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Application of a salivary immunoassay in a prospective community study of waterborne infections

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Abstract

Quantifying sporadic waterborne infections in community settings can be challenging. Salivary antibody immunoassays are a promising non-invasive tool that can be used in prospective studies of common infections, especially those involving children.

This study was conducted in a Massachusetts city, which uses a microbiologically contaminated river as its water source, during summer-early winter periods before and after construction of a new drinking water treatment plant. Monthly saliva samples (7,480 samples from 1,170 children and 816 adults) were analyzed for immunoglobulin G (IgG) responses to recombinant proteins of *Cryptosporidium*, one genogroup I (GI) and two GII noroviruses. Immunoconversion was defined as at least four-fold increase in specific antibody responses between two monthly samples with a post-conversion response above a flexible age-dependent cut-off.

Episodes of gastroenteritis (diarrhea or vomiting or cramps) were associated with 3.2 (95% confidence limits 1.1; 9.5) adjusted odds ratio (aOR) of immunoconversion to *Cryptosporidium*; episodes of combined diarrhea and vomiting symptoms were associated with 3.5 (0.8; 15.0) and 4.6 (1.7; 12.6) aORs of an immunoconversion to GI and GII noroviruses respectively. Swimming in natural water bodies or chlorinated pools was associated with 2.3 (0.4; 15.4) and 4.9 (1.6; 15.5) aORs of immunoconversion to *Cryptosporidium,* respectively. In a subset of study participants who did not use home water filters, consumption of at least some amount of non-boiled tap water reported in a monthly recall survey was associated with 11.1 (1.2; 100.0) and 0.6 (0.1; 2.5) aORs

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of immunoconversion to *Cryptosporidium* before and after the new water treatment plant construction, respectively. Among individuals who used home water filters, associations between non-boiled tap water consumption and *Cryptosporidium* immunoconversion were not significant before and after new plant construction with aORs of 0.8 (0.2; 3.3) and 0.3 (0.1; 1.6), respectively. The interaction effect of study phase and non-boiled tap water consumption on *Cryptosporidium* immunoconversions was statistically significant in the entire study population with aOR of 5.4 (1.1; 25.6).

This was the first study that has used a salivary antibody immunoassay to demonstrate significant associations between gastrointestinal symptoms and *Cryptosporidium* and norovirus infections, and between water-related exposures and *Cryptosporidium* infections.

1. Introduction

Sporadic waterborne infections in tap water consumers and in recreational water users remain a considerable public health challenge in the US (Arnold et al. 2016; Ashbolt 2015). Most previously conducted prospective epidemiological studies of sporadic water-borne infections used self-reported gastroenteritis as an outcome (Colford et al. 2006). However, obtaining sufficient statistical power to demonstrate waterborne transmission in non-outbreak settings can be problematic for studies which rely on non-specific symptoms as the outcome measure because a variety of pathogens transmitted through different routes can cause similar symptoms while many waterborne infections can be asymptomatic (Exum et al. 2016). Saliva sampling poses minimal risks and it is well tolerated by adults and children (Gammie et al. 2002; McKie et al. 2002). The use of saliva samples for quantitation of specific antibody responses to pathogens is a low cost, non-invasive alternative to the invasive blood sampling approach (Exum et al. 2016).

Cryptosporidium is a gastrointestinal protozoan parasite that is extremely chorine resistant in its environmental form, the oocyst (Collinet-Adler and Ward 2010). This parasite causes approximately half of all illness outbreaks associated with recreational water in the US, and most outbreaks associated with chlorinated swimming pools (Hlavsa et al. 2015). It has also accounted for a majority of cases of illness in drinking water-related outbreaks in the US since 1971 (Craun et al. 2010). Two species cause most infections in humans: *C. parvum* can infect a wide range of animals, including humans, while *C. hominis* (previously known as *C. parvum* genotype 1) is a specialist parasite of humans (Leoni et al. 2006; McLauchlin et al. 2000).

The incidence of reported cryptosporidiosis in the US varies from approximately 1 to 4 cases per 100,000 persons per year (Painter et al. 2015; Painter et al. 2016). The incidence is highest in children under 10 years of age; the seasonal peak of infections typically occurs in August–October (Naumova et al. 2000). A substantial proportion of *Cryptosporidium* infections can be mildly symptomatic or completely asymptomatic while most cases of clinical cryptosporidiosis are not diagnosed or not reported to passive surveillance systems; therefore, the incidence of cryptosporidiosis is drastically underreported (Painter et al. 2016). Previous studies in Canada demonstrated that *Cryptosporidium* infections were very common in contrast with the low incidence of reported cases (Ong et al. 2005).

IgG responses to *Cryptosporidium* steeply increase within approximately two weeks after infection and then gradually decline to the pre-infection level within several months (Priest et al. 2001). Research in human volunteers demonstrated that most individuals develop antibody responses to certain immunodominant antigens following experimental infection, with IgG responses being a more accurate indicator of infection than IgA responses (Moss et al. 1998). Serum IgG responses to immunodominant *Cryptosporidium* antigens have been used as an indicator of incident infections in prospective study settings (Priest et al. 2005). Salivary IgG responses (but not salivary IgA responses) to the recombinant immunodominant gp15 antigen of *Cryptosporidium* have been linked with symptoms of gastroenteritis in a community study in Massachusetts (Egorov et al. 2010). Prospective serological studies utilized seroconversion to the same antigen as a biomarker of incident *Cryptosporidium* infections (Kattula et al. 2017; Sarkar et al. 2012). The antigenically identical Cp17 (17 kDa) antigen (Priest et al. 2000) has also been applied in a prospective population-based seroepidemiological study (Priest et al. 2006).

To reduce the risk of waterborne cryptosporidiosis in the US, the Environmental Protection Agency (EPA) promulgated the Long-Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) which requires public water supply systems using surface water sources contaminated with *Cryptosporidium* oocysts to use advanced water treatment methods, such as ultraviolet (UV) light irradiation, to inactivate the *Cryptosporidium* oocysts (EPA 2006).

Noroviruses are the most common cause of infectious gastroenteritis in the US, accounting for approximately 6% of acute gastroenteritis cases in the general population and 20% of gastroenteritis cases in children (Grytdal et al. 2016; Payne et al. 2013). The presence of diarrhea and vomiting symptoms is typical of norovirus infections (Rockx et al. 2002). Two genogroups cause most human infections: genogroup II (GII) noroviruses cause most outbreaks and account for the winter seasonal peak of norovirus gastroenteritis; genogroup I (GI) noroviruses are less common but their outbreaks are more likely to be associated with water exposure (Bitler et al. 2013; Matthews et al. 2012). There are more than twenty genotypes infecting humans with an even larger number of distinct norovirus variants (Parra et al. 2017). Antibody responses to specific noroviruses exhibit varying levels of cross-reactivity with other norovirus variants, with a greater degree of cross-reactivity within each genogroup (Malm et al. 2015; Parra et al. 2017; van Beek et al. 2016). Noroviruses are not very resistant to conventional chlorine treatment (Shin and Sobsey 2008). Therefore, outbreaks of noroviruses related to drinking water tend to be associated with untreated ground water supplies or failures of surface water disinfection (Hlavsa et al. 2015; Maunula et al. 2005; Moreira and Bondelind 2017). Outbreaks of norovirus infection associated with swimming in fresh water bodies have also been reported (Zlot et al. 2015). IgG responses to noroviruses steeply increase within approximately two weeks after infection and then gradually decline to the pre-infection level within several months (Tacket et al. 2003). Salivary antibody immunoconversion or a steep increase in specific salivary antibody responses between consecutive samples can be used as an analogue to seroconversion in prospective studies in order to detect incident norovirus infections (Griffin et al. 2015; Moe et al. 2004). It has been shown that salivary IgG immunoconversion is a better indicator of incident norovirus infection than salivary IgA immunoconversion (Griffin et al. 2015).

