BEYOND DIARRHEA: FECAL-ORAL PATHOGEN TRANSMISSION AND ENVIRONMENTAL ENTEROPATHY IN IQUITOS, PERU

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Abstract

The importance of clean water and adequate sanitation is a widely recognized characteristic of healthy communities. Across the developing world, many communities are without this vital infrastructure, thereby vulnerable to enteric infections from pathogens that travel through the environment and may cause diarrhea. Looking beyond diarrhea, a more serious, long-lasting subclinical condition called environmental enteropathy (EE) may develop in the intestinal tract from enteropathogen exposure, which permanently alters the ability of the intestine to take up nutrients and the host to fight off infections.

The first manuscript of this dissertation relates water and sanitation conditions in households to child EE biomarkers in stool, urine and serum. This study found that the water and sanitation conditions were associated with fecal markers for EE in a peri-urban community of Iquitos, Peru. The results provide preliminary evidence for the hypothesis that children under 24 months of age living in unsanitary conditions will have elevated levels of fecal EE markers for gut inflammation and gut permeability that lead to stunting.

The second manuscript characterizes fecal contamination on household floors, an important transmission route for fecal pathogens that may greatly affect children under 24 months of age who spend a lot of time playing and eating off the floor. This study found that households with improved sanitation and cement floors in the kitchen area had reduced fecal contamination compared to those with unimproved sanitation and dirt floors. These findings suggest that the sanitation facilities of a home may impact the microbial load found on floors, contributing to the potential for household floors to serve as an indirect route of fecal pathogen transmission to children.

The third and fourth manuscripts present saliva as a novel and minimally invasive specimen for use in community based studies to assess microbial pressure and pathogen-specific infections. The outcome measure of salivary secretory immunoglobulin A was found to be associated with the sanitation and household characteristics of children living in peri-urban Iquitos, Peru and demonstrated an important proof of concept for future water and sanitation interventions that this marker can differentiate between households within a community.

Advisors:

Professor Kellogg J. Schwab
Assistant Professor Margaret N. Kosek
Assistant Professor Christopher D. Heaney
Associate Professor Frank C. Curriero

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Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

Background

Diarrheal diseases are a leading cause of morbidity and mortality in children under five years old, accounting for 10 percent, approximately 760,000, of all annual childhood deaths.[1] Children living in low-income countries disproportionately suffer from malnutrition, which has been shown to affect cognitive development, increase infection risk, limit physical capacity and future childbearing, reduce adult economic productivity, and increase mortality risk.[2] Interestingly, a pooled analysis of nine studies conducted between 1978 and 1998 in Africa, Asia, and the Americas showed that although interventions to improve hand washing, sanitation, and hygiene reduced diarrheal incidence by 30 percent, there was only a 2.4 percent reduction in prevalence of stunting.[3] Dietary interventions have also been unsuccessful in helping children achieve normal growth, with the growth effect achieved in the most successful studies only eliminating a third of the average deficit.[4]

Environmental enteropathy (EE) is a subclinical disorder of the small intestine characterized by an abnormal intestinal architecture and increased permeability.[5] As seen in Figure 1, enteropathy is mainly characterized by villous atrophy and intestinal inflammation. These two conditions lead to reduced intestinal barrier function and allow for increased translocation of antigenic macromolecules. The inflamed mucosal membrane with compromised tight junctions enable the passage of fecal pathogens from the intestinal lumen into the body, eliciting a subsequent systematic immune reaction (Figure 2).[6] This chronic inflammation may mediate stunting by diversion of energy and nutrients needed for growth to prioritize a host's survival and maintenance due to infection.[7] In addition, the deterioration in the absorptive surface area of the small intestine due to fusion of villi may mediate undernutrition by reduced uptake of nutrients.[5]

Figure 1. Proposed causal pathway linking enteropathy with adverse health outcomes in developing countries (Prendergast & Kelly, 2012)

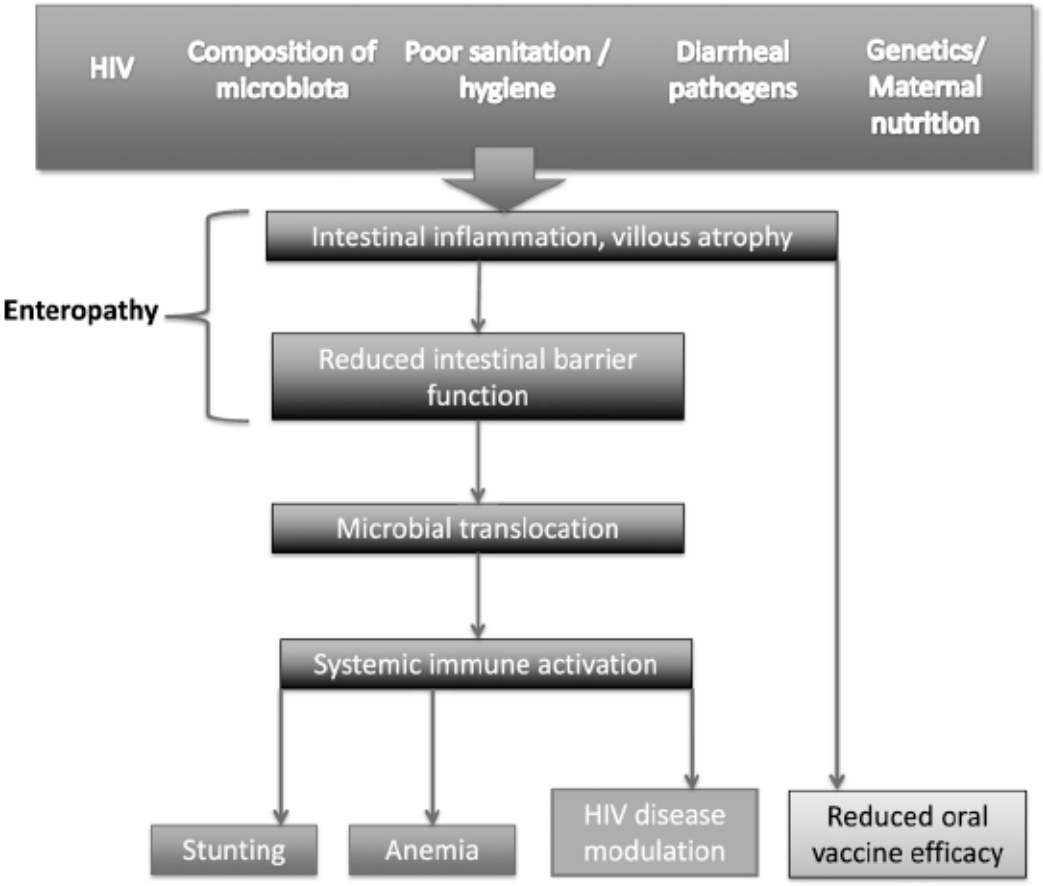
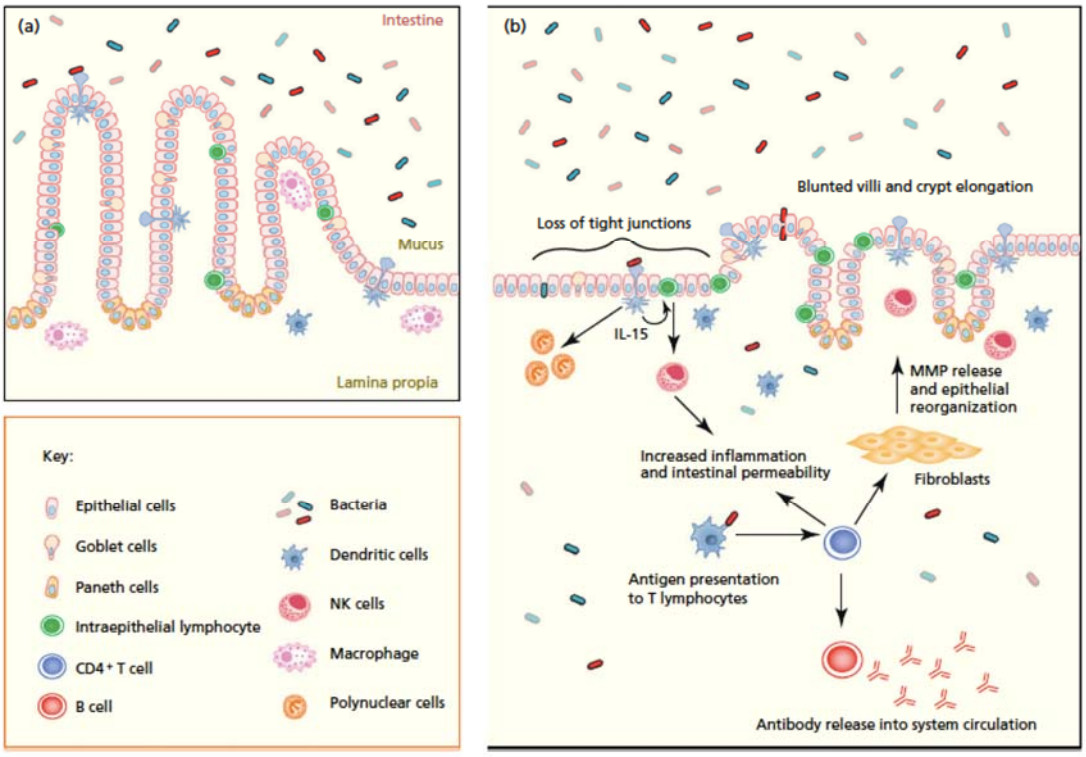


Figure 2. Proposed pathogenesis associated with environmental enteropathy (Korpe and Petri, 2012)

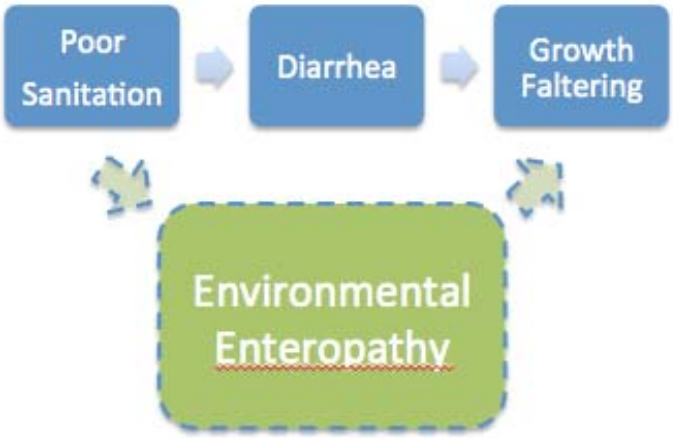


Data from many regions of the developing world suggests that diarrheal disease is not responsible for the long-term pattern of growth faltering.[3, 8] For example, Gambian infants that exhibited severe mucosal damage and inflammation had up to 43% of their observed growth faltering attributable to this intestinal permeability, which was chronic and far exceeded the 7.3% of days in their first two years of life that they spent with diarrhea.[9] Though these infants suffered from diarrhea and lost weight from these acute episodes, they tended to catch up afterward so that diarrhea prevalence was

not related to their overall growth.[10] In addition, a recent meta-analysis of data from cluster-randomized controlled trials with an intervention period of 9-12 months, found that only a small benefit on linear growth in children under five years of age came from water, sanitation and hygiene interventions[1].[11] These data indicate that EE rather than diarrhea, is the mediator between exposure to fecal pathogens and stunting (Figure 3).

[1] These specific interventions include solar disinfection of water, provision of soap, and improvement of water quality.

Figure 3. Proposed causal pathway between poor sanitation conditions and growth faltering (bottom square) versus the conventional conceptual model that poor sanitation conditions are mediated by diarrhea to cause growth faltering in children.



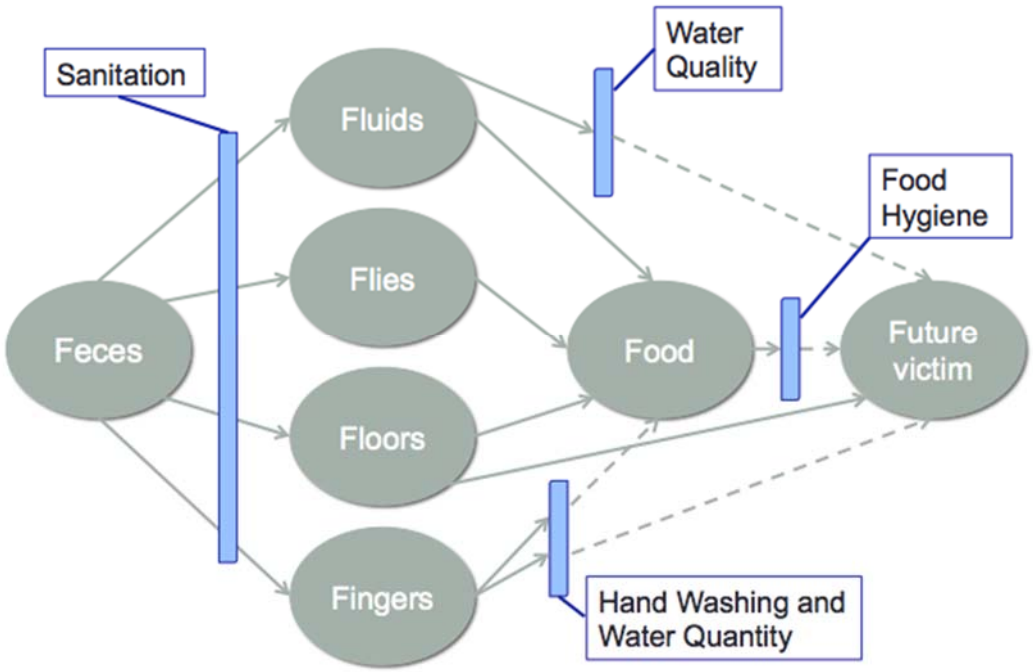
These findings suggest that the lack of improved growth following water, sanitation and hygiene interventions is due to unalterable gut dysfunction that has been established in children under five. This is further supported by the growing body of literature that shows the association between environmental factors related to poor water, sanitation and hygiene conditions and stunting.[12-16] Therefore, for those children living in poor sanitation conditions, intestinal permeability is hypothesized to be "set" at an early age and persist throughout life.[10] Exposure to pathogens at an early age is of greatest concern because this is the age when children are growing and developing rapidly and are therefore most sensitive to developmental insults.

Given the growing evidence that unhygienic environmental conditions in which children live contribute to, or perhaps causes EE, no specific organism or mechanism has been definitively identified as the major cause. It has been reported that *Helicobacter pylori* may allow other pathogens easier access to the small intestine and *Giardia intestinalis* causes an acute elevation of Giardia-specific IgM antibodies and is associated with a increased intestinal permeability, increased acute phase proteins and reduced weight gain.[10] Though both these organisms are transmitted via the fecal-oral routes of exposure, it may be that the observed associations with growth was a reflection of the levels of the overall fecal pathogen ingestion, rather than a specific effect of either *H. pylori* or *Giardia*. It is highly possible that EE comes from the frequent exposure to a combination of fecal pathogens rather than a single pathogen.

There are many fecal-oral transmission pathways, which account for important routes of exposure for the pathogens that cause diarrheal diseases. These pathways can broadly be categorized into the 'five F's' – fluids (water), fingers (hands), flies, food, and floors (Figure 4).[17] A lack of access to clean water is often implicated as the primary fecal-oral transmission route, however, a number of randomized, controlled trials investigating the

effect of drinking water on gastrointestinal health have shown no additional benefit from point-of-use interventions.[18-20] This lack of benefit is hypothesized to be because the environmental conditions from poor sanitation and hygiene allow for other sources of exposure through fecal-oral transmission pathways other than water. These other sources of exposure may nullify any potential benefit observed from improved water quality alone in a low-income setting. In addition, from an updated review of epidemiological studies on the effect of water and sanitation interventions on self-reported diarrhea episodes, no difference was found in point-of-use water interventions when blinding was taken into account.[21] These studies point to the importance of focusing in on sanitation interventions as the primary mechanism to interrupt the transmission of pathogens via the fecal-oral routes of transmission, rather than water supply interventions which may play a lesser role than once thought in reducing pathogen exposure. Figure 4 illustrates the role for each water, sanitation and hygiene intervention to interrupt the fecal-oral transmission pathways.

Figure 4. Fecal-Oral Transmission Pathways visualized through the "F" diagram and the interventions designed to interrupt these pathways (modified from Pruss et al, 2002).



To accurately assess EE it is important to have an objective measure that does not depend on a self-reported outcome, as it often occurs with diarrhea. EE has most commonly been measured indirectly with a non-invasive dual sugar permeability assay.[22, 23] The more direct measure of an intestinal biopsy would be invasive and infeasible and so investigators use an indirect measure of gut function to determine the ratio of lactulose to mannitol (L:M) excreted in urine.[16] Lactulose and mannitol characterize different conditions in the gut. The increased absorption of the lactulose disaccharide passing

through tight junctions indicates a loss of mucosal integrity while the increased passage of the mannitol monosaccharide through the transcellular routes of aqueous pores, reflect a loss of absorptive area of the hydrophilic portion of the cell.[24] Therefore, a higher ratio of the excretion percentage of lactulose to mannitol in urine is an indicator of intestinal permeability and used as a marker of EE.

Other markers of EE increasingly in use include immunoglobulin G endotoxin core antibody (IgG EndoCAb) titers, and the fecal markers of neopterin (NEO), alpha-anti-trypsin (AAT), and myeloperoxidase (MPO). The marker of IgG EndoCAb titers is measured because increased levels may indicate an infection or chronic immune stimulation. Elevated levels of IgG EndoCAb titers in the plasma reflect exposure to an endotoxin, a cell wall component of many gram-negative bacteria that could potentially cross a leaky mucosal membrane in the gut.[16] Lastly, the fecal markers of NEO, AAT and MPO represent great potential for measuring exposure to unhygienic environments and unlike the L:M ratio, their measurement reflects an alterable state of intestinal function that precedes the final "end state" of EE.[25] Each one of these three stool markers has different functions. NEO is a marker of gut inflammation, in which a TH1 response is produced

by activated T lymphocytes. A previous study found that elevated levels of NEO in stool resulted in growth failure in Gambian children.[26] In the case of intestinal inflammation or damage to the mucosa, AAT leaves the gut and thus is a classic marker of a protein losing enteropathy, which otherwise is highly resistant to permeating the mucosa and is excreted intact in the stool. MPO is a specific marker for neutrophil activity that is not elevated in the stools of breastfed children and has been associated with disease states in inflammatory bowel disease.[25] The NEO, AAT, and MPO fecal biomarkers are all affordable, commercially available, standardized assays that can be performed on normal stool to predict linear growth deficits in children.[25] Unlike the L:M test, the results can easily be carried out across laboratories with a minimal amount of equipment and technical expertise required.

Recent studies are showing that lack of cleanliness within the household are associated with EE and point to the need to go beyond diarrhea in study outcomes.[16, 27, 28] The absence of overt symptoms associated with EE explains why this under studied condition has not been previously identified as a major concern in environments with high fecal contamination.

A less explored route of exposure to fecal pathogens is the floors pathway, which may be a significant contributor to environmental contamination with fecal pathogens. A recent study of household floors in Tanzania showed that it was the dirt floors within the household rather than the latrine floors that had the highest burden of enterococci and *E. coli*.[29] Children less than 24 months of age, who are most vulnerable to enteric infection and developing EE, have been observed to have frequent behaviors of playing and eating off of the ground in high-density, low-income neighborhoods in Accra, Ghana.[30] The combination of high bacterial loads that have been found on floors in the home environment[31-33] along with the high frequency young children play on floors and engage in soil to hand to mouth activities,[34] there is a need to understand the risk that this fecal-oral pathway poses to developing enteric infections.

A challenge with using diarrheal disease as the metric to represent exposures to fecal pathogens is the use self-reporting to characterize the disease state. Self-reporting has the potential to introduce a large amount of bias, such as recall bias, courtesy bias and researcher bias. The problems with the internal validity of water, sanitation and hygiene impact evaluations using diarrhea as the study outcome have been well established in the literature.[35-37] Stool

samples are a more objective measure to classify diarrhea, however, this is challenging due to low rates of compliance and pathogen detection.[38] Blood sampling is also a preferred outcome variable to self-reported diarrhea however it is invasive, requires trained personnel, is time-consuming, and carries a risk of needlestick injuries.[39] Alternative outcome measures for water, sanitation and hygiene intervention impact evaluations that are objective and specific to the fecal pathogen contamination are therefore a necessary area of research.[40, 41]

Saliva collection is increasingly being shown to accurately diagnose infections from viral, bacterial and parasitic infections.[42] The use of antibodies in saliva is objective, simple, rapid, requires little training, eliminates the risk of needle-stick injuries, is appropriate for both children and adults, and is suitable for nonclinical settings.[43] Oral fluid is emerging as a novel method to measure exposure to fecal pathogens by the presence of antibodies in saliva, particularly in crevicular fluid. An increase in specific antibodies in saliva as a result of infection has been described in the literature as a potent measure of disease exposure and its use could enhance epidemiological studies of waterborne diseases.[44] Saliva is a mixture of secretions from salivary glands and the crevicular fluid from between the

gum margins and the teeth. Immunoglobulin concentrations in crevicular fluid are much higher than in salivary gland secretions and hence, a saliva sample that has a high proportion of crevicular fluid is most suitable for antibody detection.[45] Griffin et. al documented the first stage of a pilot, proof-of-concept project to develop a non-invasive salivary antibody technique for surveillance of waterborne infections.[38] This study collected saliva with an Oracol sampler sponge, an absorbent foam swab, (Malvern Medical Developments, Worcester, UK) as shown in Figure 5.

Figure 5. Oracol oral fluid collection device (Malvern Medical Developments, Worcester, UK)[45]

Oracol was designed to specifically target the gum area where crevicular fluid is found and the sponge is rubbed firmly along the base of the gums of the upper and lower jaw for 1 minute, using an action similar to tooth brushing.[38, 45] The sampler sponge is then tubed and returned to the laboratory at 4 degrees C, where the fluid is squeezed out and clarified by centrifugation.

