Comparing Serologic Response against Enteric Pathogens with Reported Diarrhea to Assess the Impact of Improved Household Drinking Water Quality


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Abstract. We evaluated enteric infection serology as an alternative outcome measure to diarrhea prevalence in a randomized controlled trial of household-based drinking water treatment; 492 households were randomly assigned to 5 household-based water treatment interventions or control. Individuals were followed weekly over 52 weeks to measure diarrhea prevalence. Study subjects of age ≥ 6 months and < 24 months had blood drawn at entry and exit from the study or age cohort. Serologic assays for Cryptosporidium parvum, Giardia intestinalis, enterotoxigenic Escherichia coli (ETEC), and Norovirus were done. Of 343 subjects eligible for the study, the proportions of subjects experiencing serologic responses were 56% for Norovirus, 24% for C. parvum, 10% for ETEC, and 16% for G. intestinalis. Serologic response was associated with increased diarrhea prevalence only for G. intestinalis (P = 0.0134). Serologic response to the antigens tested for G. intestinalis but not for Norovirus, C. parvum, and ETEC may be a useful health-effect measure. Larger intervention studies that yield a more marked effect on diarrheal disease, use additional and improved serologic assays, and that collect serum samples at more frequent intervals are needed.

INTRODUCTION

Enteric disease prevalence or incidence is often used as a primary outcome measure for studies that evaluate the health effect of water and sanitation interventions. In such studies, diarrhea episodes are usually ascertained through regular visits to study households by field workers who ask about diarrhea during the period since the previous visit or who review diarrhea diaries. However, measuring enteric infection prevalence or incidence in this way is subject to recall bias,1 relies on the report of an informant in the case of infants and young children, requires substantial human, transport, and material resources, and cannot distinguish pathogen-specific health effects. Furthermore, because many water and sanitation studies are not blinded, a subject’s knowledge of their intervention or control study arm assignment might affect how they report diarrhea history to the field worker.

Serologic assays have been developed that measure an individual’s antibody response to specific enteric pathogens. As such, serologic assays have the potential to provide a more objective measure of exposure to enteric infections than reported diarrhea. Serologic assays for enteric infections have been used successfully in the past to understand the role of flies and fly control in the transmission of enterotoxigenic Escherichia coli (ETEC), Shigella spp., and Norovirus.2,3

We hypothesized that enteric infection serology might be a useful alternative to diarrhea prevalence or incidence in a study designed to assess the health impact of household-based water treatment. To evaluate a battery of pathogen-specific enteric infection serologic assays as potential alternative measures of enteric infection prevalence or incidence, we conducted a nested serologic study within a randomized controlled trial of household-based drinking water treatment interventions for diarrhea prevention in rural Guatemala.

METHODS AND MATERIALS

Randomized controlled trial. The design and findings of the randomized controlled trial of household-based drinking water treatments for diarrhea prevention in rural Guatemala have been reported in detail elsewhere.4 Briefly, the study was conducted between September 2001 and August 2002 in 12 indigenous Kachiquel Mayan villages in the Department of San Juan Sacatepéquez, a region in the highlands 30 km north of Guatemala City. The infant mortality rate in this region is 47.7 per 1,000 inhabitants, and 51% of children in their first year of school meet the World Health Organization criteria for moderate or severe stunting. In this setting, water sources are highly contaminated with feces and household-based water interventions, including those used in the study, have been shown to improve drinking water quality.5 Households with an infant < 12 months of age or a mother in her last trimester of pregnancy were identified in the 12-village area. Four hundred ninety-two households were randomly assigned to 5 different water treatment groups: flocculant-disinfectant, flocculant-disinfectant plus a customized vessel, sodium hypochlorite, sodium hypochlorite plus a vessel, and control. Diarrhea was defined by the respondent, usually the mother, and was ascertained during weekly visits by field workers. Diarrhea was recorded as present or absent since the previous visit. During 1 year of observation, residents of control households had 4.31 episodes of diarrhea per 100 person-weeks, whereas the incidence of diarrhea was 24% (P = 0.04) lower among residents of households receiving flocculant-disinfectant, 29% (P = 0.02) lower among those receiving flocculant-disinfectant plus vessel, 25% (P = 0.01) lower among those receiving sodium hypochlorite, and 12% (P = 0.84) lower among households receiving sodium hypochlorite plus vessel.