The main objective of this prospective observational study was to apply a multiplexed salivary antibody assay previously developed by EPA (Augustine et al. 2016; Griffin et al. 2011; Griffin et al. 2015) to assess potential beneficial impacts of improving treatment of municipal drinking water in a selected community on waterborne transmission of *Cryptosporidium* and norovirus infections. Secondary objectives were to assess potential associations between recreational water exposures and these infections, and between episodes of gastrointestinal symptoms and these infections.

2. Methods

2.1. Study settings

This prospective cohort study was conducted in the city of Lawrence, Massachusetts (population 75,000), which uses the microbiologically challenged Merrimack River with multiple combined sewer overflow (CSO) discharge sites upstream of its water intake as the sole source of drinking water. Recent studies have suggested a potential association between CSO events and emergency room visits in eastern Massachusetts communities that derive their drinking water from sewage-contaminated rivers (Jagai et al. 2015). Prior to April 2007, the city of Lawrence had an aged (built in the 1930s) and outdated water treatment plant utilizing conventional water treatment methods (primary chlorination, coagulation, filtration and secondary chlorination). The plant could no longer reliably meet the existing drinking water quality regulations and comply with the incoming LT2ESWTR requirements. Therefore, the city built a new water treatment plant designed to comply with LT2ESWTR and to reduce drinking water-borne transmission of *Cryptosporidium*. The treatment regimen at the new plant involved chlorine dioxide disinfection, polymer-aided coagulation, flocculation and clarification in superpulsator clarifiers, granular activated carbon filtration, UV light disinfection, and secondary chlorination. This new plant became operational in April 2007. The first stage of the study was conducted from the summer 2006 to January 2007 and the second phase was conducted during the same seasons in 2008–2009.

2.2. Study design and data collection

The study enrolled a sample of local families with children. The study procedures were approved by the Institutional Review Board for the University of North Carolina at Chapel Hill. Only adult individuals who signed informed consent forms or minors whose parents or guardians provided signed assent to their participation were enrolled in the study. Minors who were 15 to 17 years old also had to sign a consent form; in addition, their parent or legal guardian had to sign an assent form.

Only households who resided in Lawrence, MA were eligible to participate. At least two participants per household were required, including an adult capable of communicating in English or Spanish and at least one child between 1 and 12 years of age. Children below one year of age were excluded because they usually lack sufficient crevicular fluid (the exudate between the teeth and gums enriched with serum immunoglobulins) and because of the presence of maternal antibodies. The study also excluded immigrants who had resided in the US for less than three years and individuals who worked or studied outside the city of Lawrence for more than 20 hours per week.

The study employed local bilingual (English and Spanish) interviewers who received training prior to data collection. All study materials were available in English and Spanish. Recruitment was conducted by going door-to-door in selected neighborhoods including subsidized housing projects for low income households and at sites frequented by local residents, such as schools, day care centers, community centers, libraries, and playgrounds.

Families were asked to remain in the study for six months. Initial recruitment started in May – June and was completed in early October. Limited recruitment continued through November to replace families that dropped out of the study. The second phase of the study excluded households which participated in the first phase. This was done because children who participated in the first phase would be systematically older in the second phase and have lower risks of infections, and because it would have been difficult to re-enroll a sufficiently high proportion of the households which participated in the first phase.

Baseline and follow-up questionnaires and saliva samples were collected via home visits by study interviewers. Data on sociodemographic variables and the use of home water filters were collected at baseline. Detailed 24-hour recall data on consumption of water and other liquids in standard “glass” units (eight ounces or 237 mL), and recall data on swimming and travel episodes, as well as illness symptoms during the previous month were collected at monthly follow-up surveys.

2.3. Saliva sampling

Oral fluid (hereafter called saliva) samples were collected at baseline and monthly follow-up surveys using Oracol™ samplers (Malvern Medical Developments, Worcester, UK). Sampling involved rubbing the gums with the sampling sponge for one minute or until it became fully saturated. The sampling method aimed to collect saliva samples enriched with crevicular fluid, which has a higher IgG content than parotid saliva (Gammie et al. 2002; McKie et al. 2002). Samples were refrigerated immediately after collection, frozen at −20° C upon delivery to the field office and shipped to the EPA laboratory in Cincinnati, OH in insulated containers with ice packs using an overnight delivery service. At the laboratory samples were separated from sampling sponges by centrifugation and stored at −80° C until analysis. Only samples that had at least 30 μL volume after processing were used in laboratory analyses.

2.4. Laboratory analysis of samples

Saliva samples were analyzed for IgG responses to *Cryptosporidium*, a GI norovirus and two GII noroviruses using an in-house multiplex fluorescent microsphere immunoassay based on xMAP® technology from Luminex Corp. (Austin, TX). In addition, salivary IgG reactivity with glutathione-S-transferase (GST) control protein and total salivary IgG content were analyzed as described previously (Griffin et al. 2011; Griffin et al. 2015).

The assay employed the recombinant gp15 (15 kDa) protein of *C. hominis* TU502 isolate, which was cloned and purified as described previously (Cevallos et al. 2000; Preidis et al. 2007). The protein contained thioredoxin, His, and S purification tags. The gp15 *Cryptosporidium* protein is an immunogenic zoite surface antigen. A previous study showed a significant correlation between antibody levels to gp15 antigens from both

Cryptosporidium species infecting humans, indicating cross-reactivity to conserved epitopes (Allison et al. 2011). Therefore, antibody responses to the gp15 antigen of *C. hominis* capture both *C. hominis* and *C. parvum* infections.

Recombinant P domains of the major capsid protein of three noroviruses, Norwalk virus (genogroup I genotype 1, GI.1), VA387 variant of GII.4 norovirus, and VA207 variant of GII.9 norovirus were kindly provided by Xi Jiang (Cincinnati Children's Hospital Medical Center, Cincinnati, OH). These antigens were produced using an *E. coli* expression system with a GST purification tag as described previously (Tan and Jiang 2005; Tan et al. 2008). Although the purification procedure involved thrombin cleavage and removal of GST, the purified norovirus proteins contained a residual amount of GST as demonstrated by reactivity with anti-GST antibodies (Griffin et al. 2011).

Analyses were conducted using two assays: a multiplex Luminex assay for IgG responses to microbial antigens and GST (the specific IgG assay), and a separate Luminex assay for analysis of total IgG content. The specific IgG assay involved distinct sets of Luminex beads coupled to different microbial antigens or GST. It utilized biotinylated donkey anti-human IgG Fc-specific detection antibody (Jackson ImmunoResearch Inc., West Grove, PA) at 30 μg/mL and streptavidin R-phycoerythrin conjugate (SAPE; Invitrogen, Carlsbad, CA) at 10 μg/mL. The total IgG assay employed goat anti-human IgG capture antibody (KPL, Gaithersburg, MD) and a similar biotinylated detection antibody at 4 μg/mL followed by incubation with SAPE at 8 μg/mL as described previously (Griffin et al. 2011).

