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PREVALENCE OF INFECTION WITH WATERBORNE PATHOGENS: A SEROEPIDEMIOLOGIC STUDY IN CHILDREN 6–36 MONTHS OLD IN SAN JUAN SACATEPEQUEZ, GUATEMALA

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Abstract. Water and sanitation interventions in developing countries have historically been difficult to evaluate. We conducted a seroepidemiologic study with the following goals: 1) to determine the feasibility of using antibody markers as indicators of waterborne pathogen infection in the evaluation of water and sanitation intervention projects; 2) to characterize the epidemiology of waterborne diarrheal infections in rural Guatemala, and 3) to measure the age-specific prevalence of antibodies to waterborne pathogens. Between September and December 1999, all children 6–36 months of age in 10 study villages were invited to participate. We collected sufficient serum from 522 of 590 eligible children, and divided them into six-month age groups for analysis (6–12, 13–18, 19–24, 25–30, and 31–36 months). The prevalence of antibodies was lowest in children 6–12 months old compared with the four older age groups for the following pathogens: enterotoxigenic *Escherichia coli* (48%, 81%, 80%, 77%, and 83%), Norwalk virus (27%, 61%, 83%, 94%, and 94%), and *Cryptosporidium parvum* (27%, 53%, 70%, 67%, and 73%). The prevalence of total antibody to hepatitis A virus increased steadily in the three oldest age groups (40%, 28%, 46%, 60%, and 76%). In contrast, the prevalence of antibody to *Helicobacter pylori* was relatively constant in all five age groups (20%, 19%, 21%, 25%, and 25%). Serology appears to be an efficient and feasible approach for determining the prevalence of infection with selected waterborne pathogens in very young children. Such an approach may provide a suitable, sensitive, and economical alternative to the cumbersome stool collection methods that have previously been used for evaluation of water and sanitation projects.

INTRODUCTION

Water is essential to life and health; however, more than one billion people worldwide do not have access to safe drinking water.[1] Waterborne diseases have been estimated to cause more than two million deaths and four billion cases of diarrhea annually.[1] Infectious diarrhea is responsible for the greatest burden of this morbidity and mortality,[2] and children less than five years of age are the most severely affected.[1] Water and sanitation interventions have the potential to reduce waterborne infections and the associated disease burden by as much as 50%.[3]

Given the potential of these interventions to decrease the burden of illness, effective means to evaluate their health impact is essential. However, water and sanitation interventions have proven difficult to evaluate because targeted enteric pathogens may be transmitted through multiple routes (i.e., contaminated drinking water, contaminated food, person-to-person contact) and because rates of disease show seasonal and secular variation.[4] Evaluations that focus on infection with specific pathogens, rather than on overall disease rates, provide more precise information about the effectiveness of these intervention programs.

Historically, epidemiologic studies that conducted pathogen-specific analysis of enteric infections have been limited. The standard technique for identifying diarrheal pathogens has been collecting stool samples with each episode, and then testing each sample for the presence of parasitic, viral, and bacterial pathogens. However, stool evaluation poses several logistical problems. First, collecting stool specimens with each diarrheal episode and testing each stool for the full range of pathogens requires extensive field and laboratory work. Second, even under optimal field conditions, a specimen from every diarrheal illness can not be obtained. Third, even when specimens are obtained, they frequently do not yield a specific identifiable pathogen. In longitudinal studies of children with diarrhea in developing countries, 16–47% of diarrheal episodes were not cultured, and 27–60% of stools from children with diarrhea yielded no pathogen.[5–8] Thus, the complexity and expense of these procedures limits the usefulness of stool collection as a means for identifying infection due to specific pathogens.

Recently improved immunodiagnostic techniques for pathogens that are often waterborne offer an attractive alternative to stool collection for assessing exposure to specific pathogens. We conducted a seroepidemiologic survey to determine the feasibility of using antibody markers as an indicator of infection with specific waterborne pathogens, to measure the age-specific prevalence of antibodies to waterborne pathogens, and to characterize the epidemiology of waterborne infections in rural Guatemala. Our goals included establishing whether serology would be an important method in the evaluation of longer term planned projects.

