with very weak responses.

Table 3 presents results of a Poisson regression that related illness events with moderately strong responses to the 15/17-kDa antigen group. The adjusted IDRs for diarrheal and gastrointestinal illness were 0.27–0.64 and were significant <1.0 (P < .05). This indicates that having a moderately strong serological response was related to a lower risk of diarrheal and gastrointestinal illness. For other illness events (i.e., not diarrheal or gastrointestinal), the adjusted IDRs were not statistically different from 1.0, indicating no evidence of protection.

Table 4 presents the results of a Poisson regression analysis for the 27-kDa antigen group. The adjusted IDRs for diarrheal and gastrointestinal illnesses were 0.31–0.80, but only 3 of 6 IDRs were statistically significant protective. Again, the IDRs for other illnesses were not statistically different from 1.0. The study phase was unrelated to the IDRs.

Because we used positive control serum samples with approximately the same intensity of response in all our serological studies, we reexamined data from a previous paired-city cross-sectional study [5]. Logistic regression examined whether a strong serological response to the 15/17-kDa antigen group was related to diarrhea lasting ≥4 days during the previous 2 months, reported at the time of the blood draw and adjusted for age (<30, 30–39, 40–49, and ≥50 years), sex, and marital status. Participants with a strong serological response were less likely to report diarrhea (city groundwater, P < .04; city surface water, P = .19).

In our previous US studies, the percentage of participants with a moderately strong serological response to the 15/17-kDa antigen varied considerably by site, ranging from a low of 19% of users of a groundwater system [5] to a high of 65% in users of a surface-water system [4]. We used this range of serological responses to calculate a population attributable risk percentage (PAR %) (table 5). This calculation estimates the reduction in occurrence of illness attributable to the serological response. It assumes that there is either a direct or indirect causal relationship between the 15/17-kDa marker and the risk of illness from Cryptosporidium infection. A higher prevalence of the marker in a population would result in a larger proportion of illness being prevented. When a direct or indirect causal relationship was assumed, the PAR % analysis suggested that a 65% prevalence of the moderately strong serological response (the highest prevalence we have observed in the United States) would reduce gastrointestinal or diarrheal illness events by 27%–64%. A 19% prevalence (the lowest level we have observed in prior studies) would reduce gastrointestinal or diarrheal illness events by 10%–34%. For example, for illnesses lasting ≥4 days, a 65% prevalence of the marker would reduce the risk of diarrheal events by at least 50%, whereas 19% prevalence would reduce the risk by at least 23%.

**DISCUSSION**

To our knowledge, this is the first study to show that a moderately strong serological response to a Cryptosporidium antigen...
group is related to a lower risk of enteric illness. There are a number of potential implications of this finding. If the illnesses prevented were cryptosporidiosis, then endemic cryptosporidiosis commonly occurred in users of the 2 surface-water systems and 1 groundwater system in our study. This suggests that modes of transmission other than drinking water may be important in these areas. Second, reanalysis of a previous study of a different population found evidence of the protective effects of a moderately strong response to the 15/17-kDa antigen group [15]. Third, 3 paired-city studies found elevated levels of serological responses to Cryptosporidium antigen groups in users of surface-derived drinking water where no outbreaks had been reported [3–5], which suggests that the users of surface-derived drinking water may be at lower risk of cryptosporidiosis. If future improvements in water treatment reduce serological responses for users of surface water, then the risk of cryptosporidiosis will likely increase. Thus, reducing low-dose waterborne exposures may increase rather than reduce the risks of diarrheal and gastrointestinal illnesses. However, we did not observe higher levels of serological responses for users of surface water versus groundwater in the present study. This may have been due to the high quality of the source water.

The 2 watersheds supplying surface drinking water were well protected and received no human or domestic animal fecal waste. The current results, with 36%–44% of study subjects having a strong serological response, are near the middle of the range of other US and non-US studies (19%–65% prevalence of moderately strong responses).