All antigens and the anti-human IgG capture antibody were covalently coupled to distinct sets of Luminex polystyrene microspheres using the standard Luminex carbodiimide coupling protocol. The 50 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer, pH 5.0 was used as coupling buffer for all proteins. Norovirus antigens were added to the coupling solution at 10 μg per 500 μL standard coupling reaction volume each, *C. hominis* gp15 protein and GST were added at 5 μg per 500 μL each, and anti-human IgG capture antibody at 25 μg per 500 μL. Coupling of antigens to Luminex microspheres was confirmed by testing with a serially diluted guinea pig anti-norovirus, mouse anti-gp15, or rabbit anti-GST antibody; for the total IgG assay, coupling confirmation involved assaying purified human IgG at serial dilutions as described previously (Griffin et al. 2011).

In the specific antibody assay, saliva samples were diluted 1:2 in the standard PBS-1% BSA Luminex assay buffer prior to analysis for the final 1:4 dilution in the microplate well. For analysis of total IgG, samples were diluted 1:10,000 in the same buffer prior to analysis (1:20,000 dilution in the well). For each assay, all samples from a specific individual were assayed on the same plate in order to minimize sample-to-sample variability in results. Analyses were conducted using the standard Luminex protocol for polystyrene beads and a Luminex-100 instrument. Median Fluorescence Intensity (MFI) of the reporter signal not corrected for blanks was used in data analysis.

2.5. Statistical data analysis

Data were analyzed using SAS statistical analysis software version 9.4 (SAS Institute, Cary, NC). Geometric mean values of duplicate samples were used in statistical analysis.

Sample-to-sample variability in saliva composition was accounted for by adjusting responses for controls, such as total salivary IgG content or response to the GST recombinant protein purification tag. Previous studies have demonstrated the use of multiplex Luminex salivary immunoassays with internal control antigens for the detection of norovirus, *Cryptosporidium* and hepatitis E virus infections (Griffin et al. 2011; Griffin et al. 2015; Pisanic et al. 2017). In this study, results for norovirus antigens were expressed as a ratio of MFI values for anti-norovirus and anti-GST IgG responses as described previously (Griffin et al. 2015). Results for *Cryptosporidium* were expressed as a ratio of an MFI value for anti-gp15 IgG response and an MFI value from the total IgG assay. Saliva samples with a total IgG concentration below the 1st percentile or an anti-GST response above the 99th percentile were excluded from data analysis to reduce the effects of abnormally high or low denominator values.

In this prospective study, individual salivary antibody responses to *Cryptosporidium* and noroviruses were not classified as positive or negative. Instead, an immunoconversion or a steep increase in salivary antibody responses between two consecutive monthly samples was used as an indicator of incident infection. Only data for individuals who had at least three valid saliva samples (at least two intervals when an immunoconversion could occur) were used in statistical analysis of immunoconversions.

Statistical data analysis was conducted in two stages. The objective of the first stage was to select most accurate definitions of immunoconversions. At the second stage, immunoconversion definitions selected at the first stage were applied in the analysis of environmental predictors of infections.

The first stage involved regression analysis of immunoconversion data against gastrointestinal symptoms. The objective of this analysis was to maximize the accuracy of immunoconversion tests taking in account sample size constraints. Noroviruses and *Cryptosporidium* are known to cause gastrointestinal symptoms in a substantial fraction of infected individuals. Therefore, gastrointestinal symptoms are statistically associated with infections in the general population. Using an immunoconversion test with less than perfect sensitivity and specificity will always result in some outcomes being misclassified. For a rare outcome, such as immunoconversion to *Cryptosporidium* or norovirus during a specific month, imperfect specificity has a much greater impact on the accuracy of the test compared to similarly imperfect sensitivity. As antibodies to unrelated pathogens are not known to cross-react with the recombinant antigens of noroviruses and *Cryptosporidium* employed in this study (Griffin et al. 2011), misclassification due to imperfect specificity was likely to be non-differential with respect to gastrointestinal symptoms (the rates of false positive results were the same in symptomatic and asymptomatic individuals not infected with noroviruses or *Cryptosporidium*). Non-differential misclassification generally produces a bias towards the null effect (Rothman 2012). In this situation, the most accurate immunoconversion test with a high specificity is likely to produce the strongest association with symptoms.

The three alternative definitions of immunoconversion that were tested all involved at least four-fold increase in the response between consecutive samples as described previously (Griffin et al. 2015; Moe et al. 2004; Monroe et al. 1993). An additional criterion included in all three immunoconversion definitions was at least three-fold increase in the antibody

response in the first post-conversion sample compared to the baseline sample, or in the very last sample in the series compared to the last pre-conversion sample as proposed in a previous salivary antibody study of recreational water contacts and norovirus infections (Wade et al. 2018). The purpose of this additional criterion was to improve the specificity of immunoconversion by excluding false positive results due to sample-to-sample variability in saliva composition. It is based on assumptions that antibody responses prior to infection should be relatively stable, while a decline in IgG antibody responses following a post-infection spike should be relatively slow.

The first immunoconversion definition was based on the above two criteria only. The second and third definitions of immunoconversions incorporated, in addition to these criteria, a minimum age-specific antibody response in the first post-conversion sample. The purpose of the third criterion was to exclude false immunoconversions due to sample-to-sample variability in low-level salivary antibody responses that could produce occasional four-fold increases between consecutive saliva samples. Specifying flexible age-dependent cut-off values was necessary because average intensities of IgG responses to *Cryptosporidium* and noroviruses increase with age in children (Blazevic et al. 2016; Egorov et al. 2010). Age-specific cut-offs were set at the upper prediction bound of a penalized B-spline regression of log-transformed IgG responses on age using the SAS procedure *transreg* as described previously (Egorov et al. 2010). The second and third immunoconversion definitions involved cut-off values based on the upper one-sided 80% and 90% prediction bounds, respectively.

Different definitions of gastrointestinal illness were used for analysis of *Cryptosporidium* and norovirus data. For analysis of *Cryptosporidium* immunoconversions, an episode of gastroenteritis was defined as symptoms of diarrhea or vomiting or abdominal cramps for at least one day. A new episode was defined as having symptoms after at least one week of being symptom-free. Since the concurrence of diarrhea and vomiting are typical of norovirus infections (Rockx et al. 2002), an episode of illness for analysis of noroviruses was defined as the presence of combined symptoms of diarrhea and vomiting for at least one day.

Antibody responses to specific noroviruses exhibit greater cross-reactivity with heterologous noroviruses within the same genogroup (van Beek et al. 2016). In this analysis, immunoconversions to GI.1 Norwalk norovirus antigen were assumed to represent infections with GI noroviruses, while immunoconversions to GII.4 VA387 or GII.9 VA207 were combined into one binary variable representing infections with GII noroviruses.

Regression analysis of immunoconversions was conducted using a generalized estimating equations (GEE) method with compound symmetry covariance structure to account for dependence of observations within households. The SAS procedure *genmod* was applied to fit regression models. To select best immunoconversion definitions for *Cryptosporidium* and noroviruses, associations between episodes of symptoms and same month immunoconversions were analyzed using univariate regression models. An immunoconversion definition with the strongest association with illness symptoms (“strongest” is defined here as a statistically significant association with the greatest effect size) was then used in further analysis of environmental predictors.