MATERIALS AND METHODS

Selection of marker pathogens. We selected bacterial, parasitic, and viral pathogens for the evaluation of pathogen-specific antibodies in sera as markers based on three conditions: 1) that the pathogens are often transmitted by water, 2) available an accurate serologic test, and 3) previous studies in other settings demonstrated a marked increase in age-specific prevalence of antibodies between 6 and 36 months of age in areas with contaminated drinking water.[9–14] We mea-

sured the IgG antibodies to provide information about long-term exposure. Based on these criteria, we chose the following pathogens: enterotoxigenic *Escherichia coli* heat-labile enterotoxin (ETEC LT), *Helicobacter pylori, Cryptosporidium parvum,* Norwalk-like viruses, and hepatitis A virus (HAV).

Site selection. We conducted the study in San Juan Sacatepéquez, Guatemala, a rural area approximately 32 kilometers west of Guatemala City. We chose communities known from previous studies to have high diarrheal rates and contaminated drinking water (Lopez MB and others, unpublished data). Water sources primarily used in these communities included shallow wells and springs. None of the communities evaluated in this study had piped or treated water or community water tanks.

Data collection. Between September 30 and December 1, 1999, we asked all families with children 6–36 months of age in 10 study villages to participate in the study. A census had recently been conducted in these villages. On the day prior to enrollment, field workers visited every household in the community where a child less than three years of age lived to provide information about the study. Mothers then brought their children to the health post for enrollment the following day and there was nearly universal participation. We confirmed the participating child's age by checking the child's birth certificate or vaccination card. Six months of age was chosen as the lower age limit to avoid the effect of the passive transfer of maternal antibody. Trained local field workers interviewed the mother and completed a questionnaire for each child. The questionnaires included information on demographics, household composition and characteristics, and water use and storage practices.

We measured weight to the nearest 0.1 kg with a digital scale (Seca Corporation, Hanover, MD) and height/length to the nearest 0.1 cm on a height/length measuring board (Shorr Productions, Olney, MD). We used upright height for children greater than or equal to two years of age and reclining length for children less than two years of age. Stunting was defined as low height-for-age or length-for-age and wasting was defined as low weight-for-height according to the United States National Center for Health Statistics/World Health Organization (WHO) international reference values. The WHO Z-score classification system was used for both malnutrition indicators (mild = Z-score < –1; moderate = Z-score < –2; severe = Z-score < –3).[15]

Laboratory methods. During the study period, we attempted to obtain a serum specimen from each participating child on the day of enrollment at the health post. We pretreated the site chosen for venipuncture with a topical anesthetic (EMLA® cream [2.5% lidocaine and 2.5% prilocaine]; Astrazeneca Pharmaceuticals, Wilmington, DE). We drew approximately 1 mL of venous blood using a 23-gauge butterfly needle and a 3-mL syringe and then transferred the blood into labeled Microtainer® tubes (Becton Dickinson, Franklin Lakes, NJ). We measured the hemoglobin level (g/dL) using the Hemocue system that measures quantitative hemoglobin using a portable battery-operated photometer and disposable cuvettes (Hemocue Inc, Mission Viejo, CA). A hemoglobin level < 11 g/dL was classified as anemia according to WHO standards.[16]

To detect the prevalence of *H. pylori,* ETEC, *C. parvum,* Norwalk-like viruses, and HAV, we measured IgG by an enzyme-linked immunoassay (EIA) according to the methods detailed later in this report. Due to a shortage of reagent, we tested only 28% (150) of the 544 samples selected at random for *C. parvum.*

Helicobacter pylori. Each sample was run in triplicate and the mean optical density (OD) was calculated to determine presence of antibodies to *H. pylori*. A pre-selected and well-characterized *cag*A+ *vac*A+ clinical isolate of *H. pylori* was grown overnight in *Brucella* broth (Gibco Laboratories, Madison, WI) with 10% fetal bovine serum (Sigma, St. Louis, MO), 5 μg/mL of trimethoprim, and 10 μg/mL of vancomycin (Sigma). Antigen extraction and protein isolation were done by gentle freeze-thaw sonication (Heat System, Farmingdale, NY).[13,17] A standard protein assay (Pierce, Rockford, IL) was used to determine the accurate and reproducible quantity of solid-phase antigen for use in our microtiter research enzyme-linked immunosorbent assay (ELISA).[18]