In unannounced evaluations of home drinking water (10 per household), free chlorine was detected in samples from 2% of control households, 27% of flocculant-disinfectant house-
holds, 34% of flocculant-disinfectant plus vessel households, 36% of sodium hypochlorite households, and 44% of sodium hypochlorite plus vessel households. Attempts were not made to isolate Cryptosporidium parvum, Giardia intestinalis, enterotoxigenic E. coli (ETEC), or Norovirus from drinking water. However, the median baseline household drinking water E. coli concentration was 63 colony-forming units/100 mL.

Nested serologic study. Selection of marker pathogens. C. parvum, enterotoxigenic E. coli (ETEC), and Norovirus were selected for analysis because they are pathogens actually or potentially transmissible by water and for which an accurate serologic test was available. Furthermore, a previous study from San Juan Sacatepéquez demonstrated that a marked increase in age-specific prevalence of antibodies against these pathogens occurred between 6–12 and 13–18 months of age in this population. A developmental assay for G. intestinalis was included because of the importance of waterborne transmission for that pathogen.

Timing of blood draws. A blood sample was drawn from all study infants ≥ 6 but < 12 months of age at study entry and at study end. Infants < 6 months of age at study entry and infants born into the study had blood drawn when they reached the age of 6 months and at study end. Children of age 12–18 months of age at study entry had blood collected at baseline and 6 months into the study. Serum was separated and frozen at −70°C for batched serologic testing.

Nutritional assessments. Height and weight of subjects were measured at study entry. Weight for age, height for age, and weight for height z-scores were calculated using the National Center for Health Statistics (NCHS) reference population.

Laboratory methods. C. parvum. Antibody levels to the C. parvum 27- and 17-kDa surface antigens were assayed by ELISA in duplicate wells using a recombinant Cp23 protein at 7 ng/well and a recombinant Cpi7 protein at 15 ng/well. Cpi7mat (AF114166) was expressed in a pGEX 4T-2 vector (Pharmacia Biotech, Piscataway, NJ) and purified by glutathione-S-transferase column chromatography as described by the manufacturer. Antibody levels of unknown samples were assigned a unit value based upon an 8-point positive control standard curve with a 4-parameter curve fit. The 1:50 dilution of the positive control serum was arbitrarily assigned a value of 6,400 units. Assays were repeated if the standard deviation for the duplicate wells was > 15% of the mean value (unless both values were considered negative). For Cp23, unit values > 116 were considered positive, and for Cpi7, unit values > 155 were considered positive. An antibody response was defined as a > 50% increase in response to one antigen and a > 10% response for the other antigen between the two samples; both responses had to be above the cutoff for positivity in the second sample. Seroconversion was defined as change from negative to positive for both antigens.

G. intestinalis. The G. intestinalis α-1 (X52485) and α-7.3 (A514360) giardins were expressed as 6× His fusion proteins in pQE81 vector (Qiagen, Valencia, CA) and purified by nickel affinity chromatography, as suggested by the manufacturer. Antibodies were assayed by ELISA in duplicate wells using both recombinant proteins at 40 ng/well. Antibody levels were assigned using a standard curve as described above for C. parvum. An arbitrary definition for a seroresponse to the G. intestinalis antigens was set as a 50% increase in unit value, with the unit value for the second sample being above 250.

Enterotoxigenic E. coli. Serologic antibodies to heat-labile enterotoxin (LT) were measured by ELISA. The LT (Sigma-Aldrich Chemical Company, St. Louis, MO) was derived from an ETEC strain of human origin. LT was diluted and coated onto Immunon-2 microtiter plates (Dynex Plastics, Chantilly, VA), incubated overnight, and washed. Test sera were added, incubated, and washed, followed by incubation with alkaline phosphatase-conjugated monoclonal anti-human antibody. Absorbance was read at 405 nm after 20 minutes of incubation. A standard curve was plotted using human serum from a culture-confirmed case of ETEC, and relative test values were derived from this curve. Seroconversion was defined as a 4-fold or greater rise between the two samples.