Analysis of environmental predictors was conducted using multivariate GEE regression models (or regular logistic regression models when fitting GEE model was not possible due to model convergence problems). Covariates were selected using a manual stepwise procedure based on their effects on parameter estimates for environmental predictors of interest and their impact on the model fit using the Akaike Information Criterion Corrected (AICc). All final models included a cubic polynomial of time in months from the middle of each data collection period (October 1, 2006 for phase 1 and October 1, 2008 for phase 2) in order to control for potential confounding effect of infection seasonality due to minor differences in cohort participation patterns between study phases. As infection rates vary with age with different patterns in children and adults, the effects of age were modeled using a piece-wise (broken stick) linear regression approach (Naumova et al. 2001) with an inflection point corresponding to the age of 18 years. Missing values of household income and education were imputed using probabilities derived from predictive logistic regression models. SAS procedure *mi* was applied to generate imputed values of these covariates. Missing data on home water filters were imputed using data on filtered and non-filtered tap water consumption.

The associations between drinking non-boiled tap water and immunoconversions were assessed using two alternative approaches. The first approach involved fitting a regression model with binary variables for study phase and tap water consumption, and an interaction effect of phase and tap water consumption. This analysis was conducted using the entire dataset and in two subsets of data stratified by the presence of water filters at home. The second approach involved assessing the effect of tap water consumption separately in each study phase. This analysis was also repeated in subsets of data stratified by the presence of home water filters.

3. Results

3.1. Descriptive statistics

The study involved a total of 1,986 individuals living in 557 households (Table 1). These included 1,170 (58.8%) children below the age of 18 years and 816 adults. The mean age of children in each phase was the same at 7.4 years while the mean age of adults increased slightly from 34.5 years in the first phase to 36.2 years in the second phase. The total number of saliva samples used in this analysis was 7,480. The mean duration of follow-up was 98.4 days. The total amount of follow-up in all participants was 535.5 person-years, including 208.5 person-years in phase 1 and 327.0 person-years in phase 2. Due to technical reasons, the number of valid results for the GII.9 VA207 norovirus antigen was slightly smaller than the above sample size for all other antigens. Only 6,977 samples from 1,855 individuals were analyzed for IgG responses to VA207. This reduced sample size corresponded to 502.9 person-years of total follow-up time.

Among all study participants, females outnumbered males (60.9% vs. 39.1%). The sex ratio among children was close to parity (51.7% females), while 74.0% of adult participants were females. The skewed sex ratio in adults was likely due to a greater willingness of adult women to participate in a study involving their children.

In general, the study participants were predominantly of Hispanic ethnicity (93.9%), which reflects the composition of the source community with a large proportion of Hispanic immigrants. English was the primary language spoken in the households of 13.6% of participants. Language data were dichotomized for regression analysis as speaking English at home vs. speaking any other language.

Most study participants had relatively low educational attainment: 10.3% of adult study participants reported having a bachelor's degree or higher education compared to 38.2% in the general adult (25 years of age or older) population of Massachusetts (Ryan and Siebens 2012). For regression analysis of environmental risk factors, education data on adult study participants were converted into a household-level binary variable with values set to one if at least one adult household member had education beyond high school and to zero otherwise.

Approximately half of study participants in both phases lived in households with total annual income below $ 25,000. This shows that the median income in the study population was considerably lower than the $ 65,400 median household income in Massachusetts in 2008 (Semega 2009). Household income data were dichotomized for regression analysis at the $ 25,000 annual income cut-off.

The average non-boiled tap water consumption increased from 399 mL/day in phase 1 to 545 mL/day in phase 2 in all participants. The overall average non-boiled tap water consumption was 400 mL/day in children between 1 and 17 years of age and 592 mL/day in adults. The total daily liquid consumption in drinks (not counting water in foods) in participants of all ages remained almost flat at 2,106 mL/day and 2,160 mL/day in phases 1 and 2, respectively. For regression analysis, non-boiled tap water consumption was dichotomized as any consumption vs. no consumption reported in a specific monthly survey. Consumption of at least some non-boiled tap water was reported in 44.9% of individual monthly survey responses in phase 1 and 50.0% of monthly surveys in phase 2 of the study. Rates of consumption of non-boiled tap water were similar in adults and children at 49.2% and 49.1% during both phases, respectively.

Counts of swimming events during the previous month by type of water body were reported in monthly recall questionnaires. For regression analysis, swimming data were dichotomized as at least one swimming event vs. no swimming during a specific month, separately for swimming pools and natural water bodies (rivers, lakes or ocean). Swimming in pools was reported in 3.6% of individual survey responses. As most monthly surveys were conducted in the fall, swimming in all types of natural water bodies was less common with only 2.5% reporting rate.

The incidence rate of gastroenteritis (diarrhea or vomiting or intestinal cramps) increased slightly from 0.37 episodes per person-year in phase 1 to 0.42 episodes per person-year in phase 2, while the incidence rate of combined symptoms of diarrhea and vomiting more than doubled from 0.06 episodes per person-year in phase 1 to 0.13 in phase 2.

3.2. Immunoconversion definitions

Descriptive statistics for the three alternative immunoconversion definitions and associations of gastroenteritis symptoms with immunoconversions from univariate regression models are presented in Table 2. For *Cryptosporidium*, application of the third definition of immunoconversion incorporating an age-specific cutoff at the 90% prediction limit from spline regression of antibody responses on age produced the strongest statistically significant association with gastroenteritis symptoms with odds ratio (OR) of 3.46 (1.23; 9.75). Therefore, the 3rd definition of immunoconversion was selected for analysis of environmental predictors for *Cryptosporidium* infections. Further multivariate regression analysis demonstrated that this association remained essentially unchanged with adjusted odds ratio (aOR) of 3.25 (1.11; 9.48) in GEE models adjusting for sex, age (piece-wise regression with inflection at the age of 18 years), cubic polynomial of time, highest education level of household adults, and household income. For this immunoconversion definition, 12.5 % (4 of 32) *Cryptosporidium* immunoconversions were associated with gastroenteritis. Conversely, 1.9 % (4 of 214) of all gastroenteritis episodes were associated with *Cryptosporidium* immunoconversions.

For GII noroviruses and for all noroviruses, the second immunoconversion definition, which included an age-specific cutoff at the 80% prediction bound from a spline regression, corresponded to the strongest statistically significant association with combined symptoms of diarrhea and vomiting (Table 2). Therefore, the 2nd immunoconversion definition was selected for analysis of environmental predictors of norovirus infections. Further regression analysis in multivariate models adjusting for sex, age (piece-wise regression), cubic polynomial of time, highest education level of household adults, and language spoken at home produced similar associations with symptoms of diarrhea and vomiting: aOR of 3.54 (0.84; 15.0) for GI noroviruses and aOR of 4.61 (1.68; 12.6) for GII noroviruses. For this immunoconversion definition, 6.2 % (13 of 210) of norovirus immunoconversions were linked with episodes of combined diarrhea and vomiting. Conversely, 23.6 % (13 of 55) of combined diarrhea and vomiting episodes were associated with immunoconversion to any of the three noroviruses during the same monthly interval. Additional analysis using this immunoconversion definition demonstrated that 7.6 % (16 of 210) of norovirus immunoconversions were linked with gastroenteritis episodes (diarrhea or vomiting or abdominal cramps) while 7.5 % (16 of 214) of all gastroenteritis episodes were linked with norovirus immunoconversions. Among gastroenteritis episodes associated with norovirus immunoconversion, 81.3 % (13 of 16) involved combined symptoms of diarrhea and vomiting. In contrast, only 25.7 % (55 of 214) of all gastroenteritis episodes in the study population involved combined symptoms of diarrhea and vomiting.