Typically, the salivary IgA response to a fecal pathogen challenge occurs before the IgG response and the IgA response tends to peak earlier than the IgG response, which will continue to increase even after the IgA response has peaked.[38] The primary function of salivary lgA is the opsonization of foreign invaders at the oral port of entrance to the body and the blockade of pathogen infectivity.[46] Total IgA and total IgG are used as an overall indication of immune response while pathogen specific IgA and IgG assays are currently under development for pathogens such as *Helicobacter pylori*, *Toxoplasma gondii*, *Cryptosporidium*, and four noroviruses.[38] It is important when carrying out the analysis to control for the different factors that affect salivary flow such as time since waking and food ingestion. The basis of the assay is that a specific antibody, if present in the specimen, cross-links antigenic sites on separate particles, agglutinating them and allowing for

visual reading.[47] Thus, the use of salivary immunoassays offers an efficient, non-invasive and economical means of determining the exposure of individuals to fecal pathogens present their environment.

Study Site

Iquitos, Peru is an excellent location for the research described in this dissertation given the Satellite Laboratory IQTLAB located in the community. The IQTLAB is equipped to carry out microbiologic, immunologic, and PCR based diagnostics. This laboratory was founded in 2002 by Dr. Margaret Kosek, Pablo Peñataro Yori, RN, MPH, and Dr. Robert Gilman of Johns Hopkins University Bloomberg School of Public Health and brings in experts from Asociación Benéfica Prisma, a non-governmental organization that has been working in Peru for over 25 years to strengthen the capacity of the poor and vulnerable to achieve social and economic development. The capabilities of the IQTLAB present great opportunity to carry out environmental microbiologic research in addition to many other relevant areas of research.

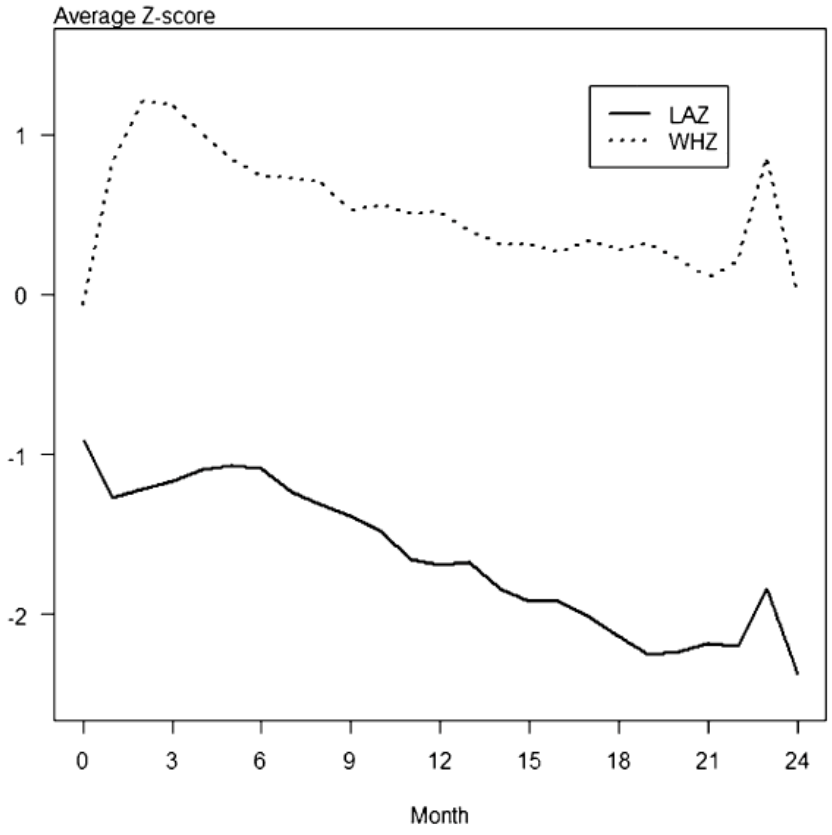
Iquitos is part of the multisite Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health

and Development (MAL-ED) cohort study, which is investigating enteric infections and nutritional status on child growth and cognitive development through the use of standard protocols of surveillance and assays implemented. The MAL-ED study includes eight sites from diverse epidemiologic settings – Fortaleza (Brazil), Dhaka (Bangladesh), Vellore (India), Bhaktapur (Nepal), Loreto (Peru), Naushahro Feroze (Pakistan), Haydom (Tanzania), and Venda (South Africa) – which span rural and urban environments and are representative of the conditions of children living in poverty across the developing world.[48] The Peru site is of particular interest for the investigation of the impacts from poor water, sanitation and hygiene conditions on environmental enteropathy given that it is also the site for a grant from the Bill & Melinda Gates Foundation on biomarkers of gut function within the grand challenges in global health initiative. The goal of the grant program is to identify and validate biomarkers that can assess gut function and guide new ways to improve the health and development of children in the developing world. The grant is pioneering the use of the fecal markers of NEO, AAT and MPO to use as a more effective way to measure the risk a child faces in developing environmental enteropathy and therefore presents an opportunity for intervention for that child. Given the cohort of children with well-characterized monthly stool samples under the MAL-ED

study, along with the work on advancing gut biomarkers, Iquitos is ideally suited to simultaneously explore the environmental exposures to fecal pathogens that may be causing these enteric infections.

Going beyond diarrhea to examine the impacts of fecal-oral diseases is ideally suited to the conditions in Iquitos, Peru. The burden of diarrheal illness is high in this peri-urban population with one study on shigella-associated diarrhea reporting a diarrheal disease incidence of 4.38 episodes per child-year.[49] These incidence rates for a stable population of children under five are relatively high when compared to the literature in the last decade.[50] In addition, the childhood stunting rates in this region are elevated to almost 50 percent while the rest of Peru is approximately 20 percent.[51] The possible attribution of stunting to fecal pathogen exposure and environmental enteropathy is further supported by the length for age z-scores (LAZ) from a birth cohort in Iquitos of children 0 to 24 months of age in Figure 6. Here the LAZ scores were one standard deviation below normal during birth but declined more severely over the next 24 months, pointing to possible environmental risk factors in this community.[52]

Figure 6. Anthropometric z scores from 0 to 24 months of age in cohort from Iquitos, Peru.[52]



The Iquitos, Peru research site is also well suited to study environmental contamination from water, sanitation and hygiene conditions. The development statistics of the study site community Santa Clara, located 15km outside of the city center of Iquitos, show high rates of diarrheal disease and stunting compared to the rest of the country as a whole. In addition the percent of the population in Santa Clara with access to clean water and improved sanitation lags behind the rest of the country with only half having access to clean water and a fifth with access to an improved toilet (Table 1).

Table 1. Development Statistics in from Santa Clara community in Iquitos Peru and the overall country statistics from Peru in 2012. (Source: Instituto Nacional de Estadística e Informática, 2012)

Indicator	Peru	Santa Clara
Life expectancy, y	72.5	69.9*
Access to clean water, % of households	77.1	46.7
Access to improved toilet/ sanitation	58.4	20.2
Children under 5 stunted, %	19.5	46.3
Children under 5 with diarrhea reported in the past week, %	13.9	35.4
Under-5 mortality rate per 1000 live births	21	60.6*

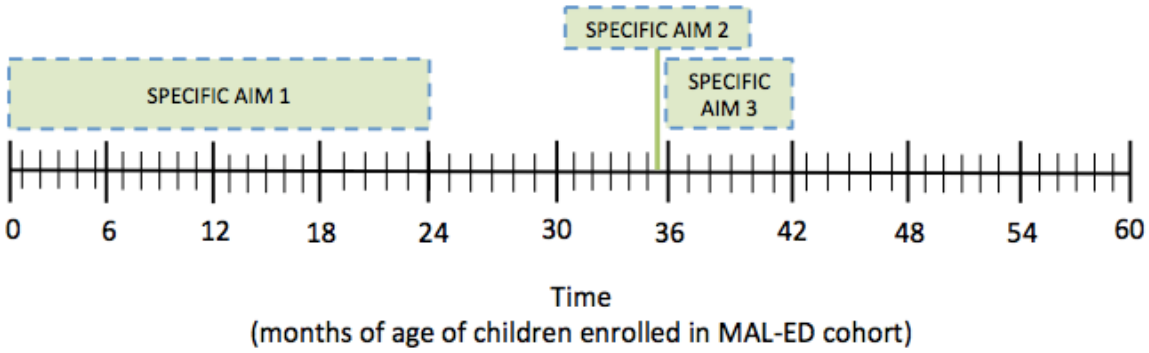
*Statistic relevant to the Department of Loreto

The maternal education levels for mothers in the region are relatively high with one study showing less than one percent were illiterate, and 48 percent had advanced past primary school level.[49] This points to the environment as a potential source of contamination rather than other socio-economic factors common in low-income settings. All combined, the development characteristics of Iquitos, Peru present a unique opportunity to study the connection between poor sanitation and the condition of environmental enteropathy.

Hypothesis and Specific Aims

The overarching hypothesis underlying the work in this dissertation is that fecal contamination in the household environment due to a lack of adequate water, sanitation and hygiene contributes to the development of environmental enteropathy in peri-urban, flood-prone communities in Iquitos, Peru. This hypothesis will be investigated with three specific aims as laid out in Figure 7.

Figure 7. Schematic diagram of sampling framework for specific aims within MAL-ED study.



Specific Aim 1

The first aim of this dissertation was to estimate the association between household water and sanitation conditions and hygiene practices and environmental enteropathy in children under five in the Santa Clara community in Iquitos, Peru. This aim included secondary data analysis of

data collected at the Peru site of the MAL-ED study to estimate the relative importance of household environmental contamination on gut inflammation. The MAL-ED longitudinal follow-up study prospectively collected diarrhea surveillance, stool samples, and growth measures on a monthly basis from 0 to 24 months of age to characterize the hypothesized pathways between enteric disease and growth. The stool samples were analyzed for three fecal gut markers (NEO, AAT, and MPO) and the L:M test. This site in the Peruvian Amazon is of particular interest for this research question for its focus on gut function where approximately 200 children were enrolled at birth.

A robust set of data was collected on the environmental household conditions from community surveys of the population that include a Follow-up Socio-Economic (FSE) status form (bi-annual) and a community census administered in 2010 and 2012. The FSE form was similarly administered to the other seven sites in the MAL-ED study and contains water, sanitation and hygiene variables along with wealth, education and other household characteristics. The census survey was only administered in Iquitos, Peru and included every household with a child enrolled in the MAL-ED study in

2010 and 2012. Most importantly, the census collected site-specific information on water storage practices throughout the community.

The outcome variables used were the gut biomarkers of intestinal inflammation, which included MPO, NEO and AAT. The sugar permeability tests were carried out to measure the lactulose to mannitol ratio which has been used in previous studies to assess the permeability of the gut to macromolecules and intestinal absorptive capacity, to characterize altered gut physiology, a key pathway leading to growth failure in children. Surveillance visits were made to households on a bi-weekly basis and stool samples were collected on a monthly basis. Additional stool samples were collected if the child was reported to be symptomatic for diarrhea at the time of a surveillance visit. Multivariate regression models evaluated the associations between the household environmental contamination variables and the various markers for EE. Mixed models were used to account for within-subject correlation over time.

Specific Aim 2

The second specific aim measured the concentration of fecal contamination on household floors and surfaces and estimated its association with

environmental enteropathy in children under five within the household. This aim characterized the level of fecal microorganisms on the floors and surfaces in the household environment in Iquitos, Peru and related this contamination to the different water, sanitation and hygiene characteristics of the home. There is a need to better understand the source, distribution, and fate of fecal contamination in households and this study examined the potential role for floors in the transmission of fecal pathogens. Two household floor sampling sites were chosen - one in the highly trafficked entrance area and another in the area of food preparation where there were more frequent water activities. Replicate samples were taken from each entrance floor sampling site within the household to quantify the within-sample variability. This provided a better characterization of fecal contamination in the household.

Specific Aim 3

The third aim of this dissertation investigated the utility of salivary immunoassays as a novel and non-invasive approach to improve assessment of recent exposure to fecal pathogens in a community-based longitudinal study. The objective of this study was to verify the application of salivary antibody tests in a pilot community study for surveillance of gastrointestinal

infections. This longitudinal antibody monitoring was then associated with the household cleanliness factors related to water, sanitation and hygiene conditions. In low-income settings the use of saliva as a non-invasive and low-cost specimen with a rapid sampling technique may vastly improve the quality of impact evaluations associated with water, sanitation and hygiene interventions.

This study was nested within the MAL-ED cohort of children in Iquitos, Peru and the saliva specimen sampling was carried out alongside the ongoing stool collection under the MAL-ED protocol. The saliva specimens were collected with the Oracol sampler sponge and analyzed for total protein and secretory IgA (SIgA) using off-the-shelf commercial kits from Salimetrics, LLC. The strengths of this study were the frequent weekly longitudinal sampling of each child, as opposed to the yearly sampling that has been done under other studies.[53-55] During saliva collection, data was collected on the other factors that can affect antibody concentration such as human patterns of diurnal Ig variability (e.g., time since last sleeping), volume of the saliva sample collected, oral health of the child, and if the child had eaten anything in the last hour and protein in the last twenty minutes before saliva sampling. The measurement for microbial exposure

was estimated using proxies for fecal contamination in the household including the types of water and sanitation infrastructure, as well as hand washing behaviors. The levels of microbial contamination found on household floors and surfaces from Specific Aim 2 were also used to estimate the microbial exposures and were related to the SIgA measures in saliva.

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Chapter 2

A longitudinal study of water, sanitation and hygiene characteristics and environmental enteropathy markers in children under 24 months in Iquitos, Peru

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Abstract

Poor child gut health and a lack of access to an improved toilet or clean water is an area of interest to understand the biological mechanisms underlying stunting. A birth cohort of 270 children from peri-urban Iquitos Peru were characterized for their household sanitation, water use, hygiene and household characteristics. These children were had monthly stool samples, quarterly urine samples and annual serum samples analyzed to derive estimates of their progression toward environmental enteropathy. This study found that sanitation conditions were associated with fecal markers for

EE (no sanitation facility compared to those with a toilet had -0.43 log myelperoxidase (MPO), 95% CI: -0.74, -0.13) as well as water conditions when comparing those with an intermittent connection versus those that had a continuous supply (MPO increased 0.36 log, 95% CI: 0.08, 0.63). These results provide preliminary evidence for the hypothesis that children under 24 months of age living in unsanitary conditions will have elevated levels of fecal EE markers for gut inflammation and permeability that lead to stunting.

Introduction

Stunting is a widespread condition for children in low and middle income countries[1] and if not reversed by 2 years old, stunting can have long-term effects on health and development. In 2011, a global estimate for stunted children under 5 years old was 165 million based on a height-for-age Z score (HAZ) –2 or lower.[2] An even larger number of children that are above the -2 HAZ threshold still experience inadequate growth, damaging the development potential and human capital of entire societies.[3] Stunting is now recognized as a major global health priority[4] and the Sustainable Development Goals recently adopted at the 2015 UN Summit state "by 2030 end all forms of malnutrition, including achieving by 2025 the

internationally agreed targets on stunting and wasting in children under 5 years of age" (Target 2.2, https://sustainabledevelopment.un.org/topics). To achieve these ambitious targets, an in-depth understanding is needed of the complex interactions between enteric infections and undernutrition that contribute to linear growth faltering.[5]

Childhood enteric infections brought on by chronic exposures to fecal pathogens, are predicted to account for 25-43% of the worldwide stunting burden.[6] Fecally contaminated environments put children at risk for chronic exposure to enteric pathogens and with 2.5 billion people who do not have access to an sanitation facility[7], a large portion of children in the developing world are at risk. Sustained episodes of acute gastroenteritis (symptomatic or not) may lead to perpetual inflammation and structural changes in the small bowel[8], a condition known as environmental enteropathy (EE), and is a key mediator in the relationship between enteric infections and linear growth. It is a subclinical condition defined by structural and functional changes to the small bowel (blunting of the finger-like villi and crypt hyperplasia)[9] and accompanied by increased intestinal inflammation, permeability, and bacterial translocation, which may lead to systemic immune activation and decreased nutrient absorptive capacity of the intestine.[8, 10-12] A murine model

has demonstrated that the etiology of EE originates from both a malnourished diet and repeated oral exposures to commensal bacteria.[13] Therefore, food security alone cannot fully explain our understanding of growth faltering and new efforts are needed to understand how fecal-oral contamination of the environment impacts the development of EE.

The evidence linking water, sanitation and hygiene conditions (WASH) with childhood stunting has increased substantially in recent years.[14, 15] Most notable is a cluster-randomized controlled trial (RCT) in Mali that demonstrated increased child growth for children with increased access to toilets [16]. The inverse relationship between fecal-oral contamination and childhood stunting has also been reported in non-randomized studies [17-21]. The increasing evidence that improved hygienic environments might contribute to improved growth outcomes for children justifies research into the WASH-EE mechanism through which these improvements in linear growth may occur.

There is scant evidence, however, linking environmental conditions to the physiologic, anatomic, and functional changes in the gut as a result of a prolonged and persistent exposure to multiple enteropathogens.[5] EE was

identified in a study of Zambian adults and found that small intestinal artcitecture (crypt depth but not villous atrophy) was associated with a hygiene score.[9] In Bangladesh, children from clean households versus contaminated homes had less intestinal permeability, though only marginally significant.[20] Also in Bangladesh, children that engaged in soil eating behaviors or had an animal corral in their sleeping quarters were found to have a higher EE score.[22, 23]. An additional challenge for EE studies is that biomarkers that can be used in environments with poor WASH conditions to obtain population based measurements of EE is an open area of investigation.[24]

The aim of this study was to explore the associations between household WASH factors and fecal, urine and serum biomarkers for EE in a longitudinal cohort. We hypothesized that reasonable improvements in water and sanitary infrastructure and hygienic practices could improve the small intestine structure and function in children younger than 24 months of age. A comprehensive set of WASH variables were examined with an in-depth characterization of water storage practices. These variables were then related

to various fecal, urine and serum biomarkers for EED at the Iquitos Peru site of the MAL-ED[2] study.

Methods and Materials

Study site and population

The study site is located along the Nanay River, a tributary of the Amazon, in three peri-urban communities - Santa Clara de Nanay, Santo Tomas, and La Union (3°47'S, 73°20'W). These communities are located about 15 kilometers outside of the city center of Iquitos in the Department of Loreto with a population of approximately 5,000 people and a population density of 4.6 people per square meter.[25] Despite Peru's success in meeting its Millennium Development goals for both access to improved water and sanitation,[26] these peri-urban communities still lag behind the country and only 50 percent of the population uses improved water sources and 20 percent has access to an improved toilet facility.[25] There is no centralized sewerage in the community and therefore, even those that have an improved toilet option such as a pour flush toilet, face a hazard from frequent overflows and lack of services to hygienically empty, transport and treat

[2] Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development

fecal matter. Water storage risks recontamination of improved drinking supplies[27] and is utilized widely throughout the community due to the intermittent supply for those connected to the piped system and frequent breakdowns of hand pumps. The municipal water system delivers water to households for hour-long intervals and hand pumps are connected to artesian wells that are often shared between households. Bleach is readily available in the community and used for water treatment. The community is vulnerable to frequent floods that inundate latrines causing overflow and putting those with onsite sanitation at greatest risk for contamination.

Childhood stunting is remarkably high in this study community when compared to the rest of Peru and beyond. For children under 5 years old in peri-urban Iquitos Peru, 46.3 percent are stunted (HAZ < -2)[25] compared to Africa and Asia where 35.6 percent and 26.8 percent of children under 5 years old are stunted, respectively.[2] A cohort study in Santa Clara found the incidence of diarrheal illness in children 12-23 months of age is 4.38 episodes per child-year,[28] which is relatively high when compared to the literature in the last decade.[29] When comparing the number of pathogens detected in the stool of children under two years old across MAL-ED sites, the Peru site was in the low range for children at three months old compared

to other sites with about 0.5 pathogens detected per stool. The Peruvian children then progressively acquired more pathogens detected per stool and by 24 months old they had about 2.0 pathogens detected per stool.[30] This frequent detection of pathogens in stool is potentially caused by widespread fecal contamination and may lead to chronic insults to the infant gastrointestinal tract.

Selection Criteria

The MAL-ED birth cohort used a prospective longitudinal design among eight country sites with historically high incidence of diarrheal disease and undernutrition to investigate the hypothesis that enteropathogen infection contributes to undernutrition by causing EE.[31] The study was designed to enroll approximately 200 healthy infants born to mothers greater than 16 years old within 17 days of birth. Enrollment was limited to one child per household and children were excluded from the cohort if they were enrolled for less than six months, had a caregiver with plans to move out of the catchment area during the first 6-months of follow-up, exhibited serious indications of disease or were of low birth weight (<1500g). Enrollment occurred over a two year period from January 2010 to February 2012 and children were followed through 24 months of age.

Household water, sanitation and hygiene risk factors

Each household with a child enrolled in the MAL-ED study was administered a socio-economic survey with questions related to water (source type, continuity of supply, point-of-use treatment and collection), sanitation access (type of facility, sharing behavior with other households), hygiene behaviors (hand washing activities, use of toilet paper), and household characteristics (floor type, roofing and wall materials, number of rooms, years of tenancy, electricity, etc). A hygiene index variable score was calculated as a cumulative score from the following four questions: i) Do you wash your hands after helping your child defecate? ii) Do you wash your hands before preparing food? iii) Do you wash your hands after going to the bathroom? and iv) Do you use toilet paper?. The hygiene index score had three levels with good indicating the interviewee answered all questions as always practicing the hygienic behaviors; average indicated that for one of the four questions the interviewee only sometimes practiced the hygienic behavior; and poor indicated that for two or more questions the interviewee only sometimes practiced the hygienic behavior. The wealth index was a sum of different possessions owned in each household giving equal weight to all possessions. The survey was adapted from questions used by the

Demographic and Health Surveys.[31] It was administered at 6, 12, 18 and 24 months of age for the children enrolled. Other variables of interest recorded were head-of-household and maternal education, monthly income level (in soles) and crowding. Breastfeeding status was recorded alongside these variables by a separate survey to characterize exclusive, mixed and fully weaned breastmilk intake.