Norovirus. Antibody to recombinant-expressed capsid proteins from Norovirus was measured using previously published methods. Dilutions of a reference serum were used to generate a standard curve relating enzyme immunoassay absorbance values to arbitrary antibody units. The unknown samples were tested at a single dilution and the standard curve was used to back-calculate antibody units. Seroconversion was defined as a 4-fold or greater rise between the two samples.

Statistical analyses. A previous study in Guatemala found that among children 6–12 months of age in the ensuing 12 months, 69% convert from negative to positive for C. parvum antibody, 56% convert from negative to positive for Norovirus antibody, and 32% convert from negative to positive for ETEC antibody. Assuming the same age-specific prevalences and an estimated 20% seroconversion in infants 3–6 months old tested 6 months later, we calculated that, among children 3–24 months, 49% will convert from negative to positive for C. parvum antibody and 41% from negative to positive for Norovirus antibody. Assuming 30% dropout and 80% power, 100 children would be sufficient to detect a ≥ 40% or difference in C. parvum antibody acquisition and a ≥ 50% difference in Norovirus antibody acquisition. One hundred children provide insufficient power to detect a difference with ETEC, but ETEC would provide a contrast between the effects on chlorine-sensitive versus chlorine-resistant organisms.

Descriptive statistics of study subjects and proportions of serologic responses were calculated as proportions, medians, and ranges. Initially, infants in households randomized to any intervention were grouped and compared with those in control households. To assess the association between serologic response and diarrhea, mean diarrhea prevalence was compared between subjects with and without each antibody response using the t test. To assess differential effects of household-based water treatments on serologic responses, infants in households randomized to flocculant-disinfectant interventions and to disinfectant interventions were compared with those in control households. Because low intervention use in this study may have blunted our ability to find intervention-specific effects on serologic responses, households were divided into those with free chlorine concentrations above and below the median. Serologic responses were compared between households with free chlorine concentrations above and below this threshold. Effects were compared using the χ²
test. Because nutritional status and diarrheal disease may be associated, we compared diarrhea prevalence of malnourished infants with normally nourished infants using the t test and compared diarrhea prevalence with Z-scores using Pearson’s correlation statistics. Furthermore, because we hypothesized that malnourished infants may have experienced more diarrhea yet have blunted serologic responses, we explored for an interaction between underweight (moderate, below –2 standard deviations [SD], and severe, below –3 SD of the median weight-for-age of the reference population), stunting (moderate, below –2 SD, and severe, below –3 SDs of the median height-for-age), and wasting (moderate, below –2 SDs, and severe, below –3SDs of the median weight-for-height) and serologic response. To do this we stratified the analysis of serologic response by nutritional state and compared the odds ratios across the strata. Analyses were done using EpiInfo version 3.01 (Stone Mountain, GA) and SAS version 9.00 (Cary, NC) software.

Ethics. The field team explained the purpose of the study to each prospective female or male head or household in Spanish or Kachiquel. Field workers emphasized that participation was voluntary and that subjects could withdraw at any time; they obtained written informed consent from those who were literate and verbal consent from those who were not. An Institutional Review Board at CDC and the Ethics Committee Review Board at the Universidad del Valle de Guatemala reviewed and approved the study protocol.

RESULTS

Characteristics of study subjects. Of 363 subjects who met the age criteria, paired sera were collected from 343 (94%) subjects eligible for the study; 179 (52%) were female. The median (range) age of study subjects at the baseline blood draw was 8 (6–12) months and 20 (6–24) months at the follow-up blood draw. Infants of age ≤ 12 months received breast milk during 97% of study weeks of the parent study, but they also received supplementary liquids in 92% and supplementary solids in 65%. By contrast, infants of 12–24 months received breast milk during 90% of study weeks, but they also received supplementary liquids in 97% and supplementary solids in 86%. The proportions of subjects experiencing serologic responses measured by study assays are summarized in Table 1.

Association between serologic responses to enteric pathogens and diarrhea. Mean diarrhea prevalence by antibody response status is summarized in Table 2. Antibody response to G. intestinalis antigen was associated with significantly higher diarrhea prevalence.