Scatter plots of antibody responses vs. age with upper 80% and 90% confidence bounds for penalized B-spline regression of antibody response on age are shown on Figure S1. The plots demonstrate age-related increases in antibody response in children. Post-conversion samples that met specific immunoconversion definitions are marked on these plots.

3.3. Descriptive analysis of immunoconversion data

Using immunoconversion definitions selected at the previous stage, incidence rates of conversions to *Cryptosporidium,* GI and GII noroviruses were 6.0, 23.3 and 20.9 per 100 person-years, respectively (Table 3). The incidence rate of immunoconversions to all noroviruses was 39.2 per 100 person-years. Incidence rates of *Cryptosporidium* immunoconversions declined steadily with age from 8.5 per 100 person-years in children between 1 and 10 years of age to 1.5 per 100 person-years in adults ages 41 to 85 years. While the unadjusted incidence rate of *Cryptosporidium* immunoconversions declined in the second phase, the effect was not significant. For GI and GII noroviruses, the lowest incidence rates of immunoconversions were observed in 21 to 40 year-old adults. The incidence rate of GII norovirus immunoconversions was significantly lower in individuals who spoke a foreign language at home (it was mainly Spanish language) than in those who spoke English potentially reflecting a greater immunity in immigrants from developing countries (Table 3). Incidence rates of norovirus immunoconversions tended to be higher in individuals living in larger households suggesting an effect of person-to-person transmission within a household. Finally, unadjusted incidence of immunoconversions to GII noroviruses (but not GI noroviruses) significantly increased in phase 2 of the study.

3.4. Multivariate regression analysis of risk factors for immunoconversion

Adjusted odds of immunoconversions to *Cryptosporidium* and GI noroviruses did not differ significantly by study phase. Immunoconversions to GII noroviruses were significantly less frequent in phase 1 compared to phase 2 with adjusted odds ratio (aOR) of 0.50 (0.32; 0.80) (Table 4).

Swimming in public pools was associated with 4.92 (1.56; 15.5) aOR of *Cryptosporidium* immunoconversions while swimming in natural water bodies was associated with 2.33 (0.35; 15.4) aOR of immunoconversion to this parasite (Table 4).

The interaction effect of non-boiled tap water consumption and phase 1 on *Cryptosporidium* immunoconversions was statistically significant with aOR of 5.40 (1.14; 25.6), $p = 0.03$. This suggests that the estimated adjusted odds ratio of *Cryptosporidium* immunoconversion due to consumption of non-boiled tap water was over five times greater before the introduction of new water treatment than after it. Parameter estimates for all variables included in this regression model are presented in Table S1. Further analysis of interaction effects of tap water consumption and study phase in the dataset stratified by the presence of home water filters demonstrated that the significant interaction effect was only observed among individuals who did not use water filters at home with aOR of 15.8 (1.10; 228), $p = 0.04$. The corresponding effect estimate in individuals with home water filters was not significant, aOR = 2.95 (0.34; 25.4), $p = 0.4$.

Analysis of data stratified by study phase and the use of home filters also demonstrated that among individuals who did not use home water filters, non-boiled tap water consumption was a risk factor for *Cryptosporidium* immunoconversion in phase 1 and not in phase 2 (after the introduction of improved water treatment) with aORs of 11.1 (1.23; 100), $p = 0.03$, and 0.58 (0.13; 2.52), $p = 0.5$, respectively (Table 4). In the analysis stratified by study phase

and use of home filters, GEE models for some strata did not converge due to the insufficient sample sizes. For consistency, results for all four strata presented in Table 4 are based on regular logistic regression analysis. When both regular logistic and GEE analyses could be completed, both models produced rather similar results with less than 15% difference in parameter estimates for environmental exposure variables (not shown).

4. Discussion

4.1. Summary of main findings

This was the first community-based prospective panel study to apply a multiplex salivary antibody assay for the detection of incident infections with *Cryptosporidium* and noroviruses. It demonstrated associations between gastrointestinal symptoms and immunoconversions to both *Cryptosporidium* and noroviruses, and between swimming in swimming pools and immunoconversions to *Cryptosporidium*. This study also provided some evidence of a reduction of drinking waterborne transmission of *Cryptosporidium* after the introduction of improved drinking water treatment in a community using a microbiologically contaminated river as its drinking water source.

Cryptosporidium and noroviruses cause acute self-resolving infections with IgG antibody responses remaining elevated for several months after the resolution of infection. In this study, immunoconversions were used to detect both symptomatic and asymptomatic infections which occurred between monthly saliva sampling episodes. The ability to detect recent infections in individuals who might no longer be infected when a saliva sample is collected is an advantage offered by the immunoconversion-based approach. The non-invasive saliva sampling is another advantage of a novel approach employed in this study. An application of saliva sampling and a multiplexed immunoassay for simultaneous analysis of IgG responses to several microbial antigens previously developed by US EPA (Griffin et al. 2011; Griffin et al. 2015) enabled a relatively low cost surveillance of incident infections in this prospective community study involving young children. The results show that the salivary antibody assay is a valuable tool for assessing water-related risk factors of sporadic symptomatic and asymptomatic *Cryptosporidium* and norovirus infections.

4.2. Incidence of and risk factors for Cryptosporidium immunoconversions

In this study, 1.9% of episodes of gastroenteritis symptoms (diarrhea or vomiting or intestinal cramps) were associated with *Cryptosporidium* immunoconversions. Population-based studies in the UK demonstrated that *Cryptosporidium* accounts only for 0.4% of acute gastroenteritis cases in the community and 1.4% of acute gastroenteritis cases in the general practice (Tam et al. 2012). Children were overrepresented in the present study driving up the observed incidence rate. The study also included the late summer – early fall peak season for cryptosporidiosis in Massachusetts (Naumova et al. 2000) further boosting the observed contribution of *Cryptosporidium* to gastroenteritis. Also, in this study only 12.5% of *Cryptosporidium* immunoconversions were associated with gastroenteritis symptoms suggesting that most infections were asymptomatic. Previous studies demonstrated that up to several percent of asymptomatic individuals harbor *Cryptosporidium*; cross-sectional serological studies have also shown high seroprevalence rates in developed countries

suggesting that infections are rather common (Collinet-Adler and Ward 2010) in contrast with a relatively low incidence rate of diagnosed symptomatic cryptosporidiosis (Painter et al. 2016).

Previous studies have demonstrated associations between recreational water contacts at public beaches and non-specific gastroenteritis symptoms in the US (Arnold et al. 2016; Wade et al. 2006; Wade et al. 2008) and between swimming in public swimming pools and sporadic cryptosporidiosis in Australia (Robertson et al. 2002). *Cryptosporidium* is the main cause of swimming pool-related outbreaks of gastroenteritis in the US (Hlavsa et al. 2015). This study showed, for the first time, that swimming in swimming pools was a risk factor for sporadic *Cryptosporidium* infections in a US community. Assessing risks of *Cryptosporidium* infections in swimmers in non-outbreak settings was enabled by the application of a non-invasive salivary immunoassay method.

Existing data on waterborne outbreaks in the US suggest that improving treatment of drinking water could be linked with reduced risk of drinking waterborne outbreaks of cryptosporidiosis: while drinking water-borne *Cryptosporidium* outbreaks used to occur regularly from 1971 to 2006 (Craun et al. 2010), no such outbreaks had been reported in 2011– 2012 (Beer et al. 2015). Research in England also demonstrated a decline in the incidence of sporadic cryptosporidiosis after the introduction of improved treatment of drinking water derived from surface sources (Goh et al. 2005).