An in-depth characterization of water storage practices was done by a community census that was administered in 2010 and 2012. The questions administered in the survey had been previously validated in a 2005 census and were shown to relate to the risk of diarrheal disease in the study community.[28] The variables of interest include the total volume of water stored in the household, types of containers used for storage and lid type (with or without lids). These questions were recorded by observation from trained field workers well acquainted with the local practices of water storage.

Stool collection and fecal marker assays

Longitudinal stool samples were collected more frequently and were less logistically burdensome than both serum and urine collection. Fecal markers

of EE - myeloperoxidase (MPO), neopterin (NEO), alpha-1-antitrypsin (AAT) - that have been found to be associated with declines in length-for-age scores across all eight sites of the MAL-ED cohort study[24] were utilized in this study for stool analysis. Stool samples were collected by field workers (without fixatives) on a monthly basis from birth to 24 months of age. Children were followed twice weekly for active surveillance for diarrheal disease and illness. Prior to stool testing all samples were stored at −70°C. Analysis for fecal markers was done on a monthly basis until 12 months of age and then at 15, 18, 21 and 24 months. Stool samples were analyzed in parallel for MPO (Alpco, Salem, New Hampshire), NEO (GenWay Biotech, San Diego, California), and AAT (Biovendor, Chandler, North Carolina) as previously described.[5] In summary, MPO and NEO were chosen as markers of gut inflammation to represent immune activation and AAT was chosen as a marker of intestinal permeability and mucosal protein wasting secondary to EE. All fecal markers are considered stable in stool specimens and resistant to degradation in the intestinal lumen.

Urine collection and dual sugar test

There is no gold standard to characterize the EE condition, however, the urinary lactulose to mannitol (L:M) dual sugar test has been used most

widely to asses intestinal barrier function and identify altered permeability (lactulose) and malabsorption (mannitol). Despite its wide use, few standards exist across studies and laboratories for administration of sugar dosages, urine collection times, assessment of analyte concentrations, or the interpretation of results.[32] In addition, administration of the five-hour "bagged" urine collections are burdensome for both families and field workers.[33] Despite these logistical and technical challenges, the dual sugar permeability test was administered under the MAL-ED protocol to each infant at 3, 6, 9, and 15 months of age.[5, 32] Urine aliquots were stored at −70°C prior to measurement of lactulose and mannitol concentrations by LC-MS/MS. The disaccharide solution was administered after stool sampling was complete to avoid inaccurate protein measurements in stool due to dilution from the watery stool caused by the L:M test.

Serum collection and plasma markers

Both known and potential biomarkers of EE are also found in serum such as alpha-1-acid glycoprotein (AGP)[34] and citrulline[6], however, collection of serum in field-based studies can be logistically challenging and limits the frequency of longitudinal samples that can be collected per child. Therefore, serum was collected and analyzed for the AGP, and citrulline markers at 7,

15, and 24 months of age. The AGP marker was included because it is often used in population based studies[34] to monitor inflammation during an infection[35] and was expected to be higher with more EE.[36] Plasma levels of AGP were determined by radial immunodiffusion.[5] Serum citrulline concentrations are potential biomarkers for overall mucosal function[37] and it is reduced in villus atrophy syndrome which has decreased epithelial cell surface area.[38]

Data Analysis

Longitudinal analyses were conducted on the entire sample enrolled up to 24 months of age. The primary outcomes of fecal, urine and plasma markers were each log-transformed for normality. Fecal markers were averaged over three month time periods (6, 12, 18 and 24 months of age) to reduce the variability from individual measurements. The means of each fecal marker were age-matched to the socio-economic survey data with water, sanitation, hygiene and household independent variables. For example, if fecal markers were averaged over months 6, 7 and 8 they were then matched to the independent variables collected from the survey administered at 6 months of age for that child. The urine and serum markers were similarly age matched to the independent variables. The relationships between WASH variables

and the EE markers was explored using mixed-effects linear regression, with a random effect specified at the child level to account for within-child correlations. The final multivariate mixed-effects models were adjusted for age, season, breastfeeding, maternal education, and wealth index. An available-data analysis was used and missing data were considered missing at random. Therefore the likelihood based modeling approach with correct specification of mean and covariance model was deemed appropriate. To assess model fit the intraclass correlation (ICC) was used to determine if there was greater variability within than between individuals. In the case of a low ICC (less than 0.10) a multivariate regression model was run to determine the R-squared and adjusted R-squared values of each model to assess the model fit. Data analyses were performed in Stata version 12.1 (College Station, TX).

Ethics Statement

All data presented in this analysis was collected as part of the Peru site MAL-ED cohort, and was approved by institutional review boards from Johns Hopkins Bloomberg School of Public Health (Baltimore, MD) and Asociación Benéfica Proyectos de Informática, Salud, Medicina, y Agricultura (A.B. PRISMA), Lima, Peru.

Results

Community profile

A total of 303 children were enrolled from the catchment area and 270 children remained in the study remained in the study to be surveyed for the 6-month baseline survey with WASH household characteristics. Between each six-month sampling period less than ten percent of the sample were lost to follow up until the children were 24 months of age. Either the 2010 or 2012 community census that was administered closest to the child's birth date was used to represent the water storage variables for a total of 258 children in the cohort. The median number of household members was 6.6 (95% CI: 6.0, 6.6) with 28.5 percent of the population had lived in their current house for less than one year (Table 1). The time-varying WASH variables that reported at least one change over the 24 months study were: i) type of sanitation facility used by the household (63.9 percent of population), ii) shared sanitation facility (46.4 percent of population), drinking water source option (55.5 percent of the population), household use of chlorine to treat their water (37.7 percent of the population), the continuity of the piped water supply (39.8 percent of the population), the

main floor type of the household (34. 2 percent of the population), household hygiene score (60.5 percent of the population); and the household location of the cooking activities (51.9 percent of the population).

The fecal marker analyte results from asymptomatic stool samples collected at the 6, 12, 18 and 24 month time points and averaged with the subsequent two months resulted in 889 observations for MPO, 892 observations for NEO and 877 observations for AAT. The median concentration for MPO, NEO and AAT all decreased across the 6, 12, 18 and 24 month time points as shown in Table S1.

Associations between sanitation variables and EE markers

The community had three main categories for the primary sanitation variable: a pour flush toilet in or near the house that flushes to a septic tank onsite (14.8 percent, n=40), no access to a sanitation facility and instead utilized the bush, field or bucket toilet (15.2 percent, n=41) and pit latrines located outside the home (58.2 percent, n=157) (Table 1). The pour flush toilet was considered to be the most hygienic option by definition of the Joint Monitoring Program that classifies a flush or pour flush toilet to a septic tank as improved.[39] The households that had either unimproved option

of no facility or a pit latrine when compared to the flush toilet both had lower markers for EE as indicated by MPO (-0.34 log, 95% CI: -0.61, -0.08) and NEO (-0.21 log, 95% CI : -0.42, 0.00) (Table 2). Meanwhile for the serum EE markers there was higher EE for households that had no facility versus those with a flush toilet to a septic (0.26 log, 95% CI : 0.09, 0.43) (Table 2). Sharing toilet facilities where a household reported two or more families using the same toilet or latrine was reported in 26.3 percent of the population (n=71) at the 6-month baseline survey. If families shared their sanitation facilities, there was an average of 2.1 families using the same toilet or latrine. For households that shared sanitation facilities compared to those that did not share, the only EE marker for which a significant association was reported was for MPO which had 0.16 higher log MPO (95% CI: 0.00, 0.33) (Table 2). In the fully adjusted multivariate regression models the relationship remained significant was for households with no sanitation facility compared to those with a pour flush toilet for MPO and the effect size increased to (-0.43 log, 95% CI: -0.74, -0.13) (Table 3).

Associations between water variables and EE markers

The main drinking water source for households in the study community was a tube well or borehole and in the 6-month survey this represented 41.5

percent (n=112) of the population (Table 1). The second most prominent type of drinking water source was a piped water connection to the household for 25.2 percent (n=68) (Table 1). A household water connection was also considered the most hygienic option in this study as these households were less likely to store water and therefore there was a lower recontamination risk. For households with a piped connection into their yard or plot, there was 0.29 log (95% CI: 0.07, 0.52) and 0.28 log (95% CI: 0.09, 0.46) higher MPO and NEO respectively, when compared to homes with household piped connections (Table 2). Similarly, for households with tube wells or boreholes as their drinking water source there was was 0.21 log (95% CI: 0.01, 0.40) and 0.16 log (95% CI: 0.005, 0.31)) higher MPO and NEO respectively, when compared to homes with household piped connections (Table 2). And lastly, for households that used a public tap or stand pipe, they had higher L:M test ratios when compared to homes with household connections (0.38 log, 95% CI: -0.002, 0.77). These relationships remained significant in the fully adjusted multivariate regression models, and their effect sizes increased, except for homes with tubewells or boreholes and MPO that was no longer significant (Table 3). An intermittent water connection was common in the community with 87.0 percent of the population reporting interruptions at the 6-month survey (Table 1). Those

that had intermittent connections had higher EE fecal markers with 0.37 log (95% CI:0.16, 0.57) higher MPO and 0.23 log (95% CI: 0.03, 0.43) higher AAT (Table 2). In the fully adjusted multivariate regression models the relationship remained significant for MPO with 0.36 log (95% CI: 0.08, 0.63) higher for those with an intermittent connection versus those that had a continuous supply (Table 3). The mean total volume of water stored per capita by household was reported to be 16.5 liters (standard deviation = 15.1 liters) (Table 1b). In the fully adjusted models, there was statistical significance for fecal EE markers where for those that stored greater volumes of water there was lower MPO (-0.33 log (95% CI: -0.58, -0.08) for the third quartile and -0.26 log (95% CI: -0.52, -0.005) for the fourth quartile) and lower NEO (-0.21 log (95% CI: -0.41, -0.01) for the second quartile and -0.26 (95% CI: -0.46, -0.07) for the third quartile) when compared to the quartile with the lowest amount of water stored (Table 3). Household treatment of water with chlorine was reported in 14.1 percent of the population (Table 1), however, there was no statistical significance with the EE markers in either the unadjusted or adjusted models (Tables 2 and 3).

Associations between hygiene variables and EE markers

The majority of the population (66.7 percent) reported a hygiene score that indicated they always practiced all hygienic behaviors at the 6-month baseline survey (Table 1). When comparing to those in the population that always practiced hygienic behaviors, higher EE serum markers were reported for those that practiced for most of the time (0.15 log (95% CI: 0.01, 0.28) for AGP and 0.11 log (95% CI: 0.001, 0.21) for citrulline (Table 2). These relationships were no longer significant in the fully adjusted models (Table 3).

Associations between household variables and EE markers

Households in the study community had two main floor types with dirt (73.0 percent) or cement (21.5 percent) and a small percentage of households with wood floors (5.6 percent) at the baseline 6 month survey (Table 1). In the unadjusted analyses, dirt floors had lower fecal and urine EE markers when compared to households with cement floors however, none were statistically significant (Table 2). In the fully adjusted model, those with dirt floors had a lower L:M test ratio (-0.35 log (95% CI: -0.61, -0.09) when compared to cement floors (Table 3). The location of cooking activities in the households varied by 72.5 percent inside the house and 25.3 percent outside the house at the 6-month baseline survey (Table 1). For households with cooking

activities performed outside the house there was lower fecal EE markers for both MPO and AAT (-0.20 log (95% CI: -0.37, -0.02) and -0.30 log (95% CI: (-0.46, -0.13) respectively) (Table 2). The relationship for cooking activities outside with AAT remained statistically significant and increased effect size in the fully adjusted model (-0.40 log, 95% CI: -0.60, -0.21) (Table 3).

Discussion

To our knowledge this is the first prospective longitudinal study to show significant associations with household WASH conditions and development of EE in a birth cohort for the first 24 months of life. After adjusting for potentially confounding covariates, the hypothesized water pathway showed higher EE for less protected drinking water sources (+0.32 log MPO and +0.28 log NEO for water piped to a yard or plot, and +0.20 log NEO for water from a tube well compared to a household piped water connection), lower EE as the water quantity stored per capita increased (-0.33 log MPO for third quartile, -0.26 log MPO for fourth quartile, -0.21 log NEO for second quartile, and -0.26 log NEO compared to the first quartile of amount of water stored), and higher EE for households that had a water supply that experienced interruptions (+0.36 log MPO). The hypothesized sanitation

pathway also showed lower EE for households that did not have access to a toilet facility and therefore defecated in places thought to be a greater distance from their household living environments (-0.43 log MPO).

Even among a relatively contained community with many shared infrastructure characteristics we were able to find a difference in the gut health of children from homes that used different types of toilet facilities and drinking water infrastructure. The longitudinal study design was able to capture the changes in the facilities used by households and account for these changes over time in relation to the development of EE over the first 24 months of life. Interestingly, the overall environmental contamination caused by open defecation did not nullify the differences between households even though households in the study community were in relative proximity to one another at the village level. This observation supports prior conclusions[20] that the household level is the appropriate unit of intervention for water and sanitation infrastructure.

The finding that the pour flush toilet sanitation option, which meets the definition for improved sanitation by the JMP was associated with higher fecal markers for EE compared to the unimproved option of no facility, is

important for this study setting. We hypothesize this finding is attributable to the common occurrence of fecal matter overflowing from the toilet storage pit and contaminating the surrounding household environment.[40] The pour flush toilets were typically located in close proximity to the households and may have created a greater risk for children to be exposed to fecal pathogens when the facilities overflowed as compared to homes where people defecated further away out in the open.[41]

This study found evidence for increased EE fecal marker of MPO for households that sometimes had interruptions in their water supply. Interruptions in water supply may force a household to use a less protected water sources or may contaminate the piped water supply from a loss of pressure and allow environmental waters to enter the pipes, which are often contaminated where sanitary improvements are lacking[42]. This finding supports the assertion that improved drinking water sources will not make meaningful contributions to public health if these systems are subject to poor reliability.[43] A low availability of water stored in liters per capita was recorded in the households of the study community with an average of 16.5 liters per capita. This is far below the recommended quantity of 50 liters per person per day to meet basic health needs for drinking, cooking and

hygiene.[44] This study found an inverse relationship with amount of water stored and fecal EE markers of MPO and NEO suggesting that a greater quantity of water available in the home improves the gut health risks associated with poor hygiene.

Households that performed their cooking activities outside had lower occurrence of EE as indicated by AAT, the marker for nutrient wasting. This highly significant finding showed the potential protective effect that solar inactivation may have for open air kitchens on child gut health. This may be explained by the greater risk of exposure to fecal pathogens in the household when cooking and water activities are performed in an enclosed, dark and humid environment where the majority of the floors are dirt and difficult to disinfect. In contrast, homes that cook outside may harbor fewer pathogens given solar inactivation[45] or wash out of fecal contamination following rain events.

This study was the first to compare associations between the WASH characteristics across five markers for intestinal inflammation, permeability, and nutrient absorptive capacity as stand alone determinants for the progression toward EE. The fecal markers of MPO and NEO showed the

greatest potential as biomarkers for the condition and had the strongest associations for both the water and sanitation variables with 18-27% of the variability in the fecal markers explained by the WASH determinants. The L:M urine marker would be considered a secondary candidate for determining EE under this analysis. There is a need to assess the utility of potential biomarkers for EE within communities of varying levels of WASH characteristics since a clear gold standard is not yet defined. This analysis provides more support to continue using MPO and NEO in exploratory WASH studies.

This study has several important limitations. First, the characterization WASH variables that were tailored to the local context only occurred at the baseline of the study from the community census. Given the importance of water storage in the community, it would have been preferred to align the community census variables for water storage with the longitudinal data collection of the MAL-ED socio-economic survey variables and the collection of the EE markers. Second, information on fecal matter storage and treatment was not available. The main sanitation variable used in this analysis was type of toilet facility and often this does not guarantee safe and sanitary removal of fecal matter from the household environment. Lastly, the

heterogeneity of the floor type variable was not captured by the socio-economic survey. The homes in this community that have cement floors mostly have cement in the entrance area while at the back of the house, where the cooking and washing activities take place, is often dirt. This is a potential source of information bias in the analysis for the relationship of floor type to the fecal markers for EE.

The strengths of the study are the longitudinal design with monthly measurements for the EE fecal markers from birth to closely track the trajectory of these markers. The questionnaire that was administered every six months also provided a close monitoring of the WASH characteristics of the home to capture any changes that the families may have had in these dynamically changing low-income households. The community census also provided detailed information on water storage which was widely practiced throughout the community. These strengths combined reduced potential biases from measurement error to understand the relationships between the WASH characteristics of the household and the development of EE in this cohort.

Conclusion

Water and sanitation conditions were associated with fecal markers for EE in this peri-urban community of Iquitos, Peru. The results provide preliminary evidence for the hypothesis that children under 24 months of age living in unsanitary conditions will have elevated levels of fecal markers for gut inflammation and permeability. Future studies are needed to examine the usefulness of these fecal markers in diverse settings where there is in-depth understanding of the water, sanitation, hygiene and household characteristics leading to increased contamination and exposure to fecal pathogens.

Table 1a. Water, sanitation, hygiene and household socio-economic characteristics of children enrolled in MAL-ED Peru site at 6, 12, 18 and 24 months of age.

WASH Household Characteristic	6 months (n) N=270	12 months (n) N= 241	18 months (n) N=213	24 months (n) N=198
Type of toilet facility that households usually use (%):				
Pour flush to septic tank	14.8 (40)	12.0 (29)	12.7 (27)	22.7 (45)
No facility/bush/field or bucket toilet	15.2 (41)	16.6 (40)	18.3 (39)	12.6 (25)
Pit latrine without flush	58.2 (157)	58.9 (142)	56.8 (121)	51.5 (102)
Flush to piped sewer system	1.8 (5)	3.7 (9)	5.2 (11)	8.1 (16)
Flush to pit latrine	1.1 (3)	2.1 (5)	1.4 (3)	2.5 (5)
Flush to somewhere else	6.3 (17)	4.2 (10)	3.3 (7)	1.5 (3)
Type of flooring material (%):				
Cement	21.5 (58)	22.8 (55)	23.6 (50)	27.3 (54)
Dirt	73.0 (197)	69.7 (168)	68.9 (146)	67.7 (134)
Wood	5.6 (15)	7.1 (17)	7.6 (16)	5.1 (10)
Tile	--	0.4 (1)	--	--
Drinking water source (%):				
Piped into dwelling	25.2 (68)	21.6 (52)	22.1 (47)	23.2 (46)
Piped into yard/plot	19.3 (52)	17.8 (43)	17.8 (38)	16.7 (33)
Public tap/stand pipe	4.8 (13)	8.3 (20)	3.3 (7)	1.5 (3)
Tube well or borehole	41.5 (112)	41.1 (99)	42.7 (91)	43.4 (86)
Protected well	1.1 (3)	0.8 (2)	1.9 (4)	3.5 (7)
Unprotected well	3.0 (8)	4.6 (11)	4.7 (10)	6.6 (13)
Surface water	1.1 (3)	2.1 (5)	0.5 (1)	0.5 (1)
Total volume of stored water in the HH per capita in liters (reported) (Mean, 95% CI)	16.9 (14.9, 18.9) (n=237)	17.3 (15.1, 19.4) (n=215)	17.4 (15.2, 19.5) (n=188)	17.2 (14.9, 19.5) (n=175)
HH uses chlorine to treat their water (%):				
No	85.9 (232)	89.2 (215)	88.3 (188)	82.3 (163)
Yes	14.1 (38)	10.8 (26)	11.7 (25)	17.7 (35)
Continuity of piped water supply (%):				

Continuous	13.0 (35)	17.4 (42)	12.2 (26)	12.6 (25)
Sometimes interrupted	87.0 (235)	82.6 (199)	87.8 (187)	87.4 (173)
Toilet facility is shared:				
No	73.7 (199)	74.7 (180)	79.8 (170)	76.3 (151)
Yes	26.3 (71)	25.3 (61)	20.2 (43)	23.7 (47)
Number of members per household (Mean, 95% CI)	6.3 (6.0, 6.6) (n=270)	5.9 (5.7, 6.2) (n=236)	5.8 (5.5, 6.1) (n=213)	5.7 (5.4, 6.0) (n=198)
Household location of cooking activities:				
Inside the house	72.5 (195)	73.0 (176)	80.6 (170)	73.7 (146)
Outside the house	25.3 (68)	25.3 (61)	18.5 (39)	20.7 (41)
Both inside and outside the house	2.2 (6)	1.7 (4)	1.0 (2)	5.6 (11)
Hygiene Score:				
4	66.7 (180)	68.9 (166)	62.3 (132)	63.6 (126)
3	14.8 (40)	14.1 (34)	14.2 (30)	17.7 (35)
0-2	18.5 (50)	17.0 (41)	23.6 (50)	18.7 (37)
Wealth Index [3-18] (Mean, 95% CI):	9.5 (9.2, 9.8)	9.4 (9.1, 9.8)	9.3 (9.0, 9.7)	9.9 (9.5, 10.3)
Duration of time family has lived in home (%):				
Less than one year	28.5 (77)	20.8 (50)	20.7 (44)	20.3 (40)
Between one year and five years	33.0 (89)	41.9 (101)	36.2 (77)	42.1 (83)
Between five years and ten years	16.7 (45)	19.5 (47)	24.9 (53)	18.8 (37)
Between ten years and twenty years	12.2 (33)	8.3 (20)	8.9 (19)	13.7 (27)
More than twenty years	9.6 (26)	9.5 (23)	9.4 (20)	5.1 (10)
Maternal education in years (Mean, 95% CI):	7.8 (7.5, 8.2) (n=268)	7.8 (7.5, 8.2) (n=237)	7.9 (7.5, 8.3) (n=211)	7.6 (7.3 8.0) (n=197)
Breastfeeding:				
Mixed	98.6 (216)	96.8 (209)	58.7 (122)	19.0 (37)
Weaned	0.9 (2)	3.2 (7)	41.4 (86)	81.0 (158)

Table 1b. Water storage variables from community census with children enrolled in MAL-ED Peru site.