Effect of flocculant-disinfectant and disinfectant interventions on serologic responses. There were no statistically sig-

<table>
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<th>Antigen</th>
<th>Serologic response</th>
<th>Mean diarrhea prevalence*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Serologic response present</td>
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<tr>
<td>C. parvum</td>
<td>Seroconversion</td>
<td>4.8040</td>
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<tr>
<td>G. intestinalis</td>
<td>Antibody response</td>
<td>5.7631</td>
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<tr>
<td>ETEC</td>
<td>Seroconversion</td>
<td>4.1262</td>
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<tr>
<td>Novovirus</td>
<td>Seroconversion</td>
<td>4.5042</td>
</tr>
</tbody>
</table>

* Not all patients had sufficient serum for all serologic tests to be done.

significant differences in serologic responses to individual enteric infections when infants living in households assigned to flocculant-disinfectant interventions were grouped and infants living in households assigned to disinfectant interventions were grouped, and each was compared with infants living in control households. Compared with control households, the relative risk of seroconversion for infants living in households assigned to flocculant-disinfectant interventions for *Norovirus* was 1.05 (P = 0.8953); for *C. parvum*, 1.15 (P = 0.7017); for *ETEC*, 0.88 (P = 0.7435); and for *G. intestinalis*, 0.61 (P = 0.2413). The relative risk of seroconversion for infants living in households assigned to hypochlorite interventions for *Norovirus* was 0.89 (P = 0.4707); for *C. parvum*, 1.24 (P = 0.5442); for *ETEC*, 0.80 (P = 0.5743); and for *G. intestinalis*, 0.80 (P = 0.5806) (Tables 3 and 4).

The median (range) free chlorine concentration in all household drinking water at unannounced visits was 0.05 (0.01–2.73) mg/L. One hundred thirteen (47%) households had drinking water free chlorine concentrations above the median, evidence that the intervention was being regularly used. We compared serologic responses of subjects living in households with drinking water free chlorine concentrations above the median with those with free chlorine concentrations below the median because higher free chlorine concentrations provide evidence of intervention use. Although there was a trend towards ETEC seroconversion being associated with evidence of intervention use, there was no such trend observed for *C. parvum*, *G. intestinalis*, or *Norovirus* (Tables 3 and 4). Similar results were found using a free chlorine concentration cutoff of ≥ 0.1 mg/L.

Relationship between nutritional status, diarrhea prevalence, and serologic responses. The median weekly diarrhea prevalence among infants with moderate or severe underweight (> 2 standard deviations below the NCHS standard) was 4.2 weeks with diarrhea per 100 person weeks compared with 4.4 for infants who were not underweight (P = 0.6477). By contrast, the median weekly diarrhea prevalence among infants with moderate or severe stunting was 4.5 compared with 4.3 for infants who were not stunted (P = 0.6918) and was 5.2 for infants with moderate or severe wasting compared with 4.3 for infants who were not wasting (P = 0.3805). When analyzed as continuous variables, wasting (r = 0.103, P = 0.0311) but not underweight (r = -0.05101, P = 0.2857) or stunting (0.00741, P = 0.8769) was associated with diarrhea incidence.

There was no association between moderate or severe stunting, wasting, or underweight and serologic response. Furthermore, when we stratified the analysis of the effect of any household-based water treatment intervention on sero-
logic response by nutritional status, we found that stratumspecific odds ratios did not differ for moderate or severe underweight, stunting, or wasting.

DISCUSSION

Although serologic responses to *C. parvum*, ETEC, and *Norovirus* were not associated with diarrhea prevalence in this study, *G. intestinalis* serologic response did show a significant association with diarrhea prevalence, suggesting that it may be useful in assessing health-effect measures for household-based water treatments. Other research used a serologic approach to demonstrate that flies play a role in the transmission of *Shigella* and ETEC, but not *Norovirus* infection, suggesting that enteric infection serology may be a useful tool in evaluating environmental interventions. The lack of an association between some serologic markers and diarrhea in our study could be that *C. parvum* and ETEC may be less likely than *Giardia lamblia* to establish chronic infections, and so antibody responses for the infections may be expected to be more short-lived and less likely to be detected by the 6-months to 1-year serum-sample collection interval of this study.