The results of the present study provided evidence of a beneficial effect of improved water treatment on *Cryptosporidium* transmission in a Massachusetts community. There was a significant interaction effect of study phase and non-boiled tap water consumption suggesting that the risk of *Cryptosporidium* infection due to tap water consumption declined after the introduction of improved water treatment. This effect was pronounced only in the subset of individuals who did not use home water filters. Similarly, in data analysis stratified by study phase, consumption of non-boiled tap water was associated with *Cryptosporidium* infections only before the introduction of improved water treatment and only in a subset of study participants who did not use home water filters. Tap water consumption was no longer associated with *Cryptosporidium* infections after the introduction of improved water treatment providing further evidence in support of health benefits of improved treatment of drinking water.

The accuracy of immunoconversion as a marker of incident infections was likely to be independent from environmental exposures such as tap water consumption and swimming. This means that outcome misclassification was non-differential (or random) with respect to environmental exposures. In the vast majority of cases, non-differential outcome misclassification biases observed associations with exposure towards the null effect diminishing the observed effect of exposure on the outcome (Rothman 2012). Improving accuracy of immunoconversions tests reduces the bias towards the null effect resulting in a more accurate estimate of an association between environmental exposures (such as swimming or consumption of tap water) and immunoconversion.

In non-outbreak settings, most individuals do not experience a *Cryptosporidium* or norovirus infection during a specific month. Therefore, an immunoconversion test deficient in specificity can produce many more false-positive than true-positive results. In other words, deficiency in specificity causes greater outcome misclassification and greater negative impact on the accuracy than a similar deficiency in sensitivity. In this situation, an immunoconversion test deficient in specificity would bias odds ratio estimates for exposure towards the null effect to a greater extent than a test similarly deficient in sensitivity. To improve the specificity, this study expanded the previously used definition of immunoconversion as a four-fold increase in antibody response between two saliva samples to incorporate an additional flexible age-based cut-off for the first post-conversion response. Regression analysis demonstrated that among the three alternative *Cryptosporidium* immunoconversion definitions the strictest one using the higher age-dependent threshold produced the strongest associations with gastrointestinal symptoms, and with water related exposures. Still, it is likely that this study underestimated the effects of water related exposures on *Cryptosporidium* infections due to remaining outcome misclassification.

4.3. Incidence of and risk factors for norovirus immunoconversions

This study used three recombinant norovirus antigens representing the GI.1 Norwalk virus and two GII norovirus variants, GII.4 VA387 and GII.9 VA207. Norovirus infections are known to produce very strong surges in IgG antibody responses to recombinant antigen of homologous noroviruses (Atmar et al. 2014). The salivary IgG immunoconversion to the Norwalk virus was previously demonstrated to have 100% sensitivity and 100% specificity in a small set of participants of a volunteer challenge study (Griffin et al. 2015). While sensitivity and specificity of similar immunoconversion tests for VA387 and VA207 noroviruses have not been evaluated, it has been shown that P particles of various norovirus variants have similar antigenic properties supporting the use of these recombinant antigens for measuring antibodies responses to homologous noroviruses (Tan et al. 2011).

However, infections with heterologous noroviruses could result in weaker observed antibody responses to the norovirus antigens used in this study due to various levels of cross-reactivity. Previous research demonstrated greater cross-reactivity in antibody responses to heterologous noroviruses within the same genogroup than noroviruses belonging to different genogroups (van Beek et al. 2016) and very little cross-reactivity of anti-Norwalk virus antibody with VA387 and VA207 norovirus antigens (Griffin et al. 2015).

Although the GI.1 Norwalk virus is the prototypical norovirus, infections with this variant are relatively uncommon (Vega et al. 2014). Similarly, GII.9 VA207 and GII.4 VA387 variants were not dominant epidemic strains when this study was conducted. It is likely that most immunoconversions detected in this study reflected infections with heterologous noroviruses, which were not represented in the multiplex immunoassay employed in this study. Quantifying an overall sensitivity and specificity of a multiplex assay involving antigens from a subset of norovirus variants in relation to all infections caused by all norovirus variants circulating in the population would require access to prospectively collected saliva samples from a large number of individuals known to be infected (or not infected) with each norovirus variant, before and after infection. Given the scarcity of

studies involving collection of pre- and post-infection saliva samples, a comprehensive evaluation of an overall assay sensitivity and specificity would be an exceptionally challenging task. Further efforts should focus on the development of a multiplexed immunoassay for noroviruses incorporating a parsimonious set of recombinant norovirus antigens that can capture infections with all common norovirus variants. Ongoing norovirus vaccine development efforts and recent advances in this area (Lindesmith et al. 2015) should guide further development of norovirus immunoassays.

As expected, norovirus immunoconversions were more common than *Cryptosporidium* immunoconversions in this study (incidence rates 39.2 and 6.0 immunoconversions per 100 person-years respectively). The proportions of gastrointestinal illness episodes linked to norovirus immunoconversions were 7.6 % for all gastroenteritis symptoms (diarrhea or vomiting or cramps) and 23.6 % for combined diarrhea and vomiting, which are typical norovirus symptoms. Previous studies in the US demonstrated that noroviruses account for 12% to 17.5% of acute gastroenteritis cases in the general population (Patel et al. 2008; Rha et al. 2013). This multiplexed assay involving three norovirus antigens might not capture all infections with heterologous noroviruses. It is also likely that, despite all efforts to improve the specificity of immunoconversion analysis, some immunoconversions detected in this study were false-positive results due to sample-to-sample variability in saliva composition or other unknown factors. This study oversampled young children which boosted the observed incidence rates of norovirus infections in the study population. On the other hand, the data collection periods for this study did not include the late winter seasonal peak of norovirus infections. Therefore, the results of this study pertaining to incidence of norovirus infections and to contribution of noroviruses to gastrointestinal morbidity should be interpreted with caution.

Human susceptibility to norovirus infections at least in part depends on the fucosyltransferase 2 (*FUT2*) genotype which determines the secretion of ABO histo-blood group antigens (Currier et al. 2015; Lindesmith et al. 2003). Approximately 20% to 30% of US residents do not have a functional copy of the *FUT2* gene. The individuals who do not secrete the oligosaccharide ligand required for some noroviruses for binding to cells are called “non-secretors”. It appears that non-secretors are resistant to the epidemic GII.4 variants and to the Norwalk (GI.1) virus but more susceptible to some other GI and GII norovirus variants (Lindesmith et al. 2003; Lopman et al. 2015). This study aimed to assess risk factors for norovirus infections in the general population, which is comprised of secretors and non-secretors. The study protocol did not include testing participants for their secretor status. It can be speculated that if the same triplex norovirus immunoassay were used in the sub-population of secretors, observed associations between gastrointestinal symptoms and immunoconversion would be slightly stronger.

In contrast with *Cryptosporidium* infections, infections with noroviruses were not associated with recreational water contacts or tap water consumption in this study. It has been shown that noroviruses are more susceptible to inactivation by chlorine than *Cryptosporidium* oocysts (Shin and Sobsey 2008). Therefore, even an outdated water treatment plant that was operated during phase 1 of this study could be efficient in inactivating waterborne noroviruses. Noroviruses are also inactivated by chlorine in public swimming pools.