Water Storage Variable	N	Mean	SD
Total volume of stored water in the HH (L) Option 1 (reported)	258	99.7	92.9
Total volume of stored water in the HH (L) Option 2 (summation by lid type)	257	94.6	90.7
No lid: Total volume of stored water in the HH (L)	257	50.3	72.3
Provisional lid: Total volume of stored water in the HH (L)	257	7.6	47.1
Secured lid: Total volume of stored water in the HH (L)	257	36.7	29.5
Total volume of stored water in the HH per capita Option 1 (reported)	258	16.5	15.1
Total volume of stored water in the HH per capita Option 2 (summation by lid type)	257	15.7	14.6
No lid: Total volume of stored water in the HH per capita	257	8.3	12.1
Provisional lid: Total volume of stored water in the HH per capita	257	1.1	6.7
Secured lid: Total volume of stored water in the HH per capita	257	6.1	4.8
Percent of water stored with no lid	256	37.2	35.2
Percent of water stored with provisional lid	256	5.6	25.2

Percent of water stored with secured lid	256	54.9	36.4
Minimum volume of container used for water storage (L) Option 1 (reported directly)	204	12.2	8.0
Minimum volume of container used for water storage (L) Option 2 (extracted from data)	257	12.4	7.4
Minimum volume of container used for water storage per capita (L) Option 1 (reported directly)	204	2.3	1.9
Minimum volume of container used for water storage per capita (L) Option 2 (extracted from data)	257	2.2	1.8

Table 2. Unadjusted mixed models for WASH household characteristics with EE biomarkers (Part 1)

WASH Household Characteristic	MPO β (95% CI) N=269, n=884	NEO β (95% CI) N= 269, n=887	AAT β (95% CI) N=268 n=871
SANITATION			
Type of toilet facility that households usually use:			
Pour flush toilet to septic tank	REF	REF	REF
No facility/bush/field or bucket toilet	**-0.34 (-0.61, -0.08)****	-0.07 (-0.28, 0.14)	-0.07 (-0.32, 0.19)
Pit latrine without flush	**-0.21 (-0.42, 0.00)***	-0.06 (-0.23, 0.11)	0.09 (-0.12, 0.29)
Flush toilet to piped sewer system	-0.33 (-0.72, 0.06)	0.05 (-0.26, 0.36)	-0.17 (-0.56, 0.21)
Flush toilet to pit latrine	-0.09 (-0.65, 0.47)	0.05 (-0.39, 0.50)	0.19 (-0.39, 0.76)
Flush toilet to somewhere else	-0.29 (-0.68, 0.11)	-0.03 (-0.35, 0.28)	0.28 (-0.11, 0.68)
Toilet facility is shared:			
No	REF	REF	REF
Yes	**0.16 (0.00, 0.33)***	0.05 (-0.09, 0.18)	0.13 (-0.03, 0.30)
WATER			
Drinking water source:			
Piped into dwelling	REF	REF	REF
Piped into yard/plot	**0.29 (0.07, 0.52)***	**0.28 (0.09, 0.46)****	0.01 (-0.22, 0.24)
Public tap/stand pipe	0.16 (-0.19, 0.51)	0.01 (-0.27, 0.29)	0.07 (-0.28, 0.42)
Tube well or borehole	**0.21 (0.01, 0.40)***	**0.16 (0.005, 0.31)***	0.06 (-0.13, 0.25)
Protected well	0.04 (-0.54, 0.62)	-0.18 (-0.65, 0.28)	-0.26 (-0.83, 0.31)
Unprotected well	**0.64 (0.27, 1.02)†**	**0.32 (0.03, 0.62)***	0.05 (-0.31, 0.41)
Surface water	-0.05 (-0.78, 0.67)	-0.08 (-0.65, 0.50)	-0.20 (-0.89, 0.49)
Total volume of stored water in the HH per capita (reported)			
Q1	REF	REF	REF
Q2	-0.006 (-0.23, 0.22)	-0.08 (-0.26, 0.10)	-0.04 (-0.26, 0.19)
Q3	-0.21 (-0.44, 0.02)	-0.14 (-0.33, 0.04)	-0.02 (-0.24, 0.21)
Q4	-0.08 (-0.30, 0.15)	0.05 (-0.13, 0.23)	0.11 (-0.11, 0.34)
HH uses chlorine to treat their water:			
No	REF	REF	REF

Yes	-0.10 (-0.31, 0.11)	0.02 (-0.15, 0.19)	-0.04 (-0.25, 0.17)
Continuity of piped water supply:			
Continuous	REF	REF	REF
Sometimes interrupted	**0.37 (0.16, 0.57)†**	0.12 (-0.04, 0.28)	**0.23 (0.03, 0.43)***
HYGIENE			
Hygiene Score:			
Always	REF	REF	REF
Most of the time	-0.04 (-0.24, 0.17)	-0.10 (-0.26, 0.06)	0.08 (-0.12, 0.28)
Sometimes	0.08 (-0.11, 0.27)	-0.06 (-0.21, 0.09)	-0.04 (-0.23, 0.14)
HOUSEHOLD			
Type of flooring material:			
Cement	REF	REF	REF
Dirt	-0.07 (-0.25, 0.11)	-0.09 (-0.24, 0.05)	-0.15 (-0.32, 0.03)
Wood	-0.07 (-0.41, 0.27)	**-0.28 (-0.55, -0.01)***	**-0.38 (-0.71, -0.06)***
Number of household members in quartiles	-0.02 (-0.06, 0.008)	-0.01 (-0.04, 0.01)	-0.02 (-0.05, 0.01)
Household location of cooking activities:			
Inside the house	REF	REF	REF
Outside the house	**-0.20 (-0.37, -0.02)***	-0.09 (-0.23, 0.05)	**-0.30 (-0.46, -0.13)†**
Both inside and outside the house	-0.25 (-0.73, 0.23)	0.25 (-0.13, 0.63)	**0.44 (-0.001, 0.88)***
Wealth Index:			
Q1	REF	REF	REF
Q2	-0.10 (-0.32, 0.13)	0.01 (-0.16, 0.19)	-0.12 (-0.35, 0.10)
Q3	-0.12 (-0.31, 0.06)	0.02 (-0.12, 0.17)	0.07 (-0.12, 0.25)
Q4	-0.06 (-0.25, 0.13)	--	-0.01 (-0.20, 0.18)
Duration of time family has lived in home:			
Less than one year	REF	REF	REF
Between one year and five years	0.07 (-0.11, 0.26)	-0.005 (-0.16, 0.15)	0.04 (-0.14, 0.23)
Between five years and ten years	0.02 (-0.20, 0.25)	0.03 (-0.15, 0.20)	0.08 (-0.14, 0.30)
Between ten years and twenty years	0.21 (-0.06, 0.48)	0.02 (-0.19, 0.24)	0.15 (-0.11, 0.41)
More than twenty years	-0.20 (-0.49, 0.09)	-0.17 (-0.40, 0.06)	-0.11 (-0.39, 0.17)

Maternal education (y)			
Low	REF	REF	REF
High	**0.15 (0.00, 0.31)***	**0.15 (0.02, 0.27)***	0.13 (-0.02, 0.28)
CHILD			
Child age (months)	**-0.06 (-0.07, -0.05)†**	**-0.07 (-0.08, -0.07)†**	**-0.07 (-0.08, -0.06)†**
Breastfeeding:			
Mixed	REF	REF	REF
Weaned	**-0.27 (-0.49, -0.04)***	**-0.43 (-0.61, -0.26)†**	**-0.55 (-0.77, -0.33)†**

* Significance at the p<0.05 level
** Significance at the p<0.01 level
† Significant difference at the p<0.001 level

Table 2. Unadjusted mixed models for WASH household characteristics with EE biomarkers (Part 2)

WASH Household Characteristic	LM β (95% CI) N=270, n=732	AGP β (95% CI) N=236, n=439	CIT β (95% CI) N=253, n=550
SANITATION			
Type of toilet facility that households usually use:			
Pour flush toilet to septic tank	REF	REF	REF
No facility/bush/field or bucket toilet	-0.17 (-0.48, 0.15)	**0.26 (0.09, 0.43)****	-0.03 (-0.17, 0.10)
Pit latrine without flush	0.01 (-0.24, 0.26)	0.02 (-0.12, 0.16)	0.03 (-0.08, 0.14)
Flush toilet to piped sewer system	-0.08 (-0.64, 0.48)	--	-0.06 (-0.25, 0.13)
Flush toilet to pit latrine	-0.62 (-1.36, 0.11)	-0.07 (-0.53, 0.39)	-0.25 (-0.52, 0.02)
Flush toilet to somewhere else	-0.24 (-0.66, 0.18)	0.20 (-0.05, 0.45)	-0.007 (-0.23, 0.21)
Toilet facility is shared:			
No	REF	REF	REF
Yes	0.02 (-0.18, 0.21)	-0.07 (-0.18, 0.05)	-0.03 (-0.12, 0.06)
WATER			
Drinking water source:			
Piped into dwelling	REF	REF	REF
Piped into yard/plot	-0.07 (-0.33, 0.19)	-0.04 (-0.19, 0.11)	0.11 (-0.008, 0.23)
Public tap/stand pipe	**0.38 (-0.002, 0.77)***	0.03 (-0.20, 0.26)	0.06 (-0.12, 0.24)
Tube well or borehole	0.03 (-0.19, 0.25)	-0.01 (-0.13, 0.110	0.10 (-0.005, 0.20)
Protected well	-0.25 (-1.18, 0.68)	0.09 (-0.31, 0.48)	0.08 (-0.20, 0.35)
Unprotected well	0.26 (-0.25, 0.76)	0.02 (-0.26, 0.29)	0.11 (-0.08, 0.310
Surface water	-0.31 (-1.05, 0.43)	0.38 (-0.09, 0.85)	-0.26 (-0.69, 0.16)
Total volume of stored water in the HH per capita (reported)			
Q1	REF	REF	REF
Q2	-0.03 (-0.30, 0.24)	-0.04 (-0.19, 0.11)	-0.07 (-0.20, 0.05)
Q3	-0.15 (-0.41, 0.11)	-0.13 (-0.27, 0.02)	-0.07 (-0.19, 0.05)
Q4	-0.09 (-0.35, 0.17)	-0.12 (-0.26, 0.02)	-0.02 (-0.14, 0.10)
HH uses chlorine to treat their water:			
No	REF	REF	REF

Yes	-0.14 (-0.40, 0.11)	0.0003 (-0.15, 0.15)	0.0009 (-0.11, 0.11)
Continuity of piped water supply:			
Continuous	REF	REF	REF
Sometimes interrupted	-0.16 (-0.40, 0.08)	0.03 (-0.10, 0.17)	0.03 (-0.08, 0.13)
HYGIENE			
Hygiene Score:			
Always	REF	REF	REF
Most of the time	-0.01 (-0.25, 0.23)	**0.15 (0.01, 0.28)***	**0.11 (0.001, 0.21)***
Sometimes	0.21 (-0.02, 0.43)	-0.05 (-0.18, 0.09)	0.09 (-0.01, 0.20)
HOUSEHOLD			
Type of flooring material:			
Cement	REF	REF	REF
Dirt	-0.04 (-0.25, 0.17)	0.02 (-0.09, 0.14)	0.006 (-0.09, 0.10)
Wood	-0.04 (-0.45, 0.37)	0.18 (-0.03, 0.40)	0.06 (-0.12, 0.24)
Number of household members in quartiles	0.02 (-0.01, 0.06)	-0.0008 (-0.02, 0.02)	-0.005 (-0.02, 0.01)
Household location of cooking activities:			
Inside the house	REF	REF	REF
Outside the house	0.04 (-0.16, 0.23)	0.05 (-0.07, 0.16)	0.04 (-0.05, 0.13)
Both inside and outside the house	**0.60 (0.01, 1.19)***	0.05 (-0.23, 0.34)	0.05 (-0.17, 0.28)
Wealth Index:			
Q1	REF	REF	REF
Q2	-0.12 (-0.35, 0.11)	-0.11 (-0.27, 0.05)	0.09 (-0.03, 0.21)
Q3	-0.20 (-0.44, 0.04)	-0.06 (-0.19, 0.06)	0.03 (-0.06, 0.13)
Q4	**-0.37 (-0.61, -0.12)****	-0.11 (-0.24, 0.02)	0.07 (-0.04, 0.17)
Duration of time family has lived in home:			
Less than one year	REF	REF	REF
Between one year and five years	-0.004 (-0.22, 0.21)	-0.05 (-0.17, 0.08)	**-0.10 (-0.20, 0.00)***
Between five years and ten years	-0.05 (-0.31, 0.22)	-0.07 (-0.22, 0.07)	0.03 (-0.09, 0.15)
Between ten years and twenty years	-0.16 (-0.47, 0.14)	-0.04 (-0.21, 0.13)	-0.02 (-0.16, 0.12)
More than twenty years	-0.05 (-0.37, 0.27)	--	-0.09 (-0.25, 0.06)

Maternal education (y)			
Low	REF	REF	REF
High	0.02 (-0.16, 0.19)	-0.09 (-0.19, 0.02)	-0.04 (-0.12, 0.04)
CHILD			
Child age (months)	**0.04 (0.02, 0.06)†**	-0.007 (-0.02, 0.0008)	**0.06 (0.05, 0.06)†**
Breastfeeding:			
Mixed	REF	REF	REF
Weaned	0.36 (-0.39, 1.12)	-0.02 (-0.21, 0.18)	0.08 (-0.05, 0.21)

* Significance at the p<0.05 level
** Significance at the p<0.01 level
† Significant difference at the p<0.001 level

Table 3. Multivariate mixed-effects models for WASH household characteristics and EE biomarkers. All models adjusted for age, season, breastfeeding, maternal education, and wealth index (Part 1).

	MPO (ng/mL)		**NEO** (nmol/L)		**AAT** (mg/g)	
N	703		705		691	
n	194		194		194	
	β (95% CI)	n	β (95% CI)	n	β (95% CI)	n
Type of toilet facility households use:						
Pour flush toilet to septic tank	REF	109	REF	109	REF	113
No facility/bush/field or bucket toilet	**-0.43 (-0.74, -0.13)****	106	-0.06 (-0.30, 0.19)	106	-0.07 (-0.37, 0.23)	105
Pit latrine without flush	-0.18 (-0.42, 0.06)	410	-0.01 (-0.21, 0.18)	412	0.07 (-0.17, 0.31)	401
Flush toilet to piped sewer system	-0.22 (-0.65, 0.22)	29	0.29 (-0.06, 0.65)	29	-0.28 (-0.74, 0.17)	26
Flush toilet to pit latrine	-0.07 (-0.73, 0.59)	11	0.14 (-0.39, 0.68)	11	0.03 (-0.66, 0.72)	10
Flush toilet to somewhere else	-0.39 (-0.83, 0.05)	27	0.04 (-0.32, 0.40)	27	0.19 (-0.27, 0.64)	26
Drinking water source:						
Piped into dwelling	REF	159	REF	159	REF	156
Piped into yard/plot	**0.32 (0.06, 0.57)***	144	**0.28 (0.07, 0.49)****	144	-0.11 (-0.37, 0.15)	140
Public tap/stand pipe	0.40 (-0.02, 0.83)	36	-0.03 (-0.37, 0.32)	36	0.12 (-0.31, 0.56)	35
Tube well or borehole	0.10 (-0.14, 0.33)	296	**0.20 (0.006, 0.39)***	298	-0.08 (-0.31, 0.16)	292
Protected well	0.19 (-0.43, 0.81)	12	-0.18 (-0.68, 0.33)	12	-0.31 (-0.97, 0.35)	11
Unprotected well	**0.47 (0.05, 0.88)***	34	**0.36 (0.03, 0.70)***	34	-0.16 (-0.58, 0.25)	36
Surface water	0.72 (-0.30, 1.74)	4	0.60 (-0.24, 1.44)	4	-0.30 (-1.35, 0.74)	4
Total volume of stored water in the HH[3]		182		182		174
Q1	REF	168	REF	169	REF	170
Q2	-0.18 (-0.43, 0.08)	169	**-0.21 (-0.41, -0.01)***	170	-0.14 (-0.39, 0.10)	165
Q3	**-0.33 (-0.58, -0.08)****	184	**-0.26 (-0.46, -0.07)****	184	-0.13 (-0.37, 0.12)	182
Q4	**-0.26 (-0.52, -0.005)***		-0.13 (-0.33, 0.08)		-0.11 (-0.36, 0.15)	
HH uses chlorine to treat their water:	REF		REF		REF	
No	-0.18 (-0.43, 0.08)	618	0.06 (-0.14, 0.26)	620	0.03 (-0.22, 0.28)	606

[3] Liters of water stored per capita reported directly by the interviewee

Yes		85		85		85
Continuity of piped water supply:						
Continuous	REF	97	REF	97	REF	94
Sometimes interrupted	**0.36 (0.08, 0.63)****	606	-0.006 (-0.23, 0.22)	608	0.20 (-0.08, 0.48)	597
Practices good hygiene composite score						
Always	REF	460	REF	461	REF	453
Most of the time	-0.04 (-0.27, 0.19)	104	-0.08 (-0.26, 0.11)	104	0.20 (-0.03, 0.44)	100
Sometimes	0.11 (-0.11, 0.33)	139	0.01 (-0.17, 0.20)	140	-0.05 (-0.28, 0.18)	138
Type of flooring material:						
Cement	REF	166	REF	167	REF	166
Dirt	-0.10 (-0.31, 0.12)	510	-0.08 (-0.26, 0.09)	511	-0.20 (-0.42, 0.02)	495
Wood	-0.03 (-0.47, 0.42)	27	-0.23 (-0.59, 0.13)	27	-0.30 (-0.73,0.13)	30
Household location of cooking activities:						
Inside the house	REF	529	REF	531	REF	518
Outside the house	-0.16 (-0.35, 0.03)	159	-0.09 (-0.24, 0.07)	159	**-0.40 (-0.60, -0.21)†**	156
Both inside and outside the house	-0.42 (-0.96, 0.12)	15	-0.09 (-0.53, 0.35)	15	0.30 (-0.21, 0.82)	17
Wealth Index						
Q1	REF	274	REF	275	REF	265
Q2	-0.24 (-0.49, 0.01)	88	0.02 (-0.19, 0.22)	88	-0.17 (-0.43, 0.09)	85
Q3	**-0.26 (-0.47, -0.04)***	174	-0.04 (-0.22, 0.13)	174	-0.07 (-0.29, 0.15)	172
Q4	-0.13 (-0.37, 0.12)	167	0.001 (-0.19, 0.20)	168	-0.17 (-0.41, 0.08)	169
Maternal education (y)						
Low	REF	395	REF	396	REF	386
High	**0.20 (0.02, 0.39)***	308	0.13 (-0.02, 0.28)	309	0.11 (-0.07, 0.29)	305
Child age (months)	**-0.06 (-0.08, -0.05)†**	703	**-0.06 (-0.07, -0.05)†**	705	**-0.04 (-0.06, -0.03)†**	691
Breastfeeding						
Mixed	REF	501	REF	502	REF	486
Weaned	-0.09 (-0.33, 0.14)	201	**-0.42 (-0.61, -0.23)†**	202	**-0.50 (-0.74, -0.26)†**	204
Seasonal effect						
Sine	**-0.24 (-0.35, -0.14)†**	703	0.06 (-0.03, 0.14)	705	0.02 (-0.08, 0.13)	691

Cosine	**0.11 (0.004, 0.21)***	703	0.02 (-0.07, 0.10)	705	-0.01 (-0.12, 0.09)	691

* Significance at the p<0.05 level
** Significance at the p<0.01 level
† Significant difference at the p<0.001 level

Table 3. Multivariate mixed-effects models for WASH household characteristics and EE biomarkers. All models adjusted for age, season, breastfeeding, maternal education, and wealth index (Part 2).