The limited association between diarrhea prevalence and serologic response in this study may have several explanations. First, although diarrhea prevalence or incidence have been the outcome measures of choice in studies evaluating household-based water treatment, mostly such studies are not blinded and it is possible that courtesy bias could lead to overestimation of the health effect measured by diarrhea and that serologic responses may, in fact, be a more accurate objective measure. Of 3 blinded studies of household-based water treatments, statistically significant reductions in diarrhea have not been observed. However, 2 studies were done in the developed world with technologies that could be more easily blinded than those in our study. Uptake of household-based water interventions was low in the health effect study within which our serologic study was nested. This was evident from the low proportion of intervention households that had detectable free chlorine levels in drinking water at unannounced visits. Low uptake of interventions led to modest diarrhea reductions compared with that which can be achieved with greater intervention use. Because there was only a modest effect of interventions on diarrhea prevalence, there was likely also a modest effect on

### Table 3

Association between water treatment interventions and serologic responses to *C. parvum* and *G. intestinalis*, San Juan Sacatepéquez, Guatemala, 2001–2002

<table>
<thead>
<tr>
<th>Intervention assignment</th>
<th>C. parvum</th>
<th>G. intestinalis</th>
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<tr>
<td>Intervention assignment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium hypochlorite alone</td>
<td>8/42 (19)</td>
<td>8/42 (19)</td>
</tr>
<tr>
<td>Sodium hypochlorite plus vessel</td>
<td>12/37 (32)</td>
<td>5/37 (14)</td>
</tr>
<tr>
<td>Sodium hypochlorite interventions combined</td>
<td>20/79 (25)</td>
<td>13/79 (16)</td>
</tr>
<tr>
<td>Flocculant-disinfectant alone</td>
<td>11/43 (26)</td>
<td>6/43 (14)</td>
</tr>
<tr>
<td>Flocculant-disinfectant plus vessel</td>
<td>8/38 (21)</td>
<td>4/37 (11)</td>
</tr>
<tr>
<td>Flocculant interventions combined</td>
<td>19/81 (23)</td>
<td>10/70 (14)</td>
</tr>
<tr>
<td>All interventions</td>
<td>39/160 (24)</td>
<td>23/159 (14)</td>
</tr>
</tbody>
</table>

### Evidence of water treatment*

Free chlorine concentration† > median 23/100 (23) 25/103 (24) 0.95 (0.8316) 15/97 (15) 17/104 (16) 0.95 (0.3585)

### Table 4

Association between water treatment interventions and serologic responses to ETEC and *Norovirus*, San Juan Sacatepéquez, Guatemala, 2001–2002

<table>
<thead>
<tr>
<th>Intervention assignment</th>
<th>ETEC</th>
<th>Norovirus</th>
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<td>Intervention assignment</td>
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<td></td>
</tr>
<tr>
<td>Sodium hypochlorite alone</td>
<td>3/54 (6)</td>
<td>23/48 (48)</td>
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<tr>
<td>Sodium hypochlorite plus vessel</td>
<td>7/51 (14)</td>
<td>24/45 (53)</td>
</tr>
<tr>
<td>Sodium hypochlorite interventions combined</td>
<td>10/105 (10)</td>
<td>47/93 (51)</td>
</tr>
<tr>
<td>Flocculant-disinfectant alone</td>
<td>7/57 (12)</td>
<td>33/50 (58)</td>
</tr>
<tr>
<td>Flocculant-disinfectant plus vessel</td>
<td>4/48 (8)</td>
<td>23/44 (52)</td>
</tr>
<tr>
<td>Flocculant interventions combined</td>
<td>11/105 (10)</td>
<td>56/94 (60)</td>
</tr>
<tr>
<td>All interventions</td>
<td>21/210 (10)</td>
<td>103/187 (55)</td>
</tr>
</tbody>
</table>

### Evidence of water treatment*

Free chlorine concentration† > median 8/113 (7) 18/126 (14) 0.50 (0.0747) 66/113 (58) 67/126 (53) 1.10 (0.4172)

*Subjects living in households with drinking water free chlorine > median concentration were classified as having evidence of intervention use.
†Free chlorine concentration was measured 10 times in all study households during unannounced visits.
serologic response to waterborne enteric pathogens, reducing our statistical power to detect an association between the two outcome measures.