Cross-reactivity and specificity of *H. pylori* whole-cell antigens has been previously described.[12,18] Optical density values at a wavelength of 492 nm were determined in triplicate for each biopsy-confirmed control patient serum, using a standard 96-well microtiter plate ELISA spectrophotometer (Fisher Scientific, Pittsburgh, PA). The mean OD values were then calculated. The ELISA cut-off values were derived using known *H. pylori*-positive and -negative control sera as previously described.[13,19] The validity of these commercial assays for use in the clinical setting in pediatric and adult populations has been previously evaluated.[17,20]

Enterotoxigenic E. coli. A research-based ELISA was developed for detecting antibodies to the LT of *E. coli.* The LT derived from an ETEC strain isolated from a human source was obtained from Sigma. One hundred microliters of an optimal concentration of LT diluted in phosphate-buffered saline (PBS), pH 7.2, was added to each well of microtiter plates (Immunlon 2: Dynatech Corporation, Chantilly, VA). The plates were covered and incubated overnight at 4°C. The optimal concentration of LT was determined by block titrations using human sera from a culture-confirmed case of ETEC infection and an alkaline phosphatase–conjugated monoclonal anti-human IgG antibody conjugate (Foodborne Disease Laboratory Section, Foodborne and Diarrheal Diseases Branch, Centers for Disease Control and Prevention [CDC], Atlanta, GA). The following day, the plates were washed three times with PBS containing 0.1% Tween 20 (PBS-T), 200 μL of 2% bovine serum albumin (BSA) in PBS was added to each well, and the plates were incubated at 37°C for at least two hours to block any unactivated sites. The blocking buffer was removed after two hours and test sera diluted in PBS-BSA containing 0.1% Tween 20 were added. The plates were incubated for one hour at 37°C, after which they were washed three times with PBS-T. One hundred microliters of an optimal concentration of alkaline phosphatase–conjugated anti-human IgG was added to each well and the plates were incubated for one hour. Finally, the plates were washed three times with PBS-T and n-nitrophenyl phosphate substrate was added. After a 20-minute incubation, the reaction was stopped with 3 N NaOH and the absorbance of each well read at 405 nm. Relative values of each test serum were determined by comparison with a standard curve (derived from serum from a culture-confirmed human case of ETEC) on each plate.

Cryptosporidium parvum. The levels of antibody to the *Cryptosporidium parvum* surface 27-kD antigen were assayed by an ELISA in duplicate wells using a recombinant Cp23 protein as previously described.[21] A total of 150 serum samples (30 from each age category) were assayed. Antibody levels of unknown samples were assigned a unit value based upon an eight-point positive control standard curve with a four parameter curve fit. The 1:50 dilution of the positive control serum was arbitrarily assigned a value of 6,400 units.[21] Unit values were expressed per microliter of serum. Assays were repeated if the standard deviation for the duplicate wells was > 15% of the mean value (unless both values were considered negative). Unit values > 116 were considered positive.

Norwalk virus. Antibody to recombinant-expressed capsid proteins from Norwalk virus was measured using previously published methods.[22] Dilutions of a reference serum were used to generate a standard curve relating EIA 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. Antibody values within two-fold were considered nominally identical.

Hepatitis A virus. Serum samples were tested at the Hepatitis Reference Laboratory at CDC for the presence of total antibody to HAV using licensed, commercially available assays (HAVAB-EIA; Abbott Laboratories, Abbott Park, IL).

Statistical analysis. Data were analyzed using Epi-Info version 6.04 (CDC, Stone Mountain, GA) and SAS version 8 (SAS Institute, Cary, NC). Study subjects were grouped into five six-month age categories, (6 to < 12, 12 to < 18, 18 to < 24, 24 to < 30, and 30 to < 36 months).

Ethics. The study procedures were explained to mothers, who provided informed consent for all participating children. The protocol was reviewed and approved by the Ethics Committee Review Board at the Universidad del Valle de Guatemala, and an Institutional Review Board at CDC.

RESULTS

Study group. We enrolled 590 children in the study; siblings from the same household were not excluded. The median age was 20.7 months (range = 6–35.9 months) and 302 (51.2%) children were male (Table 1).

Household characteristics. A total of 494 (84%) of the children's mothers had no formal education and could not read or write. The median number of persons per household was six (range = 2–14) and the median number of rooms per house was one (range = 1–8). Overall, 470 (80%) children shared a bed for sleeping. Electricity was available in 311 (53%) of the children's households.