	LM		**AGP**		**CIT** (umol/pL)	
N	565		344		440	
n	194		175		192	
	β (95% CI)	n	β (95% CI)	n	β (95% CI)	n
Type of toilet facility households use:						
Pour flush toilet to septic tank	REF	82	REF	57	REF	71
No facility/bush/field or bucket toilet	-0.28 (-0.65, 0.09)	85	0.18 (-0.04, 0.37)	53	-0.05 (-0.21, 0.11)	62
Pit latrine without flush	-0.03 (-0.32, 0.27)	340	0.03 (-0.13, 0.19)	203	0.009 (-0.11, 0.14)	254
Flush toilet to piped sewer system	-0.19 (-0.85, 0.47)	12	0.26 (-0.27, 0.78)	4	0.03 (-0.19, 0.25)	21
Flush toilet to pit latrine	-0.39 (-1.27, 0.48)	7	-0.11 (-0.66, 0.44)	4	**-0.42 (-0.74, -0.10)****	9
Flush toilet to somewhere else	-0.39 (-0.86, 0.07)	29	**0.30 (0.02, 0.59)***	17	-0.003 (-0.24, 0.24)	17
Drinking water source:						
Piped into dwelling	REF	134	REF	92	REF	103
Piped into yard/plot	-0.07 (-0.36, 0.22)	128	-0.04 (-0.21, 0.13)	76	0.12 (-0.008, 0.23)	94
Public tap/stand pipe	**0.64 (0.14, 1.14)****	35	0.11 (-0.18, 0.40)	3	0.008 (-0.12, 0.24)	24
Tube well or borehole	-0.09 (-0.36, 0.18)	227	-0.07 (-0.23, 0.09)	133	0.10 (-0.005, 0.20)	177
Protected well	-0.75 (-1.77, 0.26)	5	0.06 (-0.42, 0.54)	5	0.03 (-0.20, 0.35)	9
Unprotected well	-0.11 (-0.65, 0.44)	19	-0.04 (-0.37, 0.28)	12	0.10 (-0.08, 0.310	21
Surface water	-0.51 (-1.43, 0.40)	6	-0.41 (-1.51, 0.68)	1	**-0.73 (-1.36, - 0.11)***	2
Total volume of stored water in the HH[4]	REF	142	REF	87	REF	118
Q1	-0.12 (-0.40, 0.17)	122	-0.06 (-0.22, 0.11)	73	-0.07 (-0.21, 0.06)	98
Q2	-0.26 (-0.54, 0.01)	154	-0.10 (-0.26, 0.06)	93	-0.05 (-0.18, 0.07)	108
Q3	-0.26 (-0.55, 0.04)	147	-0.15 (-0.32, 0.02)	91	-0.006 (-0.14, 0.12)	116
Q4						
HH uses chlorine to treat their water:						
No	REF	501	REF	311	REF	386

[4] Liters of water stored per capita reported directly by the interviewee

Yes	-0.12 (-0.43, 0.19)	64	0.05 (-0.15, 0.25)	33	0.02 (-0.12, 0.15)	54
Continuity of piped water supply:						
Continuous	REF	82	REF	52	REF	65
Sometimes interrupted	0.05 (-0.29, 0.39)	483	0.09 (-0.11, 0.28)	292	-0.04 (-0.18, 0.10)	375
Practices good hygiene composite score						
Always	REF	387	REF	232	REF	292
Most of the time	0.11 (-0.17, 0.39)	78	0.14 (-0.02, 0.30)	54	**0.13 (0.01, 0.25)***	75
Sometimes	0.19 (-0.08, 0.46)	100	-0.05 (-0.22, 0.11)	58	0.07 (-0.06, 0.20)	73
Type of flooring material:						
Cement	REF	125	REF	83	REF	102
Dirt	**-0.35 (-0.61, -0.09)****	419	-0.001 (-0.17, 0.13)	243	0.02 (-0.10, 0.13)	322
Wood	-0.06 (-0.60, 0.48)	21	0.06 (-0.21, 0.33)	18	0.12 (-0.13, 0.37)	16
Household location of cooking activities:						
Inside the house	REF	417	REF	249	REF	318
Outside the house	0.08 (-0.14, 0.31)	134	0.02 (-0.12, 0.16)	84	0.04 (-0.06, 0.14)	109
Both inside and outside the house	**0.72 (0.13, 1.31)***	14	-0.02 (-0.35, 0.31)	11	0.09 (-0.16, 0.34)	13
Wealth Index						
Q1	REF	137	REF	127	REF	167
Q2	-0.09 (-0.35, 0.18)	151	-0.04 (-0.23, 0.15)	40	0.06 (-0.07, 0.20)	56
Q3	**-0.28 (-0.56, 0.00)***	150	-0.03 (-0.18, 0.12)	91	0.04 (-0.07, 0.15)	112
Q4	**-0.47 (-0.78, -0.16)****	112	-0.02 (-0.19, 0.15)	86	0.08 (-0.04, 0.21)	105
Maternal education (y)						
Low	REF	315	REF	197	REF	258
High	0.20 (-0.009, 0.40)	250	-0.04 (-0.16, 0.08)	147	-0.02 (-0.12, 0.07)	182
Child age (months)	**0.04 (0.01, 0.06)†**	565	-0.01 (-0.02, 0.003)	344	**0.05 (0.04, 0.06)†**	440
Breastfeeding						
Mixed	REF	558	REF	296	REF	310
Weaned	-0.14 (-1.11, 0.82)	5	0.002 (-0.23, 0.23)	47	0.09 (-0.05, 0.23)	129
Seasonal effect						
Sine	-0.03 (-0.15, 0.09)	565	0.002 (-0.08, 0.08)	344	-0.02 (-0.07, 0.04)	440

Cosine	-0.09 (-0.21, 0.04)	565	-0.02 (-0.10, 0.05)	344	-0.02 (-0.07, 0.04)	440

* Significance at the p<0.05 level
** Significance at the p<0.01 level
† Significant difference at the p<0.001 level

Table S1. Median concentrations of fecal markers of EE - myeloperoxidase (MPO), neopterin (NEO), alpha-1-antitrypsin (AAT)

	MPO (ng/mL)	NEO (nmol/L)	AAT (mg/g)
6 months	14,363	3,782	0.53
12 months	9,047	2,758	0.44
18 months	6,215	1,623	0.35
24 months	4,002	1,037	0.19

Table S2. Unadjusted analysis for water storage variables in each household with log-transformed EE using mixed-effects models (Part 1).

Risk Factor		MPO β (95% CI) (N=727, n=232)	NEO β (95% CI) (N=727, n=232)	AAT β (95% CI) (N=727, n=232)
Total volume (L) Option 1 (reported)				
	Q1	REF	REF	REF
	Q2	0.07 (-0.24, 0.38)	-0.04 (-0.32, 0.24)	**0.37 (0.04, 0.69)**
	Q3	-0.10 (-0.40, 0.20)	-0.15 (-0.42, 0.11)	0.005 (-0.30, 0.31)
	Q4	-0.02 (-0.32, 0.28)	0.22 (-0.04, 0.49)	**0.37 (0.07, 0.68)**
Total volume (L) Option 2 (summation by lid type)				
	Q1	REF	REF	REF
	Q2	0.06 (-0.26, 0.38)	-0.06 (-0.35, 0.23)	0.29 (-0.04, 0.63)
	Q3	-0.17 (-0.46, 0.13)	-0.16 (-0.42, 0.11)	0.07 (-0.23, 0.37)
	Q4	-0.15 (-0.44, 0.15)	0.20 (-0.07, 0.46)	0.27 (-0.04, 0.57)
No lid: Total volume (L)				
	Q1	REF	REF	REF
	Q2	-0.23 (-0.56, 0.10)	0.03 (-0.27, 0.32)	0.19 (-0.15, 0.53)
	Q3	-0.09 (-0.39, 0.21)	-0.15 (-0.42, 0.12)	-0.01 (-0.32, 0.29)
	Q4	-0.16 (-0.45, 0.12)	-0.002 (-0.26, 0.26)	**0.30 (0.004, 0.59)**
Secured lid: Total volume (L)				
	Q1	REF	REF	REF
	Q2	0.04 (-0.25, 0.34)	0.07 (-0.20, 0.33)	0.27 (-0.03, 0.58)
	Q3	0.10 (-0.21, 0.41)	0.10 (-0.18, 0.38)	**0.35 (0.03, 0.67)**
	Q4	0.36 (0.05, 0.66)	**0.29 (0.01, 0.57)**	0.30 (-0.02, 0.62)
Total volume per capita Option 1 (reported)				
	Q1	REF	REF	REF
	Q2	0.10 (-0.22, 0.41)	-0.05 (-0.33, 0.23)	0.10 (-0.22, 0.43)
	Q3	0.06 (-0.24, 0.36)	-0.05 (-0.32, 0.22)	0.07 (-0.24, 0.38)

	Q4	-0.05 (-0.35, 0.25)	0.18 (-0.09, 0.45)	**0.35 (0.04, 0.65)**
Total volume per capita Option 2 (summation by lid type)				
	Q1	REF	REF	REF
	Q2	0.003 (-0.31, 0.31)	-0.09 (-0.37, 0.19)	0.31 (-0.008, 0.63)
	Q3	-0.08 (-0.39, 0.23)	-0.10 (-0.38, 0.18)	0.13 (-0.19, 0.44)
	Q4	-0.15 (-0.46, 0.17)	0.14 (-0.14, 0.42)	**0.43 (0.10, 0.75)**
No lid: Total volume per capita				
	Q1	REF	REF	REF
	Q2	-0.10 (-0.42, 0.21)	0.03 (-0.26, 0.31)	0.26 (-0.07, 0.59)
	Q3	-0.11 (-0.40, 0.19)	**-0.26 (-0.52, 0.01)**	-0.05 (-0.36, 0.25)
	Q4	-0.24 (-0.52, 0.05)	0.10 (-0.16, 0.36)	**0.29 (-0.005, 0.59)**
Secured lid: Total volume per capita				
	Q1	REF	REF	REF
	Q2	-0.06 (-0.49, 0.37)	0.12 (-0.27, 0.51)	0.02 (-0.43, 0.47)
	Q3	0.12 (-0.16, 0.40)	0.06 (-0.19, 0.32)	-0.05 (-0.34, 0.25)
	Q4	**0.29 (0.02, 0.56)**	**0.28 (0.03, 0.52)**	0.13 (-0.15, 0.42)
Percent of water stored with no lid				
	Q1	REF	REF	REF
	Q2	-0.22 (-0.54, 0.10)	-0.06 (-0.35, 0.23)	0.25 (-0.08, 0.58)
	Q3	0.01 (-0.28, 0.31)	-0.05 (-0.32, 0.22)	0.04 (-0.28, 0.35)
	Q4	-0.25 (-0.54, 0.03)	-0.03 (-0.29, 0.23)	0.21 (-0.08, 0.51)
Percent of water stored with secured lid				
	Q1	REF	REF	REF
	Q2	0.28 (-0.01, 0.58)	-0.06 (-0.32, 0.21)	-0.10 (-0.41, 0.21)
	Q3	**0.26 (-0.004, 0.53)**	-0.03 (-0.28, 0.21)	-0.20 (-0.48, 0.08)
	Q4	--	--	--
Minimum volume of container (L) Option 1 (reported directly)				
	Q1	REF	REF	REF
	Q2	0.27 (-0.03, 0.58)	-0.07 (-0.62, 0.49)	0.31 (-0.31, 0.93)
	Q3	**0.35 (0.03, 0.67)**	0.004 (-0.21, 0.22)	0.08 (-0.17, 0.33)
	Q4	0.30 (-0.02, 0.62)	0.62 (-0.73, 1.97)	-0.68 (-2.21, 0.85)

Minimum volume of container (L) Option 2 (extracted from data)			
Q1	REF	REF	REF
Q2	-0.07 (-0.49, 0.34)	0.009 (-0.37, 0.39)	-0.28 (-0.71, 0.16)
Q3	0.14 (-0.08, 0.37)	0.02 (-0.18, 0.23)	0.04 (-0.19, 0.28)
Q4	0.34 (-1.34, 2.03)	0.06 (-1.46, 1.58)	-0.16 (-1.93, 1.62)
Minimum volume of container per capita (L) Option 1 (reported directly)			
Q1	REF	REF	REF
Q2	-0.05 (-0.36, 0.27)	0.08 (-0.20, 0.37)	0.10 (-0.22, 0.43)
Q3	0.27 (-0.06, 0.60)	0.01 (-0.29, 0.31)	0.10 (-0.23, 0.45)
Q4	0.16 (-0.18, 0.50)	0.06 (-0.25, 0.37)	0.19 (-0.16, 0.54)
Minimum volume of container per capita (L) Option 2 (extracted from data)			
Q1	REF	REF	REF
Q2	0.23 (-0.06, 0.52)	-0.04 (-0.30, 0.22)	-0.20 (-0.49, 0.10)
Q3	**0.32 (0.02, 0.61)**	0.001 (-0.27, 0.27)	-0.14 (-0.44, 0.16)
Q4	0.10 (-0.22, 0.42)	0.06 (-0.23, 0.35)	**0.38 (0.06, 0.71)**

*Significance at the p<0.05 level
** Significance at the p<0.01 level
† Significant difference at the p<0.001 level

Table S2. Unadjusted analysis for water storage variables in each household with log-transformed EE using mixed-effects models (Part 2).

Risk Factor		LM β (95% CI) (N=652, n=237)	AGP β (95% CI) (N=393, n=211)	CIT β (95% CI) (N=485, n=223)
Total volume (L) Option 1 (reported)				
	Q1	REF	REF	REF
	Q2	-0.10 (-0.37, 0.17)	-0.14 (-0.29, 0.005)	-0.03 (-0.16, 0.10)
	Q3	0.004 (-0.26, 0.26)	**-0.15 (-0.30, -0.01)***	-0.04 (-0.16, 0.08)
	Q4	0.004 (-0.26, 0.26)	-0.11 (-0.25, 0.03)	-0.04 (-0.16, 0.08)
Total volume (L) Option 2 (summation by lid type)				
	Q1	REF	REF	REF
	Q2	-0.11 (-0.38, 0.17)	**-0.18 (-0.33, -0.03)***	-0.04 (-0.17, 0.09)
	Q3	-0.02 (-0.28, 0.23)	-0.14 (-0.28, 0.004)	0.006 (-0.11, 0.13)
	Q4	-0.009 (-0.27, 0.25)	-0.09 (-0.23, 0.05)	-0.05 (-0.17, 0.07)
No lid: Total volume (L)				
	Q1	REF	REF	REF
	Q2	0.14 (-0.14, 0.42)	0.03 (-0.12, 0.18)	**-0.16 (-0.28, -0.03)***
	Q3	0.13 (-0.12, 0.39)	-0.04 (-0.19, 0.11)	-0.04 (-0.16, 0.08)
	Q4	0.12 (-0.13, 0.37)	-0.06 (-0.19, 0.08)	-0.03 (-0.15, 0.09)
Secured lid: Total volume (L)				
	Q1	REF	REF	REF
	Q2	-0.29 (-0.66, 0.08)	0.12 (-0.08, 0.33)	-0.15 (-0.33, 0.03)
	Q3	-0.05 (-0.29, 0.20)	-0.08 (-0.20, 0.05)	0.01 (-0.10, 0.12)
	Q4	-0.02 (-0.25, 0.22)	-0.03 (-0.17, 0.10)	-0.07 (-0.18, 0.04)
Total volume per capita Option 1 (reported)		REF	REF	REF
	Q1	-0.03 (-0.30, 0.24)	-0.04 (-0.19, 0.11)	-0.07 (-0.20, 0.05)
	Q2	-0.15 (-0.41, 0.11)	-0.13 (-0.27, 0.02)	-0.07 (-0.19, 0.05)
	Q3	-0.09 (-0.35, 0.17)	-0.12 (-0.26, 0.02)	-0.02 (-0.14, 0.10)

	Q4			
Total volume per capita Option 2 (summation by lid type)				
	Q1	REF	REF	REF
	Q2	-0.15 (-0.42, 0.12)	-0.04 (-0.19, 0.11)	-0.03 (-0.15, 0.09)
	Q3	-0.15 (-0.41, 0.12)	-0.10 (-0.26, 0.04)	-0.02 (-0.14, 0.11)
	Q4	-0.14 (-0.41, 0.14)	-0.10 (-0.24, 0.05)	0.03 (-0.10, 0.15)
No lid: Total volume per capita				
	Q1	REF	REF	REF
	Q2	0.25 (-0.04, 0.53)	0.03 (-0.12, 0.18)	**-0.17 (-0.29, -0.04)****
	Q3	0.15 (-0.10, 0.40)	-0.05 (-0.19, 0.10)	-0.06 (-0.17, 0.06)
	Q4	0.05 (-0.20, 0.30)	-0.05 (-0.19, 0.09)	-0.006 (-0.12, 0.11)
Secured lid: Total volume per capita				
	Q1	REF	REF	REF
	Q2	-0.08 (-0.34, 0.18)	0.03 (-0.11, 0.17)	-0.11 (-0.23, 0.006)
	Q3	-0.005 (-0.28, 0.27)	-0.06 (-0.22, 0.09)	-0.02 (-0.14, 0.11)
	Q4	-0.15 (-0.41, 0.12)	-0.004 (-0.16, 0.15)	-0.12 (-0.24, 0.006)
Percent of water stored with no lid				
	Q1	REF	REF	REF
	Q2	0.26 (-0.01, 0.54)	-0.01 (-0.16, 0.14)	**-0.19 (-0.32, -0.06)****
	Q3	0.05 (-0.20, 0.31)	-0.02 (-0.16, 0.13)	-0.02 (-0.13, 0.10)
	Q4	0.11 (-0.14, 0.36)	-0.04 (-0.18, 0.10)	-0.04 (-0.16, 0.08)
Percent of water stored with secured lid				
	Q1	REF	REF	REF
	Q2	-0.02 (-0.28, 0.24)	0.02 (-0.12, 0.17)	0.05 (-0.07, 0.17)
	Q3	-0.04 (-0.28, 0.19)	0.05 (-0.07, 0.18)	-0.03 (-0.14, 0.08)
	Q4	--	--	--
Minimum volume of container (L) Option 1 (reported directly)				
	Q1	REF	REF	REF
	Q2	-0.18 (-0.74, 0.37)	0.17 (-0.11, 0.45)	-0.21 (-0.46, 0.04)
	Q3	-0.001 (-0.22, 0.22)	0.004 (-0.11, 0.12)	-0.007 (-0.11, 0.09)
	Q4	-0.66 (-2.12, 0.80)	-0.56 (-1.28, 0.16)	-0.48 (-1.15, 0.18)

Minimum volume of container (L) Option 2 (extracted from data)			
Q1	REF	REF	REF
Q2	-0.07 (-0.43, 0.29)	-0.04 (-0.24, 0.15)	-0.05 (-0.21, 0.12)
Q3	-0.04 (-0.23, 0.16)	0.05 (-0.06, 0.16)	**0.10 (0.007, 0.19)***
Q4	-0.04 (-1.44, 1.37)	0.43 (-0.58, 1.45)	-0.48 (-2.62, 1.66)
Minimum volume of container per capita (L) Option 1 (reported directly)			
Q1	REF	REF	REF
Q2	-0.33 (-0.62, -0.04)	-0.05 (-0.20, 0.10)	0.002 (-0.13, 0.13)
Q3	-0.17 (-0.47, 0.13)	-0.14 (-0.30, 0.02)	-0.02 (-0.15, 0.12)
Q4	-0.17 (-0.48, 0.14)	0.03 (-0.14, 0.19)	-0.04 (-0.18, 0.10)
Minimum volume of container per capita (L) Option 2 (extracted from data)			
Q1	REF	REF	REF
Q2	-0.17 (-0.42, 0.08)	-0.09 (-0.23, 0.04)	0.01 (-0.10, 0.13)
Q3	-0.19 (-0.44, 0.06)	-0.11 (-0.27, 0.05)	0.11 (-0.02, 0.24)
Q4	-0.03 (-0.31, 0.25)	0.009 (-0.13, 0.15)	0.09 (-0.03, 0.21)

*Significance at the p<0.05 level
** Significance at the p<0.01 level
† Significant difference at the p<0.001 level

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Chapter 3

Floors and toilets: Association of floors and sanitation practices with fecal contamination in Peruvian Amazon peri-urban households

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Abstract

Over two billion people worldwide lack access to an improved sanitation facility that adequately retains or treats feces. This results in the potential for fecal material containing enteric pathogens to contaminate the environment, including household floors. This study aimed to assess how floor type and sanitation practices impacted the concentration of fecal contamination on household floors. We sampled 189 floor surfaces within 63 households in a peri-urban community in Iquitos, Peru. All samples were analyzed for

colony forming units (CFUs) of *E. coli* and households were evaluated for their water, sanitation and hygiene characteristics. Results of multivariate linear regression indicated that households with improved sanitation and cement floors in the kitchen area had reduced fecal contamination to those with unimproved sanitation and dirt floors (Beta: -1.18 $\log_{10}$ *E. coli* CFU/900 cm^2; 95% confidence interval [CI]: -1.77, -0.60). Households that did not versus did share their sanitation facility also had less contaminated kitchen floors (Beta: -0.65 $\log_{10}$ *E. coli* CFU/900 cm^2; 95% CI: -1.15, -0.16). These findings suggest that the sanitation facilities of a home may impact the microbial load found on floors, contributing to the potential for household floors to serve as an indirect route of fecal pathogen transmission to children.

Introduction

Diarrheal diseases are a leading cause of malnutrition and death in children under five years old, accounting for 10 percent of all deaths (approximately 760,000 children annually).[1] Children living in low-income countries disproportionately suffer from malnutrition, which has been shown to increase mortality risk, affect cognitive development, increase infection risk, limit physical capacity and childbearing, and reduce adult economic

productivity.[2] Fecal contamination in the environment due to a lack of sanitation leads to high rates of diarrhea and is hypothesized to impact malnutrition through environmental enteropathy (EE), a condition in the gut caused by exposure to enteric pathogens that lead to alterations in intestinal structure, function, and local and systemic immune activation.[3] EE is also considered to negatively impact growth. A growing body of evidence supports the contribution of environmental factors related to poor water, sanitation and hygiene conditions to stunting in children.[4-6]

There are many fecal-oral transmission pathways, which account for important routes of exposure for the pathogens that cause enteric infection. These pathways can broadly be categorized by the F-diagram, which depicts the concept that human-derived enteric pathogens are transmitted through food, flies, floors, fingers, and fluids.[7] A lack of access to clean water is often implicated as the primary fecal-oral transmission route; however, a number of randomized, controlled trials investigating the effect of drinking water on gastrointestinal health have shown no additional benefit from point-of-use interventions.[8-11] This lack of benefit from clean water is hypothesized to stem from the additive contributions of poor sanitation and hygiene, which allow for exposures through alternative fecal-oral

transmission pathways and negate any potential benefit observed from improved water quality alone. In addition, a recent review of epidemiological studies on the effect of water and sanitation interventions on self-reported diarrhea episodes revealed no difference in point-of-use water interventions when blinding was taken into account.[12] These studies emphasize the importance of investigating other transmission routes to understand which fecal-oral pathways pose the greatest risk for ingestion of pathogens.