Although chlorine-based household drinking water interventions would be expected to have little effect on infections due to chlorine-resistant pathogens such as *C. parvum* and *G. intestinalis*, the flocculant-disinfectant evaluated in this study has been shown to accomplish > 3-log reductions of *Cryptosporidium* oocysts, an effect on water quality that would not be anticipated with disinfectant alone. Despite anticipated effects of the flocculant-disinfectant on chlorine-resistant pathogens, based on water quality studies, there was no statistically significant differential effect seen in households randomized to flocculant-disinfectant compared with households randomized to sodium hypochlorite in this study. The lack of observed differential effects in our study could have been due to limited statistical power because of the relatively small number of infants studied. When we divided study households according to evidence of intervention use based on free chlorine concentration in drinking water, we saw a trend toward household members with evidence of intervention use being less likely to seroconvert to ETEC but not to other pathogens. This trend is consistent with the known relatively high chlorine-susceptibility of ETEC compared with *C. parvum* and *G. intestinalis* and with the predominance of *Norovirus* transmission by non-waterborne routes described in the United States. It is likely that a substantial proportion of infants in this study experienced serologic response to enteric pathogens before study enrollment. An earlier study from Guatemala showed that at the age of 6–12 months, the seroprevalence of antibodies for *Norovirus* was already 24%, for ETEC was 48%, and for *C. parvum* was 27%. Seroprevalent infection at baseline may have limited the utility of serology as a health outcome measure in our study of infants and would certainly curtail the value of serology as an outcome measure in older age groups. It is also possible that the presence of maternal antibody present at birth could have elevated antibody levels in the first sample, making accomplishment of a significant antibody rise at the second sample less likely. Because breastfeeding occurred in 90% of study weeks, even for subjects of age 12–24 months, it is unlikely that the effect of neutralizing factors in breast milk changed substantially between the first and second blood draws. Appropriate timing of collection of serum samples relative to an infectious episode is of critical importance. Research using the *Cryptosporidium* serology among Peruvian children suggests that the time interval between serum samples in our study may have been too long to detect immunoglobulin peaks associated with individual infections. Transmission of enteric pathogens by routes other than water would blunt the measured health effect in a study evaluating interventions focused only on water. Indeed, another study suggested that transmission from the environment may be important for children of age 1–4 years. Furthermore, while little is known about the transmission routes for *Norovirus* in developing countries, in the United States *Norovirus* is thought to be transmitted predominantly by the foodborne route. It is likely that numerous pathogens other than those examined by the serologic assays in this study contributed to diarrhea and that the proportion of diarrhea attributable to each evaluated pathogen may have been small. Our ability to evaluate only 4 pathogens serologically among a small group of infants likely hampered our power to detect an association of serology with diarrhea.

Another disadvantage of a serologic approach is that it measures infection rather than disease. Preventing disease is a more important health goal than preventing infection. In an environment highly contaminated with enteric pathogens such as San Juan Sacatepéquez, preventing infection may be more difficult than preventing disease, and, therefore, a serologic approach may be too sensitive. Methods for quantifying reported diarrhea, such as diarrhea incidence and longitudinal diarrhea prevalence, have been shown to be associated with health outcomes such as weight gain and mortality, respectively. Testing for a range of enteric infections using serologic methods is expensive, although this study did not aim to compare the cost of measuring reported diarrhea with serologic approaches.

Diarrheal disease has been associated with poor nutritional status as well as with other long-term adverse health outcomes. We observed an association between diarrhea prevalence and wasting in this study but no association between diarrhea prevalence and underweight or stunting. Despite the association with wasting, stratified analysis showed no effect of moderate or severe stunting, wasting, and underweight on serologic responses. These findings suggest that serologic responses to enteric infections are preserved even in the face of moderate and severe malnutrition.

Our study suggests that serologic response to antigens testing for *G. intestinalis* but not for *Norovirus*, *C. parvum*, and ETEC is a useful health effect measure for household-based intervention studies examining the health effect of water interventions. However, serology does offer a potential objective measure of health effect, requires blood draws at intervals rather than intensive diarrhea surveillance, and offers the potential to evaluate pathogen-specific effects. As such, we believe that further research on serologic approaches is warranted. Larger intervention studies in other locations that yield a more marked effect on diarrheal disease, use additional and improved serologic assays, and that collect serum samples at more frequent intervals are needed.

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