Water practices. Families stored drinking water in 586 (99%) of the households. The drinking water storage container was covered in 410 (70%) and open in 175 (30%) of the households. To obtain drinking water, 540 (92%) dipped a cup or bowl into the water storage container. Household water treatment was uncommon: 61 (10%) reported boiling water and 37 (6%) reported using chlorine.

Anthropometry. Stunted growth was common, with 359 (60.8%) children having at least moderate stunting based on Z-scores. In contrast, wasting was much less common, with only 121 (20.5%) children having mild or moderate wasting based on Z-scores (Table 1).

Laboratory results. A total of 114 (19.4%) children met the WHO criteria for anemia (Table 1). We obtained sufficient serum to measure IgG by EIA in 544 of the 590 enrolled children. We excluded 22 of these children from the analysis because their ages could not be confirmed. Overall seroprevalence varied for the different pathogens (Table 2), as did the pattern for age of acquisition (Figure 1). For ETEC and Norwalk virus, the prevalence of antibodies was lowest in children 6–12 months old compared with the four older age groups, with the steepest increase in antibody acquisition between six and 18 months of age (ETEC = 48%, 81%, 80%, 77%, and 83%; Norwalk virus = 27%, 61%, 83%, 94%, and 94%). The seroprevalence of *C. parvum* followed a similar pattern, with a slightly more gradual increase in antibody (27%, 53%, 70%, 67%, and 73%). The prevalence of total antibody to HAV increased steadily in the three oldest age groups (40%, 28%, 46%, 60%, and 76%). In contrast, the prevalence of antibody to *H. pylori* remained relatively constant in all five age groups (20%, 19%, 21%, 25%, and 25%).

Risk factors for infection. We evaluated risk factors for infection including sex, feeding practices, nutritional status, household characteristics, and water storage and treatment practices. None of these factors were strongly associated with infection (relative risk > 1.5).

TABLE 1

Characteristics of the study population in San Juan Sacatepéquez, Guatemala, 1999

Characteristic	No. (%)
Age group in months	
6 to <12	111 (18.8)
12 to <18	127 (21.5)
18 to <24	147 (24.9)
24 to <30	104 (17.6)
30 to <36	101 (17.1)
Male sex	302 (51.2)
Anthropometry	
Stunting	
Mild	133 (22.5)
Moderate	226 (38.3)
Severe	188 (31.9)
Total	547 (92.7)
Wasting	
Mild	108 (18.3)
Moderate	13 (2.2)
Severe	0 (0.0)
Total	121 (20.5)
Anemia	
Hemoglobin < 11 g/dL	114 (19.4)

DISCUSSION

In these Guatemalan communities, serology appeared to be an efficient and feasible approach for determining the history

TABLE 2

Overall seroprevalence of marker pathogens in 522 children ages 6–36 months in San Juan Sacatepéquez, Guatemala, 1999

Pathogen	Overall No. (%)
Norwalk virus	388 (72.4)
Enterotoxigenic *Escherichia coli*	268 (45.4)
Hepatitis A virus	256 (48.8)
Helicobacter pylori	95 (22.1)
*Cryptosporidium parvum**	88 (58.7)

* Total tested = 150.