One of the pathways that has not been well characterized in communities with significant fecal contamination are household floors. This transmission pathway is especially important for infants (7-12 months) who are more likely to remain indoors and spend more time playing on the floor than older children.[13] Younger children are also more likely to engage in pica (i.e. soil consumption), object-to-mouth, and hand-to-mouth activity than older children.[14, 15] These behaviors, combined with immature immune responses, render the youngest children most vulnerable to enteric infections. Despite its importance, limited research has focused on floors as a critical pathway for pathogen transmission. The few studies conducted have highlighted the importance of quantifying fecal indicator bacteria on household floors and

surfaces to understand the distribution of fecal matter.[16-18] One limitation of these studies is that no duplicate samples were processed at the sample collection level to understand if the fecal contamination is significantly associated with location within a household. Repeat samples are also necessary to characterize between sample variability and understand if the fecal contamination within a household is consistent or varies over time and displays a "patchiness" as has been demonstrated in quantifying bacteria in beach sands.[19]

Our study reports on the *Escherichia coli* bacteria levels of the main floor surfaces in the homes of children near Iquitos, Peru enrolled in the Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development (MAL-ED) cohort study. The use of *E. coli* concentration as a fecal indicator bacteria within the household has been shown to be effective in a number of studies[16-18, 20] as well as at this study site in Iquitos Peru.[21] The aim of this study was to compare concentrations of *E. coli* recovered from household floors according to characteristics of household sanitation. A secondary aim was to characterize the variability of recovery of *E. coli* within households.

Methods

Study Setting and Population

This study was nested within the MAL-ED cohort in three peri-urban communities of Iquitos, Peru: Santa Clara de Nanay, Santo Tomas, and La Union (3°47'S, 73°20'W). In order to be eligible for the floor sampling study, a household had to have a child less than 48 months of age who was still enrolled in the MAL-ED study at the time of sampling.

Prior work has shown that these communities lag behind the rest of the Peru in terms of development indicators.[22] Only 20.2 percent of the population had access to an improved sanitation facility while 58.4 percent of the overall Peru population had access. Similarly, 46.7 percent of households in the study communities had access to clean water versus 77.1 percent in all of Peru. Child growth also lagged behind with 46.3 percent of children under 5 years old being stunted versus 19.5 percent in Peru. Children under 5 years old who were reported to have diarrhea in the past week was 35.4 percent versus 13.9 percent in Peru.[22] The households were low-income with the mean monthly per-capita income at $28 US dollars.[23] The temperature ranges between 21.9 and 32.4 degrees Celsius with an average of 25.8

degrees Celsius.[22] Rainfall is frequent and occurs throughout the year on about half of all days with the heaviest rainfall in January.[22]

The communities are located proximal to the Nanay River, which is a major branch of the Amazon river system. The river levels rise until March and, at the time of initiation of the study, the Nanay River was receding and no flooding was apparent within any of the households visited. There is no centralized sewerage infrastructure in the community and hence open ditches are used to drain storm and gray water away from the home. The frequent flooding in this riverine community also leads to fecal matter from latrines being released into the environment.

Classification of Floors and Sanitation Practices

During each household visit, a household questionnaire was administered in Spanish prior to floor sampling. The questionnaire was based on the Demographic and Health Surveys[3] and was a shortened version of the standardized questionnaire. In addition, study staff conducted a standardized visual inspection of floor type by room within households and noted the materials used as either dirt, wood, cement or tile.

The questionnaire assessed the primary exposure variable of the type of sanitation facility used by household members and whether or not this facility was shared. The options for type of sanitation facility were: i) no facility/open field; ii) pit latrine; iii) pour flush toilet to a septic; iv) flush to somewhere else; or v) ventilated improved pit latrine. Responses to water and hygiene questions provided covariate data on the household's primary water source, mode of water treatment, time it takes to fetch water, hygiene behavior and crowding. Information was also collected on socio-economic factors such as housing construction materials, length of tenancy, electricity access, maternal education, and monthly income. Given the propensity for households to keep free-ranging or corralled chickens in this community, participants also were interviewed regarding the presence of chickens in the home to evaluate the influence of chicken feces on the bacterial contamination of household floors.

Floor Sampling

From August to September 2015 household floors were sampled for *E. coli* bacteria using a modified dry electrostatic cloth method based on one designed for household settings.[24] Samples were collected from highly trafficked areas, namely the household entrance and the kitchen, which has

been shown to have higher levels of fecal bacteria than the bathroom areas.[17, 25] These areas were also selected for high likelihood of fecal pathogen exposure for children under five years of age who spend large amounts of time in play near the entrance and near the primary caregiver engaged in cooking activities. The first area sampled was the entryway floor, typically located at the front of the house and closest to the open drains that conveyed untreated wastewater and had a tendency to overflow during periods of rainfall. The second area sampled was the kitchen floor area, typically located at the back of the house (Figure 1). If there was a latrine or toilet, it was most commonly in the back of the house, closest to the kitchen area. The kitchen area was also observed to be the area of the house where most water use and storage activities took place, creating a potentially favorable environment for bacterial growth. Duplicate samples were taken side by side at the entryway location to investigate the heterogeneity of fecal contamination across floors. To assess the potential influence of different floor material types (e.g. dirt, wood, cement) on fecal contamination, we recorded information about the floor material types at the household entrance and in the kitchen area at the time of sampling.

Prior to field collection, sterile packets of dry electrostatic cloths (Swiffer™; Proctor & Gamble, Cincinnati, OH) were separated, quartered, and individually wrapped in autoclave paper (Fisher Scientific™, Pittsburgh, PA). Wrapped packets were then sterilized by autoclaving. For each collection an adapted protocol from Davis et al. (2012) was used where a prepared cloth was passed over a 30 cm by 30 cm floor surface with medium pressure to maximize the amount of pick-up from the surface [24]. The cloth was then placed into a sterile 700 mL Whirlpak bag (Nasco, Fork Atkinson, WI) and 5 mL of sterilized Milli-Q ultra-pure water to guard against microbial desiccation during transport. Collected material on the cloth buffered the water on contact to prevent bacterial osmotic shock (data not shown). Samples were stored in a cooler on ice at 4°C during field collection and transported to the laboratory. Samples were processed within six hours of collection.

Microbiological evaluation

For elution, 100 ml of sterile 0.1% Peptone buffer was added into the Whirlpak bag containing the cloth and vigorously shaken for one minute. The cloth was aseptically removed and *E. coli* in the buffer were enumerated following USEPA Method 1604[26] using m-coliblue24 commercial media

(HACH, Loveland, U.S.A.). Positive *E. coli* were identified as blue colonies. Pre, intermittent and post blanks were run to confirm the absence of cross contamination of samples. To obtain a countable number of colonies (i.e. 20-200), undilute, 10-fold, 100-fold and 1000-fold dilutions of eluate for samples collected on dirt floors and undilute, 10-fold and 100-fold for samples collected on wood and cement floors were processed, enabling a detection range of 0 to 200,000 colony forming units (CFU) per 900 $cm^2$of floor area to be enumerated.

Data Analysis

The data were processed and visualized using R software version 3.0.3 (R-FSC, Vienna, Austria) and subsequent statistical analysis was conducted using Stata version 12.1 (College Station, TX). The primary independent variable of sanitation facility was categorized into "improved" and "unimproved" sanitation facilities as defined by the Joint Monitoring Program (JMP) for Water Supply and Sanitation.[27] The JMP classifies improved facilities as those that ensure hygienic separation of human excreta from human contact and include facilities that flush or pour flush to a piped sewer system, septic tank or pit latrine. Unimproved facilities on the other hand, do not ensure hygienic separation of human excreta from human

contact and include pit latrines without a slab. For the purposes of this study, those households that did not have a toilet facility were also categorized as "unimproved".

Water source, water treatment and floor type covariates were analyzed as categorical variables. A hygiene index variable score was calculated as a cumulative score from the following four questions: i) Do you wash your hands after helping your child defecate? ii) Do you wash your hands before preparing food? iii) Do you wash your hands after going to the bathroom? and iv) Do you use toilet paper? The hygiene index score had three levels with good indicating the interviewee answered all questions as always practicing the hygienic behaviors; average indicated that for one of the four questions the interviewee only sometimes practiced the hygienic behavior; and poor indicated that for two or more questions the interviewee only sometimes practiced the hygienic behavior. *E. coli* concentrations were $\log_{10}$-transformed and reported as $\log_{10}$ *E. coli* CFU/900 cm^2.

Two-sample t-tests with equal variances and Pearson Chi-squared analysis were used to compare household characteristics across improved and unimproved sanitation facility types. Unadjusted linear regression analyses

were conducted to evaluate associations between water, sanitation, hygiene (WASH) and household characteristics with $\log_{10}$ *E. coli* CFU/900 cm^2 in the entrance and kitchen areas. Using generalized linear models we conducted a stratified analysis by sanitation type (improved versus unimproved). For this analysis of the relation between floor types and the levels of $\log_{10}$ E. coli CFU in strata of household sanitation type (unimproved and improved), observations in the entrance and kitchen of each house were combined. We adjusted for potential confounding covariates in linear regression models using a backward elimination approach. A final parsimonious covariate adjustment set was selected based on considerations of sample size and the minimization of the Akaike Information Criterion (AIC).[28] Interaction terms between sanitation type and floor type were included to determine if the association between sanitation and $\log_{10}$ *E. coli* CFU/900 cm^2 was modified by floor type. Beta coefficients and 95% confidence intervals were estimated and represent the $\log_{10}$-unit change in *E. coli* CFU/900 cm^2 per unit of each of the independent variables (household sanitation type, floor type, etc). Pearson correlation coefficients and 95% confidence intervals were calculated to estimate the variability between duplicate floor samples within the same household.

Ethics

The study protocol and questionnaires were approved by the institutional review boards from Johns Hopkins Bloomberg School of Public Health (Baltimore, MD) and Asociación Benéfica Proyectos de Informática, Salud, Medicina, y Agricultura (A.B. PRISMA), Iquitos, Peru. All participants gave written consent prior to household sampling.

Results

Table 1 illustrates that, among 63 household visits during the study period, 189 samples were collected, representing 63 entrance floor samples, 63 additional samples (duplicates) from adjoining areas to the primary entrance floor samples, and 63 samples from the kitchen floor. There were a total of 31 households that were classified as having unimproved sanitation and 32 households with improved sanitation facilities. In the entrance area there were 36 homes with dirt floors, 3 with wood floors and 24 with cement floors. In the kitchen area there were 46 homes with dirt floors, 4 with wood floors and 13 with cement floors. One household in each category for sanitation type had ceramic tile in either the entrance and kitchen area. These households were categorized as having a cement floor for analysis due to the common composition and construction characteristics between the

local tile and cement. There were no missing data for the $\log_{10}$ *E. coli* CFU/900 cm^2outcome variable and less than ten percent of data were missing when all variables were considered in the full model.

Figure 2 depicts the $\log_{10}$ *E. coli* CFU/900 cm^2 in both the entrance and kitchen areas of the home by floor type. The entrance area of homes had an average of 3.40 $\log_{10}$ *E. coli* CFU (standard deviation=1.00) per 30 by 30 cm sample and the kitchen areas had significantly higher levels of $\log_{10}$ *E. coli* with 3.91 $\log_{10}$ *E. coli* CFU (sd=1.00) (p-value = 0.005). Within the entrance areas, dirt floors had statistically significantly higher levels of $\log_{10}$ *E. coli* CFU than cement floors (3.75 vs 2.86, p-value<0.001) and within the kitchen areas, dirt floors also had statistically significantly higher levels of $\log_{10}$ *E. coli* CFU than cement floors (4.27 vs 2.96, p-value=0.002) and wood floors (4.27 vs 2.89, p-value=0.023). Lastly, when comparing dirt floors between the entrance and kitchen areas within a household, the levels of $\log_{10}$ *E. coli* CFU in the kitchen area were statistically significantly higher than in the entrance (4.27 vs 3.75, p-value=0.013).

Household characteristic differences by sanitation type

For households with unimproved versus improved sanitation facilities, there were significant differences in household characteristics (Table 1). Households with unimproved versus improved sanitation had a higher percentage of dirt floors in both the entrance (77.4 vs 37.5, $p<0.01$) and kitchen (87.1 vs 59.4, $p<0.05$ level) and a more frequent reporting of chickens in the home (45.2 vs 12.5, $p<0.01$). Households with improved versus unimproved sanitation had a higher percentage of cement floors in both the entrance (56.3 vs 19.4, $p<0.01$) and kitchen (31.3 vs 9.7, $p<0.05$). There were no significant differences across sanitation type for other household WASH characteristics such as sharing sanitation facilities, type water connection, time to fetch water, household chlorine use to treat drinking water, crowding, income, education, electricity connection, wall and roof type and tenancy in the house.

Unadjusted analysis of household WASH characteristics and E. coli levels on floors

Linear regression models comparing individual household WASH characteristics and the levels of $\log_{10}$ *E. coli* CFU demonstrated significant associations in both the entrance and kitchen areas (Table 2). Households with improved sanitation had lower levels of $\log_{10}$ *E. coli* CFU/900 cm^2 on floors when compared to homes with unimproved sanitation in both the

entrance and kitchen (Beta: -0.63 (95% CI: -1.12, -0.15); and Beta: -0.80 (95% CI: -1.27, -0.33) respectively). For shared sanitation, households that reported not sharing their sanitation facility versus those did share had lower levels of $\log_{10}$ *E. coli* CFU/900 cm^2 (Beta: -0.70; 95% CI: -1.27, -0.13) in the kitchen area. Household entrance and kitchen areas with cement floors also had lower levels of $\log_{10}$ *E. coli* CFU/900 cm^2 when compared to household entrance and kitchen areas with dirt floors (Beta: -0.89 (95% CI: -1.38, -0.40); and Beta: -1.31 (95% CI: -1.83, -0.79) respectively). For every additional minute that interviewees reported needing to fetch water, corresponding increases in the concentrations of $\log_{10}$ *E. coli* CFU/900 cm^2 on entrance floors (Beta: 0.06; 95% CI: 0.02, 0.10) and kitchen floors (Beta: 0.05; 95% CI: 0.01, 0.09) were observed. Table 2 illustrates that wall type, crowding, electricity access, maternal education and housing tenancy were independently associated with increases in $\log_{10}$ *E. coli* CFU/900 cm^2.

To further understand the relationship between floor type and sanitation type, the stratified data by sanitation type (improved versus unimproved) are shown in Figure 4. The lowest $\log_{10}$ *E. coli* CFU/900 cm^2 were found in the homes with both improved sanitation and improved floor types (defined by their ability to be disinfected such that wood and cement floors are

combined into the improved category and dirt as unimproved). The reduction in $\log_{10}$ *E. coli* CFU/900 cm^2 among households with unimproved sanitation was -0.60 (95% CI: -1.03, -0.17) when comparing wood or cement (improved) floors to dirt floors (unimproved). An even greater reduction of -1.17 $\log_{10}$ *E. coli* CFU/900 cm^2 (95% CI: -1.68, -0.66) was observed among households with improved sanitation when comparing wood or cement floors to dirt floors (Table 3).

Adjusted analysis of household WASH characteristics and E. coli levels on floors

Two multivariate linear regression models were run for the entrance and kitchen floor areas with predictor variables that included both the sanitation type (improved or unimproved) as an interaction term with floor type and the variable for whether the sanitation facility was shared (Table 4). The models adjusted for time to fetch water, presence of chickens in the household, crowding, maternal education and wall type. For the entrance floor area, households with improved sanitation and cement floors had lower $\log_{10}$ *E. coli* CFU/900 cm^2 on their floors when compared to households with unimproved sanitation and dirt floors (Beta: -0.43; 95% CI: -1.08, 0.21). For the kitchen floor area, households with unimproved sanitation and wood floors and households with improved sanitation and cement floors

both had statistically significantly lower $\log_{10}$ *E. coli* CFU/900 cm^2 on their floors when compared to households with unimproved sanitation and dirt floors (Beta: -2.36 (95% CI: -3.86, -0.86) and (Beta: -1.18 (95% CI: -1.77, -0.60) respectively). Households that did not share their sanitation facility also had significantly reduced $\log_{10}$ *E. coli* CFU/900 cm^2 on their kitchen floors (Beta: -0.65; 95% CI: -1.15, -0.16) when compared to kitchen floors in households that did share their sanitation facility. The significant covariates in the adjusted model for the kitchen area included lack of chickens in the household (Beta: -0.63; 95% CI: -1.12, -0.15; indicating lower $\log_{10}$ *E. coli* CFU/900 cm^2 for those without versus with a presence of chickens) and maternal education (Beta: -0.08; 95% CI: (-0.15, -0.004; indicating lower $\log_{10}$ *E. coli* CFU/900 cm^2 in homes for every year increase in of education). The significant covariates in the adjusted model for the entrance area, were time to fetch water (Beta: 0.05; 95% CI: 0.003, 0.09; indicating higher $\log_{10}$ *E. coli* CFU/900 cm^2 for every minute increase in time to fetch water) and maternal education (Beta: -0.10; 95% CI: -0.19, 0.00; indicating lower $\log_{10}$ *E. coli* CFU/900 cm^2 for every year increase in of education).

Variability of recovery of E. coli within households

For the entrance area where side-by-side samples were collected to understand the distribution of *E. coli* bacteria across floor surfaces, the Pearson correlation coefficient between the initial and duplicate samples was 0.83 with a p-value < 0.001 (n=63) (Figure 3). The 95% confidence interval for the Pearson correlation coefficient ranged from 0.73 to 0.89 indicating a homogenous spread of bacteria across the sampling area.

Discussion

This study found evidence that household floors carried differential loads of fecal contamination depending on the type of sanitation facility and whether or not that sanitation facility was shared. The kitchen area had a higher level of *E. coli* than the entryway, which is consistent with previous studies that reported that the kitchen area is the location of greatest contamination.[17, 25] Additionally, the kitchen areas of these households were most commonly in the back, in closest proximity to the sanitation facility (if sanitation facilities were onsite) (Figure 1). This makes the kitchen area the most likely first point of contact for a household member after defecation and may therefore increase the bacterial loads within this area of the house. Homes with dirt floors were also found to have higher levels of bacteria than homes with cement floors. This supports the findings from previous interventions that

replacing dirt floors with cement floors may significantly improve child gut health.[29]

The sanitation facility was the household characteristic found to have the most significant and consistent relationship with the levels of bacteria on kitchen floors. These findings support the potential for sanitation interventions targeting hygienic containment of human waste to reduce exposures to fecal pathogens in the home. In the study communities, a flush toilet to a septic was a more hygienic sanitation option than the pit latrine, which was simply a hole in the ground (either covered or uncovered). Those who shared sanitation facilities were also more likely to have floors contaminated with *E. coli* in the kitchen area. This provides evidence in support of the definition for "shared" sanitation facilities being characterized as "unimproved" by JMP. The underlying assumption by the JMP that there is little commitment or incentive for users to keep a shared facility clean may in fact hold true in this community despite contrary evidence in other settings.[16]

Among homes with the same sanitation type, there was a reduction in fecal contamination when comparing unimproved (dirt) to improved (either wood

or cement) floors however, the magnitude of reduction was greater among homes with improved sanitation. Interestingly, the reduction of fecal contamination was not as large with only one of the two fecal-oral transmission pathways was interrupted (improved sanitation or an improved floor). This highlights the importance of interrupting additional fecal-oral transmission pathways, such as floors, during a sanitation intervention to most effectively reduce exposures to fecal pathogens in the home

This study also found that the presence of chickens in homes significantly increased the *E. coli* contamination on floors. Similar to people, either pathogenic or commensal *E. coli* can be identified in the chicken gastrointestinal tract, and chickens can be either asymptomatic carriers or exhibit disease.[30] Study staff frequently observed the presence of chicken droppings on surfaces in the home when chickens were present, suggesting the potential for direct fecal contamination from the birds.

This was the first study to use a dry electrostatic cloth as the sampling method for *E. coli* on floor surfaces in low-resourced settings. Other studies that sampled for *E. coli* either collected soil or used a cotton swab. One study in Tanzania examined household floors across different locations in

the home by quantifying the number of *E. coli* from a layer of soil 10 cm by 10 cm by 1 cm thick.[17] Another study in Cambodia sampled the floor surface around the base of household latrine and a floor surface near the kitchen sink using a swab method over the sample surface of 4 cm^2.[18] In comparison to these studies, the concentrations of *E. coli* contamination found of the dirt floors of these Peruvian homes were approximately 5[18] to 80[17] times more contaminated. This may be due to the efficiency of the sampling method used or may additionally or alternately reflect a higher typical bacterial load among homes in this community. The climate in the Peruvian Amazon provided an ideal environment for Gram-negative bacterial growth with consistently hot and humid weather year round and regular precipitation with dark and shady spaces inside the houses. Dirt floors in homes further promote bacterial growth and are difficult to disinfect due to the organic material and complex matrix. Therefore, fecal pathogens that reach household floors have a high chance for survival in the environment with increased potential for transmission to children.

This study also found evidence for the consistency in the contamination of floors across the entrance floor area as evidenced by the side-by-side sampling. This finding enhances confidence that the concentrations of *E.*

coli measured on floors represents a spatially-typical exposure for children. It also highlights the utility of the use of a dry electrostatic cloth sampling method as reproducible. Previous research on beach sand contamination found that on a micro-spatial scale, fecal indicator bacteria can vary greatly over short distances.[19] The strong correlations between the side-by-side measurements taken on the entrance floors suggest that the *E. coli* are more evenly distributed across households.