Similarly, noroviruses in sewage discharged into the Merrimack River upstream of the drinking water intake for Lawrence could have been inactivated by chlorine treatment most of the time except during CSO events when untreated sewage was discharged directly into the river by upstream communities. While it could be expected that CSO events would be linked with norovirus infections in individuals who swam in the Merrimack River, most of data collection for this study occurred in the fall after the end of the swimming season in Massachusetts. A specially designed study would be needed to assess recreational water-borne transmission of noroviruses during CSO events.

The incidence rate of immunoconversions to GI noroviruses did not change in phase 2 compared to phase 1 while immunoconversions to GII noroviruses increased significantly in phase 2. There was also an increase in diarrhea and vomiting symptoms, which are typical of norovirus infections. GI noroviruses are more likely to contaminate water sources while GII norovirus infections are mainly transmitted via person-to-person contacts or contaminated food; waterborne transmission makes only a minor contribution to epidemiology of GII noroviruses (Bitler et al. 2013; Matthews et al. 2012). Incidence rates of infections with specific norovirus variants are known to vary from year-to-year. The number of reported norovirus outbreaks in the United States declined from 505 (with 14,753 cases) in 2006 to 356 (with 9,175 cases) in 2008 (Centers for Disease and Prevention 2009, 2011), while syndromic surveillance data for Boston, MA suggest similar rates of norovirus infections in these two years (Desai et al. 2012). The GII.4 noroviruses, which are currently most common, have been evolving especially rapidly with new variants constantly emerging to overcome the herd immunity (Buesa et al. 2008; Parra et al. 2017; Siebenga et al. 2009). Antibody responses to various heterologous GII noroviruses could have various degrees of cross-reactivity with the GII norovirus antigens employed in this study further affecting the observed temporal patterns. In addition, the pattern of norovirus variants detected in clinical cases of gastroenteritis can differ substantially from the pattern of noroviruses circulating in the community (Kazama et al. 2016). It is likely that the observed increase in GII norovirus immunoconversions as well as episodes of diarrhea and vomiting symptoms in phase 2 of the study reflected a year-to-year variability in infections with certain GII norovirus variants in the Lawrence, MA population due to factors other than drinking water quality.

5. Conclusions

This prospective community study demonstrated an application of non-invasive multiplexed salivary antibody immunoassay for the detection of incident infections with *Cryptosporidium* and noroviruses. Significant associations were detected between gastrointestinal symptoms and immunoconversions to these pathogens. It was the first study to demonstrate that swimming in swimming pools was a risk factor for sporadic *Cryptosporidium* infections in the US, and to produce some evidence that introducing an improved treatment of drinking water derived from a contaminated river reduced waterborne transmission of *Cryptosporidium*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1.

Descriptive statistics of the study population

Factor or parameter	Both phases	Phase 1	Phase 2
Study participants	1,986	876	1,110
Households	557	264	293
Total N of valid saliva samples	7,480	3,428	4,052
Duration of follow-up in days, mean (SD)	98.4 (29.3)	86.9 (24.4)	107.5 (29.6)
Total amount of follow-up time for all participants, person-years	535.5	208.5	327.0
Gender			
Males	766 / 39.1%	346 / 39.7%	420 / 38.7%
Females	1,192 / 60.9%	526 / 60.3%	666 / 61.3%
Age in years, mean (SD)			
Children under 18 years of age	7.4 (4.5)	7.4 (4.6)	7.4 (4.5)
Adults	35.5 (11.6)	34.5 (10.8)	36.2 (12.1)
Age category, N / % of participants			
Children under 18 years of age	1,170 / 58.8%	544 / 62.1%	626 / 56.3%
Adults	816 / 41.2%	332 / 37.9%	484 / 43.7%
Race, N / % of participants			
Black	112 / 5.6%	56 / 6.4%	56 / 5.0%
White	290 / 14.6%	148 / 16.9%	142 / 12.8%
Mixed or other	213 / 10.7%	197 / 22.5%	16 / 1.4%
Not reported	1,371 / 69.0%	475 / 54.2%	896 / 80.7%
Ethnicity, N / % of participants			
Hispanic	1,864 / 93.9%	809 / 92.4%	1,055 / 95.0%
Non-Hispanic	106 / 5.3%	51 / 5.8%	55 / 5.0%
Not reported	16 / 0.8%	16 / 1.8%	0 / 0.0%
Language spoken at home, N / % of participants			
English only	270 / 13.6%	121 / 13.8%	149 / 13.4%
Spanish or Portuguese	1,536 / 77.3%	716 / 81.7%	820 / 73.9%
Other or not reported	180 / 9.1%	39 / 4.5%	141 / 12.7%
Annual household income, N / % of participants			
Less than $ 25,000	972 / 48.9%	462 / 52.7%	510 / 45.9%
Between $ 25,000 and $ 49,000	390 / 19.6%	95 / 10.8%	295 / 26.6%
Between $ 50,000 and $ 74,000	88 / 4.4%	18 / 2.1%	70 / 6.3%
Between $ 75,000 and $ 99,000	16 / 0.8%	8 / 0.9%	8 / 0.7%
$ 100,000 per year or more	3 / 0.2%	3 / 0.3%	0 / 0.0%
Not reported	517 / 26.0%	290 / 33.1%	227 / 20.5%
Education, N / % of adult participants			
Did not complete high school	267 / 32.7%	114 / 34.3%	153 / 31.6%
High school diploma	255 / 31.3%	98 / 29.5%	157 / 32.4%
Less than 4 years of college	133 / 16.3%	71 / 21.4%	62 / 12.8%
Bachelor’s degree	44 / 5.4%	15 / 4.5%	29 / 6.0%

Factor or parameter	Both phases	Phase 1	Phase 2
Graduate or professional degree	43 / 5.3%	15 / 4.5%	28 / 5.8%
Not reported	74 / 9.1%	19 / 5.7%	55 / 11.4%
Home filters, N / % of participants			
Any water filter	1,079 / 54.3%	470 / 53.7%	609 / 54.9%
No filter	892 / 44.9%	391 / 44.6%	501 / 45.1%
Not reported	15 / 0.8%	15 / 1.7%	0 / 0.0%
Swimming, N / % of monthly surveys			
Swimming in pools	200 / 3.6%	80 / 3.1%	120 / 4.1%
Swimming in lakes, rivers	75 / 1.4%	17 / 0.7%	58 / 2.0%
Swimming in the ocean	90 / 1.6%	26 / 1.0%	64 / 2.2%
Consumption of non-boiled tap water, N / % of monthly surveys			
No	2,705 / 49.2%	1,279 / 50.1%	1,426 / 48.5%
Yes	2,617 / 47.6%	1,145 / 44.9%	1,472 / 50.0%
Not reported	172 / 3.1%	128 / 5.0%	44 / 1.5%
Liquid consumption per 24 hours in mL, mean (SD)			
Plain bottled water	336 (591)	389 (601)	291 (578)
Boiled tap water and drinks made of it	286 (501)	255 (421)	313 (561)
Non-boiled tap water and drinks made of it	478 (730)	399 (661)	545 (778)
Total liquid consumption in drinks including tap and bottled water, soda and milk	2,136 (1,164)	2,106 (1,165)	2,160 (1,162)
Episodes of gastrointestinal symptoms, N / incidence rate per person-year			
Diarrhea	147 / 0.27	46 / 0.22	101 / 0.31
Vomiting	112 / 0.21	35 / 0.17	77 / 0.24
Abdominal cramps	20 / 0.04	17 / 0.08	3 / 0.01
Gastroenteritis (diarrhea or vomiting or cramps)	214 / 0.40	77 / 0.37	137 / 0.42
Combined diarrhea and vomiting	55 / 0.10	13 / 0.06	42 / 0.13

Table 2.