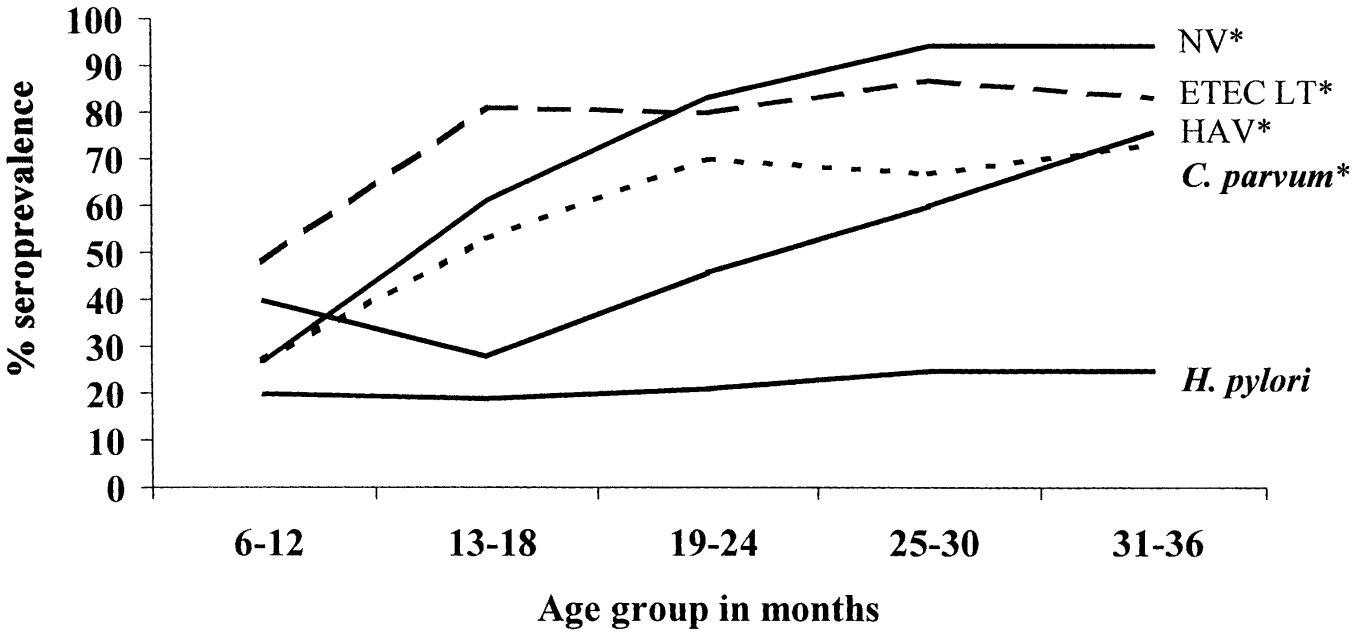

FIGURE 1. Age-specific seroprevalence rates in San Juan Sacatepéquez, Guatemala, 1999. NV = Norwalk virus; ETEC LT = enterotoxigenic *Escherichia coli* labile enterotoxin; HAV = hepatitis A virus; *C. parvum* = *Cryptosporidium parvum*; *H. pylori* = *Helicobacter pylori*. $*P < 0.01$ by chi-square test for trend.

of infection with selected waterborne pathogens in very young children. Serology measures antibodies that accumulate over time making it easy to assess past infection, which is difficult to do with active stool surveillance. The cross-sectional design allowed us to collect data in much less time than would be required to collect stool specimens during or following each episode of illness for each child. Stool collection would have required multiple home visits and complicated arrangements for transporting specimens to the laboratory, thereby increasing the cost of the study. Instead, over the course of the study, we collected all of the specimens at the health post during each child's single visit for study enrollment and transported them to the laboratory for analysis. The use of an anesthetic cream for venipuncture improved the acceptability of the procedure at local health posts and may have contributed to our high rate of success in obtaining serum. In addition, serology is more sensitive than stool testing because infection from asymptomatic episodes and missed episodes of diarrhea are detected. Furthermore, infection with some enteric pathogens such as *H. pylori* and HAV may not cause diarrhea and the pathogens may not be easily isolated from stool specimens. Thus, methods of identifying infection such as serology (or breath tests for *H. pylori*) are the most practical way of determining whether these infections occurred in study participants.

Although not as accurate as a seroconversion incidence study conducted over a defined time period, this study compared the different age-specific prevalence of infection. Norwalk-like viruses, ETEC, *C. parvum,* and HAV were common, and the prevalence of infection increased with age. The choice of the age of study subjects was critical because seroconversion in this population most commonly occurred between six and 18 months of age. These results demonstrate the importance of selecting very narrow age bands for analysis in populations with similar frequent exposure to fecal contamination. In addition, the study suggests that a single age group followed over time could be used to capture changes in prevalence of infection for all four of these pathogens that may result from a water and sanitation intervention. Choosing young children for conducting such evaluations is logical because infants begin with a low prevalence of infection and rapidly accumulate antibody in the absence of intervention. Thus, evaluation of newly introduced interventions can provide useful feedback over time intervals as short as 12 months.