The main limitation of this study was that *E. coli* is an indicator organism for fecal contamination and may have limited accuracy for determining the presence of pathogens.[31] *E. coli* represents a large group of fecal bacteria from both human and animal sources and may come from relatively low-risk sources of fecal pollution.[32] Many *E. coli* are commensal, while other more pathogenic species, such as enteroviruses, norovirus, Cryptosporidium spp. and Giardia spp., have different survival rates in the environment than *E. coli*.[33, 34] Therefore, the presence virulent strains or other pathogenic microbes may or may not be accurately indicated by the detection of *E. coli*. The strengths of the study were that it used a novel sampling technique of the dry electrostatic cloth with high recovery efficiencies during the elution process. As the evidence base increases for the importance of the floors

pathway, this study highlights the need for rigorous methodological evaluation of household bacterial sampling strategies and methods in the context of environmental enteropathy. Another strength of the study was the analysis of within sample variability. This analysis showed the high correlations between samples taken side-by-side and therefore increased the confidence that the fecal contamination measured in this study is an accurate reflection of the levels of microbial pressure within the home.

This study demonstrated that household floors are a potential pathway for transmission of fecal pathogens and demonstrated that households with unimproved sanitation facilities and shared facilities had higher loads of *E. coli* bacteria. The high loads of *E. coli* bacteria suggest that this route of exposure is especially important for children less than 12 months of age who spend most of their time on the floor and partake in hand-to-mouth activity. These results suggest that interventions, such as covering dirt floors with cement and excluding chickens from contact with surfaces in the home, hold promise to reduce chronic exposure to fecal pathogens that may be implicated in diseases such as environmental enteropathy. This study also highlights the importance of a multidisciplinary approach to the reduction of fecal contamination that extends current drinking water interventions to

interrupt the transmission of pathogens in the environment by other pathways.

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Figure 1. Floor plan of typical household in the study communities

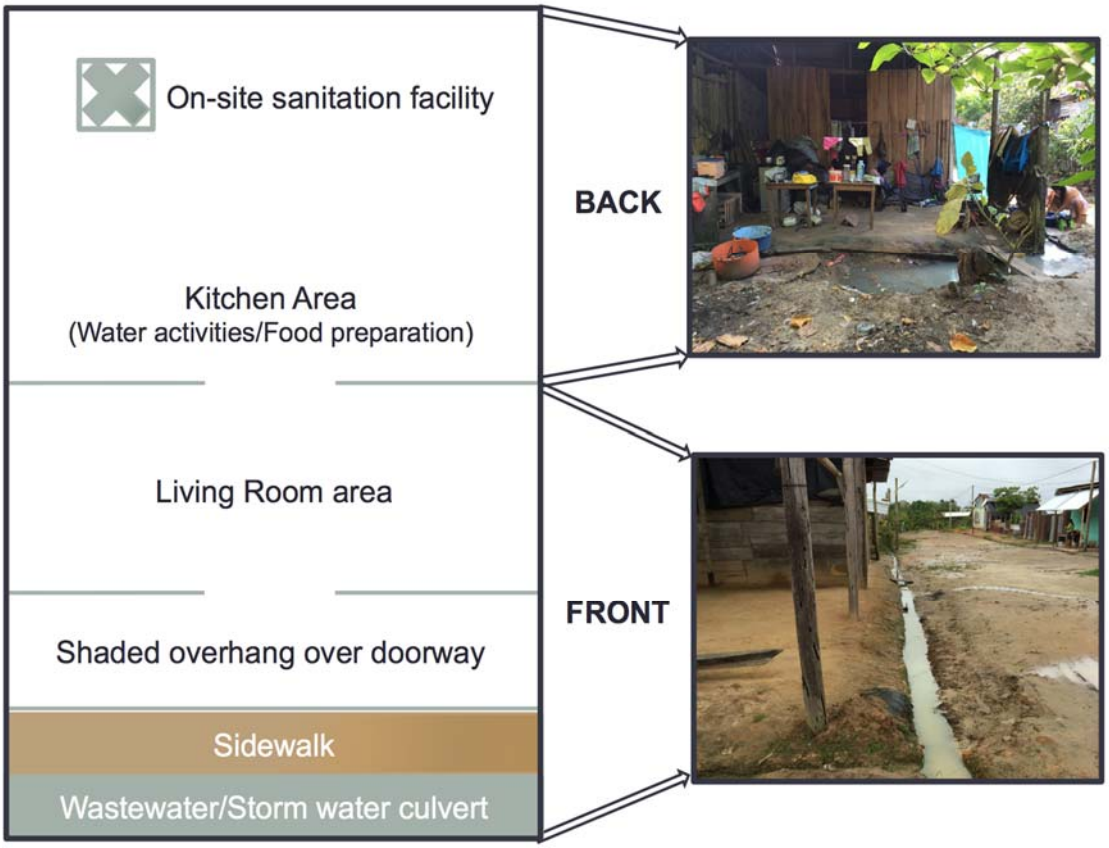
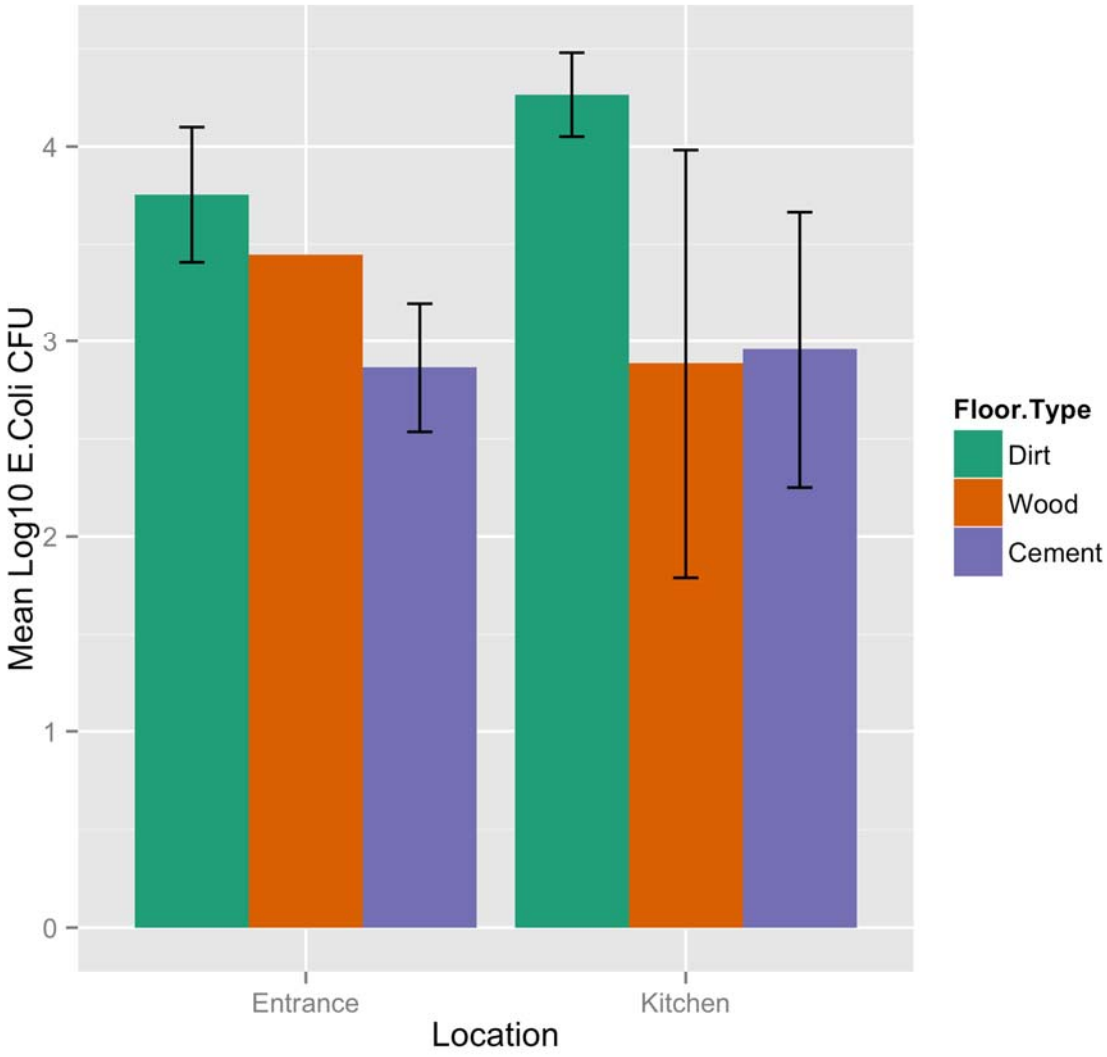


Figure 2. Concentrations of E.coli in Entrance and Kitchen by Floor Type (Mean log10-transformed colony forming units (CFU/900cm2), error bars represent 95% confidence intervals)



*Error bars represent 95% confidence intervals

Figure 3. Pearson correlation coefficient of log-10 transformed E.coli colony forming units per 900 cm2 from entrance floor duplicate samples taken side by side

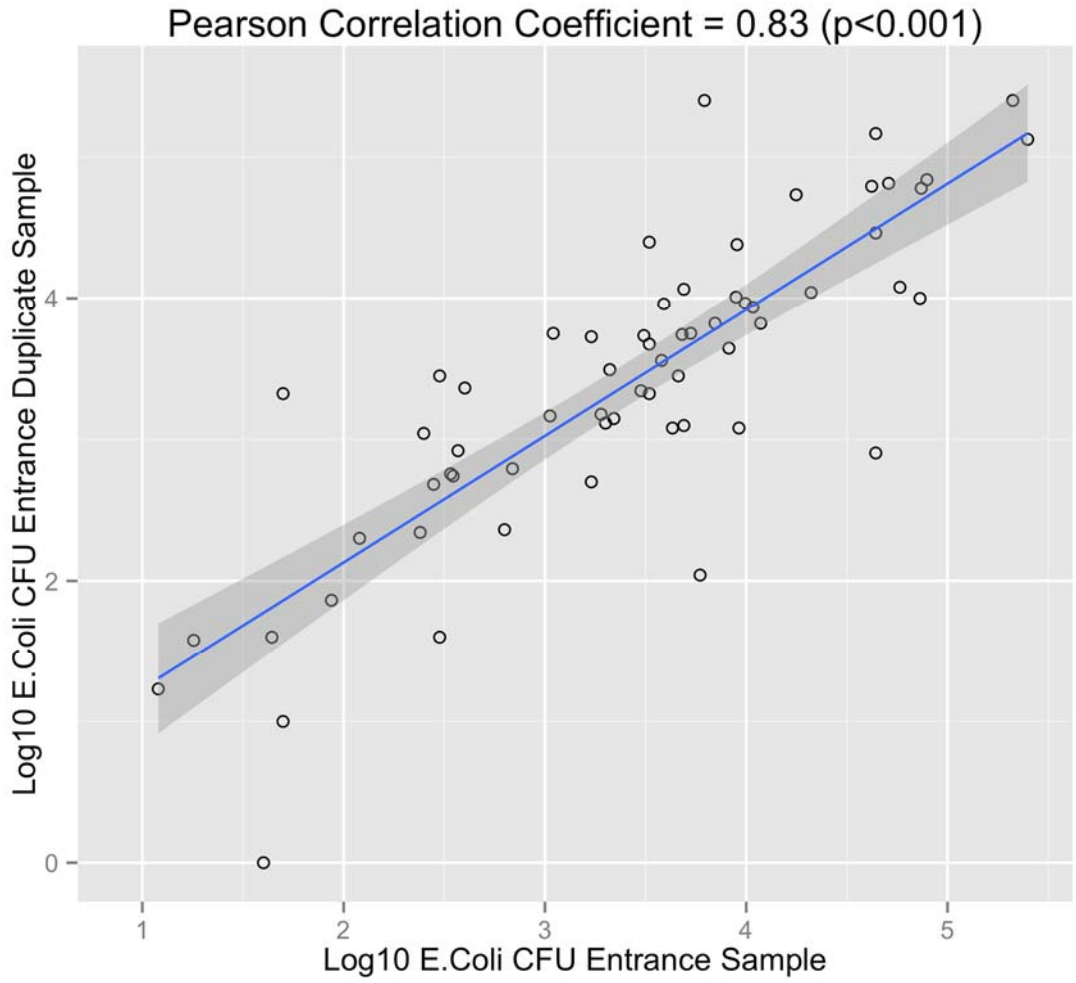


Figure 4. Log10 E.coli CFU per 900 cm2 by Sanitation and Floor Type

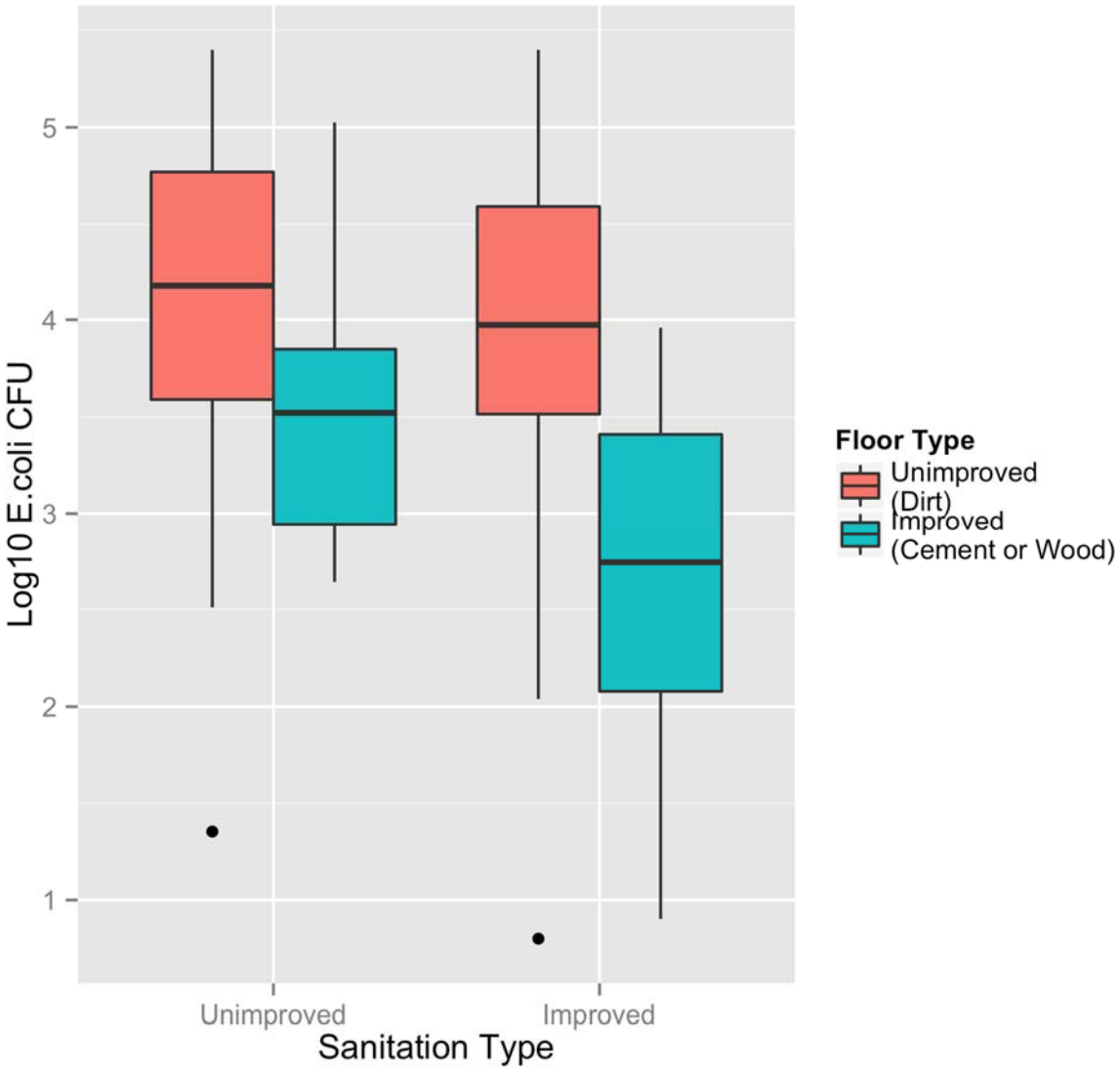


Table 1. Household characteristics by sanitation type (Pearson Chi-squared tests and two-sample t-tests with equal variances performed)

	Unimproved Sanitation Facility (N=31)	Improved Sanitation Facility (N=32)
Sanitation facility is shared (n=58)	38.5%	21.9%
Entrance floor type**:		
Dirt (n=36)	77.4%	37.5%
Wood (n=3)	3.2%	6.3%
Cement (n=24)	19.4%	56.3%
Kitchen floor type*:		
Dirt (n=46)	87.1%	59.4%
Wood (n=4)	3.2%	9.4%
Cement (n=13)	9.7%	31.3%
Drinking water source:		
Faucet in house (n=2)	3.3%	3.1%
Public tap (n=8)	9.7%	15.6%
Community hand pump (n=44)	71.0%	68.8%
Open well (without top) (n=1)	3.2%	0.0%
Surface water (n=2)	0.0%	6.3%
Other (n=6)	12.9%	6.3%
Time to fetch water in minutes (n=62)	8.6 (6.2, 11.1)	5.9 (4.0, 7.7)
Household uses chlorine to treat water (n=63)	25.8%	25.0%
Presence of chickens in HH** (n=63)	45.2%	12.5%
Crowding (Number of people sleeping in HH/ Number of rooms) (n=62)	1.9 (1.5, 2.4)	1.7 (1.2, 2.2)
Hygiene Score:		
Good (n=41)	64.5%	65.6%
Average (n=11)	12.9%	21.9%
Poor (n=11)	22.6%	12.5%
Monthly income per capita (in USD) (n=61)	26.1 (19.8, 32.3)	27.7 (20.2, 35.3)
Maternal Education (years) (n=62)	6.6 (5.5, 7.6)	8.1 (7.0, 9.2)
Electricity connection (n=62)	77.4%	93.5%
Wall type:		
Wood (n=48)	83.9%	68.8%
Concrete (n=14)	12.9%	31.3%
Other (n=1)	3.2%	0.0%
Roof type:		
Tin (n=60)	93.6%	96.9%
Palm (n=2)	3.3%	3.1%
Other (n=1)	3.3%	0.0%
Tenancy in household:		
Less than a year (n=13)	32.3%	9.4%

Between one and five years (n=19)	22.6%	37.5%
Between five and ten years (n=14)	25.8%	18.8%
Between ten and twenty years (n=9)	12.9%	15.6%
More than twenty years (n=8)	6.5%	18.8%

* Significant difference at the $p<0.05$ level, Pearson Chi-squared
** Significant difference at the $p<0.01$ level, Pearson Chi-squared

Table 2. Relation of household characteristics with log10-transformed E.coli colony forming units (CFU) per 900 cm2 in entrance and kitchen areas.

	Entrance Log10 *E. coli* CFU/900cm2 Beta[1] (95% CI)	Kitchen Log10 *E. coli* CFU/900cm2 Beta (95% CI)
Sanitation Type:		
Unimproved (n=31)	REF	REF
Improved (n=32)	-0.63 (-1.12, -0.15)**	-0.80 (-1.27, -0.33)†
Shared Sanitation Facility:		
Shared (n=17)	REF	REF
Unshared (n=41)	-0.53 (-1.09, 0.03)	-0.70 (-1.27, -0.13)*
Floor Type (Entrance, Kitchen):		
Dirt (n=36, n=46)	REF	REF
Wood (n=3, n=4)	-0.31 (-1.42, 0.81)	-1.38 (-2.27, -0.51)**
Cement (n=24, n=13)	-0.89 (-1.38, -0.40)†	-1.31 (-1.83, -0.79)††
Drinking water source:		
Community hand pump (n=44)	REF	REF
Faucet in house (n=2)	-0.04 (-1.54, 1.46)	1.10 (-0.36, 2.56)
Public tap (n=3)	0.33 (-0.47, 1.13)	0.003 (-0.77, 0.78)
Open well (without top) (n=1)	-0.43 (-2.53, 1.67)	-1.05 (-3.09, 1.00)
Surface water (n=2)	0.38 (-1.12, 1.89)	-0.46 (-1.92, 1.00)
Other (n=6)	0.22 (-0.68, 1.13)	0.48 (-0.40, 1.36)
Time to fetch water in minutes (n=62)	0.06 (0.02, 0.10)**	0.05 (0.01, 0.09)*
Household uses chlorine to treat water:		
No (n=47)	REF	REF
Yes (n=16)	0.08 (-0.51, 0.66)	-0.004 (-0.59, 0.59)
Presence of chickens in HH:		
Yes (n=18)	REF	REF
No (n=45)	-0.38 (-0.93, 0.18)	-0.53 (-1.08, 0.02)
Crowding (Number of people sleeping in HH/ Number of rooms) (n=62)	0.22 (0.02, 0.42)*	0.16 (-0.04, 0.36)
Hygiene Score:		
Good (n=41)	REF	REF
Average (n=11)	0.26 (-0.43,0.95)	0.10 (-0.59, 0.79)
Poor (n=11)	0.18 (-0.51, 0.87)	0.39 (-0.30, 1.08)
Monthly income per capita (in USD) (n=61)	0.002 (-0.01, 0.02)	-0.0004 (-0.01, 0.01)
Maternal Education (years) (n=62)	-0.09 (-0.17, -0.01)*	-0.04 (-0.13, 0.04)
Electricity connection:		
Yes (n=53)	REF	REF
No (n=9)	0.78 (0.07, 1.49)*	0.67 (-0.05, 1.39)

Wall type:		
Wood (n=48)	REF	REF
Concrete (n=14)	-0.88 (-1.45, -0.31)**	-1.05 (-1.61, -0.52)††
Roof type:		
Tin (n=60)	REF	REF
Palm (n=2)	1.16 (-0.26, 2.58)	0.41 (-1.04, 1.85)
Tenancy in household:		
Less than a year (n=13)	REF	REF
Between one and five years (n=19)	-0.72 (-1.44, -0.01)*	-0.40 (-1.13, 0.32)
Between five and ten years (n=14)	-0.78 (-1.54, -0.02)*	-0.76 (-1.53, 0.02)
Between ten and twenty years (n=9)	-0.49 (-1.35, 0.37)	-0.33 (-1.21, 0.54)
More than twenty years (n=8)	-0.86 (-1.74, 0.03)	-0.61 (-1.52, 0.30)

* Significance at the p<0.05 level

** Significance at the p<0.01 level

†Significant difference at the p<0.001 level

††Significant difference at the p<0.0001 level

[1] The beta coefficient represents the log10-unit change in E. coli CFU/900 cm2 between the exposed and unexposed (REF) categories. For the continuous independent variables the beta coefficient represents the log10-unit change in E. coli per increase in a unit change of the variable.