Summary statistics for immunoconversions and associations with gastrointestinal symptoms by immunoconversion definition from univariate GEE models

Pathogen	Immunoconversion definition [a]	Total N of immunoconversions	N / % of immunoconversions with symptoms [b]	% of episodes of symptoms with immunoconversion	Unadjusted OR of immunoconversion after an episode of symptoms
Cryptosporidium	1	63	7 / 11.1%	3.3%	3.07 (1.42; 6.63) *
	2	43	5 / 11.6%	2.3%	3.20 (1.30; 7.87) *
	3	32	4 / 12.5%	1.9%	3.46 (1.23; 9.75) *
GI norovirus	1	182	8 / 4.4%	14.5%	3.77 (1.02; 14.0) *
	2	125	6 / 4.8%	10.9%	3.80 (0.74; 19.6)
	3	102	4 / 3.9%	7.3%	2.67 (0.29; 25.0)
GII noroviruses	1	177	7 / 4.0%	12.7%	3.42 (1.21; 9.64) *
	2	112	7 / 6.3%	12.7%	5.25 (1.76; 15.7) *
	3	72	3 / 4.2%	5.5%	3.58 (0.73; 17.6)
All noroviruses	1	294	13 / 4.4%	23.6%	4.26 (1.74; 10.5) *
	2	210	13 / 6.2%	23.6%	6.15 (2.42; 15.6) *
	3	155	7 / 4.5%	12.7%	3.88 (0.99; 15.2)

[a] Immunoconversion definitions: (1) Four-fold increase in antibody response between monthly samples; (2) same as in 1 and post-conversion responses above the 80 % prediction bound of spline regression on age; (3) same as in 1 and post-conversion responses above the 90 % prediction bound of spline regression on age.

[b] Symptoms definitions: gastroenteritis (diarrhea or vomiting or abdominal cramps) for *Cryptosporidium,* combined diarrhea and vomiting symptoms for noroviruses.

* $p < 0.05$

Table 3.

Salivary antibody immunoconversions by sociodemographic factors and study phase, total number / incidence rate per 100 person-years with *p* values for trend using the exact two-sided Cochran-Armitage test for trend

Factor	Category	*Cryptosporidium*	*p* value for trend	GI norovirus	*p* value for trend	GII noroviruses	*p* value for trend
All		32 / 6.0	NA	125 / 23.3	NA	112 / 20.9	NA
Age, years	1–10	20 / 8.5		67 / 28.4		56 / 23.7	
	11–20	5 / 5.6		28 / 31.5		20 / 22.5	
	21–40	6 / 4.2		15 / 10.4		24 / 16.7	
	41–85	1 / 1.5	0.03	15 / 22.4	0.01	12 / 17.9	0.2
Sex	Male	9 / 4.4		50 / 24.2		38 / 18.4	
	Female	23 / 7.2	0.2	75 / 23.4	0.9	74 / 23.1	0.2
Study phase	1	17 / 8.2		51 / 24.5		33 / 15.8	
	2	15 / 4.6	0.5	74 / 22.6	0.3	79 / 24.2	0.0005
Language spoken at home	English	10 / 8.1		32 / 25.8		40 / 32.2	
	Any other	22 / 5.3	0.2	93 / 22.6	0.3	72 / 17.5	0.0005
Household income	Less than $ 25,000	21 / 6.2		82 / 24.0		71 / 20.8	
	$ 25,000 or more	11 / 5.7	1.0	43 / 22.1	1.0	41 / 21.1	0.7
Highest education of adult household members	High school or less	22 / 7.1		88 / 28.3		70 / 22.5	
	More than high school	10 / 4.4	0.3	37 / 16.5	0.007	42 / 18.7	0.4
Total household size, individuals	2–3	7 / 5.2		27 / 20.0		22 / 16.3	
	4–5	17 / 6.2		49 / 17.8		59 / 21.4	
	6–9	8 / 6.4	0.6	49 / 39.2	0.001	31 / 24.8	0.09
Home water filter present	No	16 / 6.5		62 / 25.2		58 / 23.6	
	Yes	16 / 5.5	0.6	63 / 21.5	0.4	54 / 18.6	0.2

Table 4.

Risk factors for *Cryptosporidium* and norovirus immunoconversions: adjusted odds ratio (95% confidence interval) from multivariate models

Predictor variable	*Cryptosporidium*	GI norovirus	GII noroviruses
Study phase 1 vs. phase 2 [a]	1.28 (0.58; 2.80)	1.04 (0.64; 1.69)	0.50 (0.32; 0.80) *
Swimming in pools [b]	4.92 (1.56; 15.5) *	1.50 (0.50; 4.50)	0.92 (0.31; 2.69)
Swimming in natural water bodies [b]	2.33 (0.35; 15.4)	0.20 (0.03; 1.14)	1.30 (0.37; 4.51)
Interaction effect of drinking non-boiled tap water and phase 1 [c]			
All study participants	5.40 (1.14; 25.6) *	1.06 (0.44; 2.59)	1.21 (0.50; 2.94)
Individuals who did not have water filters at home	15.8 (1.09; 228) *	0.82 (0.24; 2.77)	1.07 (0.33; 3.44)
Individuals who had water filters at home	2.95 (0.34; 25.4)	1.80 (0.51; 6.44)	1.68 (0.43; 6.61)
Effect of drinking non-boiled tap water in stratified analysis [d]			
Phase 1, all participants	2.17 (0.77; 6.13)	1.25 (0.63; 2.47)	1.52 (0.73; 3.16)
Phase 1, individuals who did not have home water filters #	11.1 (1.23; 100) *	0.72 (0.31; 1.66)	1.67 (0.59; 4.72)
Phase 1, individuals who had home water filters #	0.83 (0.21; 3.33)	2.13 (0.85; 5.34)	1.40 (0.48; 4.04)
Phase 2, all participants	0.40 (0.12; 1.27)	1.24 (0.69; 2.20)	1.13 (0.70; 1.84)
Phase 2, individuals who did not have home water filters #	0.58 (0.13; 2.52)	1.01 (0.49; 2.05)	1.74 (0.90; 3.37)
Phase 2, individuals who had home water filters #	0.29 (0.05; 1.58)	1.29 (0.67; 2.50)	0.71 (0.36; 1.40)

[a] Adjusted for sex, number of individuals in the household, age (piece-wise regression), cubic polynomial of time, highest education level of household adults, income (for *Cryptosporidium* only), and language spoken at home (for noroviruses only).

[b] Adjusted for sex, study phase, number of individuals in the household, age (piece-wise regression), cubic polynomial of time, highest education level of household adults, income (for *Cryptosporidium* only), and language spoken at home (for noroviruses only).

[c] Adjusted for sex, study phase, number of individuals in the household, language spoken at home, age (piece-wise regression), cubic polynomial of time, highest education level of household adults, income (for *Cryptosporidium* only), language spoken at home (for noroviruses only), and drinking tap water.

[d] Adjusted for sex, number of individuals in the household, age (piece-wise regression), cubic polynomial of time, highest education level of household adults, income (for *Cryptosporidium* only), language spoken at home (for noroviruses only), and drinking tap water.

* $p < 0.05$

Regular logistic regression models were used for these strata.