Unlike the other pathogens, the prevalence of antibodies to HAV decreased among the 13–18-month-old children relative to the 6–12-month-old children. This decrease likely reflects loss of passively-transferred maternal antibody, which presumably persists longer for HAV than for the other pathogens studied. Passively transferred maternal antibody is detectable in infants born to mothers positive for antibody to HAV, but most children have lost it by 12–15 months of age. Because of this phenomenon, the prevalence of antibody to HAV among infants less than 12 months of age is not a good indicator of current hygienic conditions.

While evidence of early infection with *H. pylori* occurred frequently, it was less common than for the other infections. The other marker pathogens demonstrated a rapid increase in the first two years of life while antibody to *H. pylori* remained relatively constant among children in all age groups. In most studies from developing countries, *H. pylori* seroprevalence increases with age during childhood.[23] However, most of these seroprevalence studies do not present information on very young children in narrow age groups that can be compared with our findings.

In studies that did evaluate *H. pylori* seroprevalence in the same age groups, results were variable. An investigation in South Africa similarly found a relatively constant rate of infection between one and three years of age.[24] In contrast, investigators in Pakistan noted a consistent increase in sero-

prevalence in the first three years of life.[25] In Thailand, seroprevalence increased steadily between six months and six years of age.[26] In studies in Alaskan native, Bolivian, and Taiwanese children using the same validated serology assay, similar discrepancies in the very young were identified.[19,27–29] An increase in seroprevalence rates were seen in the Alaskan natives and the Bolivian cohorts, but a more constant but elevated infection rate was seen in the Taiwanese cohort. In these populations, whether differences in seroprevalence can be explained by variation in persistent maternal antibody crossing through breast milk, or early and high exposure rates to a significant antigenic load, remains to be determined.

Due to a shortage of reagent, we analyzed only a fraction (28%) of the serum samples for *C. parvum.* However, the samples that were analyzed suggested that *C. parvum* may be a good outcome measure in future intervention studies because acquisition of antibody increased steeply among the three youngest groups of children.

Due to the cross-sectional nature of the study, we could not capture seasonal differences in infection rates. Repeat testing would be required to assess such differences. In addition, while serologic evaluation provides a sensitive method to efficiently assess exposure to waterborne pathogens, it does not provide a direct measure of clinically significant disease. However, preventing seroconversion would be expected to prevent disease; thus, on a population basis, serology could provide an estimation of health impact following a water and sanitation intervention.

Although we looked for risk factors for infection, we found that in this setting of highly contaminated water, no single factor substantially affected the risk of infection. Given the cross-sectional nature of the study and the homogeneous practices of the families who participated (i.e., virtually all breastfed), there may not have been sufficient power to demonstrate an exposure-outcome association. Moreover, several exposure variables such as water source, hand washing, and latrine use were not well captured through our questionnaire. An intervention study in which environmental exposures could be more easily separated may be able to detect such associations.

We expect that a serologic approach using these newer assays is likely to be successful in other settings where children are commonly exposed to human enteric pathogens. Studies in other countries have demonstrated similar rates of seroprevalence in young children for individual pathogens. For example, in South Africa, more than 80% of the children had antibodies to Norwalk virus by 36 months of age.[9] In Ecuador, 90% of children had antibodies to ETEC LT by the age of two years.[10] In Peru, there was rapid acquisition of antibody to *C. parvum* among children < 36 months of age.[11] In Brazil, serologic evidence of infection with HAV increased markedly between six and 36 months of age.[12]

In conclusion, these results demonstrate the feasibility of using a serologic approach for evaluating water and sanitation interventions targeted at young children. We were able to obtain sufficient serum from a large population of infants and very young children for testing of antibody markers to multiple pathogens. Sharp enough differences in acquisition of antibodies to Norwalk-like viruses, ETEC, and *C. parvum* exist to suggest that these pathogens would be useful markers. Given the very young ages at which infection with these pathogens occurred in these communities, safe water interventions should be introduced to infants by 12 months of age to reduce the childhood burden of waterborne infections.

Researchers who elect to use serology for the evaluation of interventions in similar populations should enroll sufficient numbers of children in narrow age groups and analyze sequential serologies in the same children over time to estimate incidence. We envision that serology could be used to evaluate water and sanitation interventions in a variety of settings and a longitudinal evaluation of water and sanitation interventions using serology would be the next step in establishing the benefit of this approach. Serology has the potential to provide an alternative to more cumbersome stool collection methods that have more often been used for evaluation.

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