It is unclear whether the serological responses to the 15/17-kDa antigen group directly reduce the risk of illness or whether other mechanisms associated with serological responses are the primary causes of protection. The 15/17-kDa antibody response declines more rapidly after infection than does the 27-kDa antibody response [16]. Therefore, the stronger relationship between the increased 15/17-kDa antibody response and reduced illness risk may simply reflect the higher correlation of this marker with the time since the most recent infection.

Although this is the first study to specifically relate serological responses to the risk of diarrheal and gastrointestinal illnesses, it is not the first to have found evidence of protective immunity to cryptosporidiosis. This evidence was seen in 2 previous outbreak investigations. Outbreak investigations in Talent and Medford, Oregon [17], and Collingwood, Ontario [14], found evidence that the residents were at lower risk of illness than were visitors who drank city water. In Collingwood, visitors accounted for such a large fraction of illnesses that local physicians questioned whether city drinking water could have caused an outbreak. In Talent, local residents attending a wedding party were at much lower risk of illness than were out-of-town visitors [18]. Because Talent residents had such low attack rates of cryptosporidiosis, Talent drinking water was delivered to Medford residents, to reduce the occurrence of cryptosporidiosis in Medford. This, unfortunately, resulted in a high attack rate of cryptosporidiosis among Medford residents who drank the Talent water [18]. The Collingwood and Talent residents also had, on average, more-intense serological responses than did residents of neighboring towns [17]. Unfortunately, neither of these studies was able to directly relate illness episodes with serological responses.

We made several important assumptions in reaching these conclusions. Serological responses to the Cryptosporidium antigens were assumed to specifically reduce the risk of Cryptosporidium-related illnesses. We do not, however, have direct evidence that the illness prevention resulted from Cryptosporidium infection. It is possible, although unlikely, that the Cryptosporidium serological responses are protective for illness caused by other pathogens, especially if the pathogen shares antigens with Cryptosporidium. At this time, we are unaware of any other organisms that share these antigens and that commonly infect humans in North America. Alternatively, it is unlikely that these serological responses are related to complete protection from all Cryptosporidium-related illnesses. If those with a strong serological response were still at some risk of cryptosporidiosis, then the total burden of Cryptosporidium-related illnesses among adults would be larger than estimated.

The current study suggests that sources other than drinking water may commonly transmit Cryptosporidium. In fact, food and other modes of parasite transmission may be at least as important as drinking water and may be more likely to transmit higher dose exposures.

Cryptosporidium oocysts will likely remain ubiquitous in our environment and eliminating or even significantly reducing human exposures may not be possible. Contaminated drinking water has been a source of epidemics, but, by inducing pro-

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<th>Table 5. Population-attributable risk percent for different prevalences of moderately strong serological responses to the 15/17-kDa antigen group.</th>
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<td>Illness type and duration, days</td>
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<tr>
<td>19% prevalence of marker</td>
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<td>Diarrheal</td>
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<td>Gastrointestinal</td>
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*a Fraction of illnesses prevented by protective effects associated with a 19% or 65% prevalence of moderately strong serological responses to the 15/17-kDa antigen group. 

*b Calculated only if the incidence density ratio was statistically significant <1.0.
tective immunity, it may also protect people from cryptosporidiosis epidemics. In fact, it is possible that the emergence of cryptosporidiosis as a serious epidemic disease in Western countries resulted largely from reduced levels of low-dose exposure and protective immunity. Protective immunity likely declined after improvements in sanitation and drinking-water treatment. Because new drinking-water treatment technologies are available that can more completely remove or inactivate waterborne pathogens, minimizing future public-health risks of enteric illness will require informed decisions, to balance the public-health risks and benefit of these new technologies. It is possible that, if these technologies reduce exposure and, therefore, further reduce protective immunity, they may not prevent waterborne cryptosporidiosis and may even increase the burden of disease from infection. We recognize the potential implications of these finding and that additional studies are needed. However, if our interpretations are correct, then the complete removal of pathogens from drinking water may decrease the risk of waterborne enteric illnesses but increase the risks from nonwaterborne exposures to the same pathogens. Therefore, in evaluating low-dose pathogen exposures, public-health agencies should consider both the potential benefit from protective immunity, as well as the illness risks from waterborne-pathogen exposures.

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References