Table 3. Relation between floor type and Log10 E.coli CFU per 900 cm2 by sanitation type

	Improved Sanitation Type[2] (n=64)	Unimproved Sanitation Type[3] (n=62)
Floor Type[1]:		
Unimproved	REF (n=31)	REF (n=51)
Improved	**-1.17 (-1.68, -0.66)**†† (n=32)	**-0.60 (-1.03, -0.17)**** (n=11)

[1] Improved floor type is classified as either cement or wood and unimproved as dirt.
[2] Among homes with improved sanitation, Beta 0 for dirt floors = 3.90 log10-transformed CFU versus 2.74 log10-transformed CFU for cement or wood floors
[3] Among homes with unimproved sanitation, Beta 0 for dirt floors = 4.12 log10-transformed CFU versus 3.52 log10-transformed CFU for cement or wood floors

** Significance at the p<0.01 level
††Significant difference at the p<0.0001 level

Table 4. Adjusted regression model of household characteristics with log10-transformed E.coli colony forming units (CFU) per 900 cm2 in entrance and kitchen areas (models adjust for time to fetch water, presence of chickens in the household, crowding, maternal education and wall type).

	Entrance Log$_{10}$ *E. coli* CFU			Kitchen Log$_{10}$ *E. coli* CFU		
N	56			56		
R-Squared (Adjusted R-squared)	0.392 (0.241)			0.651 (0.564)		
	β (SE)	95% CI	p-value	β (SE)	95% CI	p-value
Primary independent variables:						
Sanitation Type with Floor Type:						
Unimproved with Dirt	REF	REF	REF	REF	REF	REF
Unimproved with Wood	-1.13 (0.92)	(-2.99, 0.74)	0.230	**-2.36 (0.75)**	**(-3.86, -0.86)**	**0.003**
Unimproved with Cement	-0.51 (0.57)	(-1.66, 0.64)	0.372	0.40 (0.52)	(-0.65, 1.45)	0.444
Improved with Dirt	0.45 (0.36)	(-0.28, 1.18)	0.271	0.32 (0.26)	(-0.20, 0.83)	0.222
Improved with Wood	0.25 (0.69)	(-1.14, 1.64)	0.721	-0.74 (0.47)	(-1.68, 0.20)	0.119
Improved with Cement	-0.43 (0.32)	(-1.08, 0.21)	0.183	**-1.18 (0.29)**	**(-1.77, -0.60)**	**<0.001**
Shared Sanitation Facility:						
Shared	REF	REF	REF	REF	REF	REF
Unshared	-0.40 (0.31)	(-1.02, 0.22)	0.203	**-0.65 (0.25)**	**(-1.15, -0.16)**	**0.011**
Adjustment covariates:						
Time to fetch water in minutes	**0.05 (0.02)**	**(.003, 0.09)**	**0.038**	0.03 (0.02)	(-0.002, 0.07)	0.063
Presence of chickens in HH:						
Yes	REF	REF	REF	REF	--	--
No	-0.63 (0.24)	(-1.12, -0.15)	0.185	**-0.63 (0.24)**	**(-1.12, -0.15)**	**0.012**
Crowding	-0.06 (0.13)	(-0.32, 0.19)	0.622	-0.17 (0.10)	(-0.37, 0.02)	0.084
Maternal education (years)	**-0.10 (0.05)**	**(-0.19, 0.00)**	**0.048**	**-0.08 (0.04)**	**(-0.15,-0.004)**	**0.040**
Wall type:						
Wood	REF	REF	REF	REF	REF	REF
Concrete	-0.11 (0.35)	(-0.81, 0.60)	0.766	-0.33 (0.25)	(-0.84, 0.18)	0.198

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Chapter 4

Use of pathogen-specific antibody biomarkers to estimate waterborne infections in population-based settings

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Abstract

This review discusses the utility of pathogen-specific antibody biomarkers for improving estimates of the population burden of waterborne infections, assessing the fraction of infections that can be prevented by specific water treatments, and understanding transmission routes and the natural history and ecology of disease in different populations (including asymptomatic infection rates). The application of pathogen-specific antibody response data to estimate incidence and prevalence of acute infections and their utility in assessing the contributions of waterborne transmission pathways are discussed. Advantages and technical challenges associated with the use of serum versus minimally-invasive salivary antibody biomarkers in cross-sectional and prospective surveys are discussed. We also highlight challenges and outline future directions for research and development of antibody-based and other immunological biomarkers of waterborne infections.

Introduction

Waterborne infections cause an estimated two million deaths and four billion episodes of diarrheal illness per year worldwide [1]. Waterborne diseases will continue to be of broad public health importance as peri-urban populations rapidly expand at a pace that exceeds developing countries' abilities to

invest in infrastructure [2]. While most of these illnesses occur in developing countries, industrialized countries also bear a substantial burden of waterborne diseases [3]. For high-income countries, if investments in water supply and sewer systems do not enable proper maintenance and timely replacement of aging infrastructure, the risk of waterborne infections is likely to increase [4].

Waterborne disease outbreaks are defined as two or more persons experiencing a similar illness after exposure to water where epidemiologic evidence implicates water as the probable source of the outbreak [5]. Waterborne pathogens that result in human infections include bacteria (e.g., *Campylobacter* spp., *Shigella* spp.), viruses (e.g., norovirus, rotavirus) and protozoa (e.g., *Cryptosporidium* spp., *Giardia* spp.) and these pathogens may be conveyed to humans via drinking and/or recreational water transmission routes [6]. The health outcome most commonly associated with exposure to waterborne pathogens is acute gastrointestinal illness (AGI). AGI is defined in various ways and definitions used in epidemiological research range widely [7, 8]. One commonly used definition is: diarrhea (3 or more loose stools in a 24-hour period), vomiting, nausea, stomach ache, fever, and/or interference with regular activities (missed time from work or

school or missed regular activities as a result of illness) [8-10]. Other illnesses caused by waterborne pathogens include viral hepatitis (hepatitis A and E viruses [11]), skin and soft tissue infections and sepsis (*Vibrio* spp, *Staphylococcus aureus*[12]), primary amoebic meningoencephalitis (*Naegleria fowleri* [13]) and pneumonia (*Legionella pneumophila*[14]).

In this review we summarize the latest evidence on use of pathogen-specific antibodies as biomarkers (defined as "any substance, structure, or process that can be measured in the body or its products and can influence or predict the incidence of outcome or disease" [15]) of infection for the waterborne pathogens that cause the greatest population burden of AGI in the United States (norovirus, Shiga toxin-producing *E. coli*, and *Cryptosporidium* spp.)[16] and in developing countries globally (rotavirus, *Cryptosporidium* spp., *Shigella*, *Giardia* spp., *Vibrio cholerae*, and *Campylobacter* spp.)[17, 18]. We also include hepatitis A and E viruses because these pathogens are the most common causes of feces-transmitted acute viral hepatitis worldwide (Table 1)[19, 20]. Such pathogen-specific antibody biomarkers represent promising tools to identify causative agents in population-based studies of AGI, including waterborne disease outbreak investigations, surveillance studies, and observational and randomized intervention studies to test

hypotheses related to transmission routes, water treatments, and disease ecology. Because not all individuals who become infected with waterborne pathogens will experience symptoms of AGI – i.e., a waterborne infection may be asymptomatic (without clinical disease) or symptomatic (clinical disease observable) [21] – immunological biomarkers of host response can be used to identify a causative pathogenic agent and estimate symptomatic and/or asymptomatic waterborne disease burden. Knowledge of the waterborne pathogens responsible for asymptomatic infections can improve estimates of waterborne infections in source populations and advance understanding of upstream risk factors and transmission routes. Not knowing these can hinder the development of effective prevention strategies to reduce waterborne outbreaks and/or contamination events (e.g., via infrastructure improvements prior to onset of symptoms).

We review the challenges in measuring population burdens of infection that can be attributed to waterborne versus other transmission routes (contaminated food, hygiene, sanitation, person-to-person and animal-to-person contact). Antibodies as biomarkers of waterborne infections are then discussed to highlight their current and future utility in population-based settings. Antibody responses to specific pathogens are described as they

relate to measuring immunoconversions (defined as a change from antibody negative to antibody positive in serial samples or a four-fold increase in antibody titer in serial samples), rates, and time-intervals of infection. The use of antibody biomarkers in serum are presented, followed by the discussion of novel salivary antibody biomarkers and their potential to improve upon estimates of waterborne infections. The utility of antibody biomarkers for detection of acute and chronic infections in population-based settings is discussed, including how estimates of the incidence of acute short-term infections can be obtained within the context of both cross-sectional and prospective study designs. Finally, the technical challenges involved with using minimally-invasive saliva samples as a matrix for the detection of pathogen-specific antibodies are presented along with future directions for salivary immunoassay work.

Challenges with epidemiologic estimates of waterborne AGI in population-based settings

The outcome most commonly employed in epidemiologic studies of waterborne disease is self-reported AGI symptoms. Because most AGI symptoms are self-limited, only a small proportion of the individuals who experience AGI actually seek medical care and have a stool sample submitted for testing. Furthermore, clinical diagnostic laboratories are not

always able to identify a pathogenic agent responsible for AGI symptoms [22]. Thus only a small proportion of AGI disease will be captured by studies of, or reporting systems involving, patient populations seeking a clinical diagnosis (Figure 1). AGI symptoms are also non-specific, with numerous pathogens and transmission routes that must be investigated in order to determine the etiologic agent. These features of AGI symptoms mean that epidemiologic studies that rely upon AGI as a primary outcome may not provide an accurate estimate of the population burden of disease. The ability to determine a host's immunologic response to specific pathogens that are responsible for waterborne infections could improve the specificity and decrease the misclassification of AGI in epidemiologic studies. Biomarkers of pathogen-specific host immunologic response could improve studies of the effects of improved water treatment and/or source water protection as well as advance understanding of pathogen exposure (e.g., spatial and temporal distribution) and modifiable factors that are associated with progression from asymptomatic to symptomatic states of infection (e.g., natural history and ecology of disease) in populations. For example, objective biomarkers of asymptomatic waterborne infections have helped identify low water pressure at the faucet as an important risk factor for self-

reported diarrhea in the control group of a case-control study of sporadic cryptosporidiosis [23].

Most evidence of waterborne transmission in developed countries comes from outbreaks of infectious diseases. In the United States, the Centers for Disease Control and Prevention (CDC) as well as state and local authorities investigate outbreaks and attempt to identify the source. CDC publishes the biannual Morbidity and Mortality Weekly Report on outbreaks associated with drinking and recreational water sources. For example, in 2011-2012 for drinking water a total of 32 outbreaks were reported and associated with 431 illnesses, 102 hospitalizations and 14 deaths [16]. For recreational water in 2011-2012, there were 90 outbreaks that resulted in at least 1,788 cases, 95 hospitalizations, and one death [24].

Knowledge of the pathogen-specific etiology of waterborne infections would help identify different risk factors and transmission routes, which can improve the evidence base for decision-making about management and prevention strategies. A classic example of this is the massive waterborne outbreak of cryptosporidiosis in Milwaukee in 1993 when the chlorine-based disinfectant used had little effect on *C. parvum* oocysts and the drinking

water treatment plants consequently had to investigate alternative disinfectants such as UV light [25]. Another example is a study of the presence of enteric viruses in non-disinfected drinking water from municipal wells and their relation with community incidence of AGI [26]. In this study the authors noted a positive association between norovirus genogroup I (GI) and AGI. But the associations between the presence of other enteric viruses – adenovirus and echovirus serotypes – and AGI were not statistically significant. This lack of association could be due to misclassification and/or the non-specificity of AGI as an outcome in epidemiologic studies (e.g., potential influence of measurement error due to participant self-reporting of AGI symptoms).

Waterborne outbreaks usually occur from causative factors such as weather events, wastes from animals, agriculture, or humans and failures in water treatment [27]. Drinking water associated outbreaks are often caused by contaminated source waters, inadequacies in treatment, or contamination occurring within the distribution system [28]. Whereas, recreational water associated outbreaks have been attributed to swimming in waters impacted by inadequate chlorination or other disinfection (swimming pools), fecal contamination shed by swimmers (swimming pools and natural waters),

runoff from publicly-owned treatment works (POTW) wastewater effluents, sanitary and combined sewer overflows of untreated sewage, private on-site septic systems, agricultural production, and wildlife [29].

Most cases of waterborne infections are sporadic or diffuse, low-level outbreaks. Ingestion of waterborne pathogens can also result in a completely asymptomatic infection depending on the interplay of pathogen-specific and host-specific factors, such as a pathogen's virulence and a host's immune response [30]. They may be caused by deficiencies in drinking water treatment, resulting in contamination with waterborne pathogens, and transmission to consumers [31]. Waterborne pathogens that are resistant to chlorination (especially *Cryptosporidium* spp.)[32] or physical removal (especially viruses) can pass through the water treatment barrier and contaminate tap water even when water quality indicators based on surrogate bacteria (total and/or fecal coliforms, *E. coli*) are within the regulatory limits [33]. Viruses, such as noroviruses, can filter through the soil, contaminate shallow ground water sources and present a health risk in drinking water systems that are ground water supplied and do not use chemical disinfection [26]. Individual sporadic cases of AGI usually cannot be linked to a specific

source in the framework of routine surveillance, contributing to the underestimation of waterborne infections in the population.

Antibody biomarkers of waterborne infection

Specific antibody responses can be used as biomarkers of infection in epidemiological studies to estimate the prevalence and incidence of infections and to assess the contribution of waterborne transmission. Different pathogens result in different temporal distributions of antibody response and infection. Both symptomatic and asymptomatic infections typically cause an antibody response in the host [31]. A pre-existing antibody response can be a factor affecting host's susceptibility to re-infection or the probability of developing symptoms if infection occurs [34]. The presence of antibodies specific to the pathogen of interest in biological samples (e.g. serum, saliva, stool, breastmilk) is an indication of current or prior infection [31]. The different immunoglobulin isotypes (IgG, IgA, IgM) have different utility as estimates of population disease frequency and burden. Single time point measurements of pathogen-specific IgG have utility as an estimate of historical/prior exposure or prevalent infection whereas IgA and/or IgM have utility as an estimate of acute-phase or incident infection [35, 36]. Immunoconversion is used to detect incident infections in prospective

survey settings. This change from an antibody-negative sample to an antibody-positive sample in a time series of two or more samples, also defined as a four-fold increase in antibody titer in a time series of two or more antibody-positive samples, is used to measure new, acute cases in a defined population over a defined time period [37-39].

Serologic antibody response

Serum is the most accurate and widely used matrix to monitor population immune responses to pathogens. Sera can be collected by sampling the population or residual blood banks can be used. However, there are significant drawbacks to both since blood collection requires trained individuals to visit participants [40] and may be cost prohibitive along with low response rates that have been shown in Europe due to the invasive nature of blood collection [41, 42]. Its application in prospective studies and especially in studies involving children is problematic due to high attrition and low compliance [43]. Relying on previously collected samples from sera banks overcomes these issues however they are usually anonymous with limited data available on the patient and importantly, their background as it pertains to water, sanitation, and hygiene-related behaviors and activities [44]. However, a number of studies have successfully used sero-epidemiological

methods in the context of waterborne disease [45, 46]. Frost et al. found that people who live in cities using surface-derived drinking waters had an increased risk of *Cryptosporidium* infection compared to those using drinking water from municipal groundwater sources [45]. And in the context where sanitation conditions are poor and clean water supplies are limited, Priest et al. found IgG antibody responses during *Cryptosporidium* infections with *C. parvum*, *C. felis*, and *C. meleagridis* and with four different subtypes of *C. hominis* [46].

Salivary antibody response

The utility of novel salivary antibody biomarkers as a measure of host immune response to specific pathogens has the potential to improve upon estimates of waterborne infections that rely on invasive collection of serum. Saliva collection is minimally invasive and can be self-collected and returned by mail[47], allowing for a larger sampling of the population than is possible with serum. Saliva is a mixture of secretions from salivary glands. Oral fluid contains saliva (enriched with secretory IgA), crevicular fluid (flows from between the gum margins and teeth), and is enriched with serum antibodies [48]. Some oral fluid sampling techniques are specifically designed

to collect samples enriched with crevicular fluid for measurements of systemic antibody responses [49-51].

Salivary assays have been used to identify various viral, bacterial and parasitic infections [52] (see Table 2). Measuring antibodies in saliva is appropriate for both children and adults, and is suitable for population-based surveillance settings [38]. Salivary immunoassays have been developed for pathogens such as *Helicobacter pylori, T. gondii, Cryptosporidium*, and noroviruses [50]. Griffin *et al*. (2011) applied the Luminex xMAP microsphere-based technology (Luminex Corp., Austin, TX) assay to measure antibodies to multiple pathogens within a single saliva sample volume [50]. The Norwalk virus assay developed in Griffin et al. (2011) was subsequently validated using samples from a human volunteer challenge study [51]. A similar salivary immunoassay is being applied to measure the incidence of norovirus infections following recreational water exposures at beaches in Puerto Rico, Iowa, and Wisconsin where saliva has been collected as part of the Environmental Protection Agency's National Epidemiologic and Environmental Assessment of Recreational Water Study[53].

An important challenge in using saliva to measure immunologic responses is the greater inter- and intra-individual variability in saliva composition and immunoglobulin levels. While saliva contains a high level of secretory IgA (SIgA) antibodies, there can be significant diurnal, age, and oral health-related variability[54], making these factors important to consider in community-based field studies. The salivary concentrations of IgG and IgM isotypes are lower than in serum. Thus, a salivary antibody assay targeting IgG has to be sensitive enough to quantify low intensity antibody responses. Typically it is necessary to assay saliva at relatively low dilutions, where matrix effects (e.g. inhibition, high background signal) can be pronounced in some pathogen-specific antibody assays [55]. For each pathogen-specific antibody target it is critical to optimize the conditions that may influence assay performance and sensitivity and specificity [51].

There is scant evidence on the temporal patterns of salivary antibody responses to infection with a specific pathogen (peak levels and rates of decline for different antibody isotypes). Our current understanding of generalized trajectories (Figure 2) comes from prospective studies using serum or saliva from individuals with confirmed infections, such as volunteer challenge studies for norovirus[38, 56, 57], *Cryptosporidium*[58], *Giardia*

lamblia[59, 60], and *Shigella*[61]. The pattern of antibody isotypes may be used in diagnostic and research settings to provide information on the infection state (acute versus convalescent) and to assess the timing of infection [31]. Typically, the IgA and/or IgM response to a waterborne pathogen ramps up before the IgG response [34, 56, 57]. The generalized trajectories of different antibody isotype levels during a transient acute infection from a waterborne pathogen are depicted in Figure 2. After the convalescent stage, IgG pathogen-specific antibodies may remain detectable for weeks to years, depending on the causative agent, and may remain elevated above pre-infection levels [34, 62]. There can be vast differences in these temporal patterns of antibody responses depending on the pathogen causing the infection. Thus, an area of future work is to develop population-based antibody infection curves for specific waterborne pathogens.

Platforms and assay types

Various immunoassay platforms have different costs, quantitation levels, dynamic ranges and multiplexing potentials [63]. The most basic of these platforms is the indirect enzyme immunoassay, however the low through-put and high sample volume requirements make it less desirable for population based analyses where multiple pathogens are being analyzed and sample

volume is limited. Multiplex immunoassays, such as those based on the Luminex (Luminex Corp., Austin, TX) microbead suspension fluorescence immunoassay platform, require a low sample volume to analyze multiple pathogen-specific antibody analytes simultaneously. They are also less labor intensive because more data are generated per test/analyte, and thus are more cost-effective [50, 51, 64-67]. Another immunoassay platform that is used and allows multiplexing is the Meso Scale Discovery (MSD; Rockville, MD) electrochemiluminescence (ECL) platform. Platforms that facilitate multiplexing can be used to expand the range of available options for testing the signal of pathogen-specific antibody responses as well as background signals. The adjustment of the pathogen-specific antibody signal for background signals, such as those produced by total IgA or total IgG or by antigen tags such as glutathione-S-transferase (used during antigen purification), can improve the performance of antibody assays [50, 51]. Multiplexing of these target signals can also reduce excess use of biospeciment sample volume because all signals can be measured in the one sample volume in a single reaction well. Thus multiplexing testing platforms can facilitate a broader application of antibody testing in community-based epidemiologic investigations of diverse waterborne pathogens.

Applications of pathogen-specific antibody biomarkers in population-based studies of waterborne infections

To improve current epidemiologic estimates of AGI from waterborne pathogens in population-based settings, pathogen-specific antibody biomarkers can be used. For chronic infections, antibody responses can be positive or negative, and can be validated against diagnostic tests. The proportion of IgG positive results in serum or saliva can serve as a direct measure of infection prevalence in the population [68, 69]. In contrast, for acute short-term infections, such as noroviruses and *Cryptosporidium*, the presence of pathogen-specific antibodies in serum or saliva may indicate an ongoing infection or more commonly a past infection with or without symptoms. Thus, the concept of "positive" antibody response to an acute short-term infection or seroprevalence of positive responses, often reflect the proportion of results above an arbitrary threshold, such as a detection limit of the method or by standardizing response intensities to the response of a reference sample of positive control sera [70-73] or saliva.

One approach to estimating incidence of acute infections using antibody data is to use immunoconversion in prospective study settings as a marker of new infections. The sensitivity and specificity of an immunoconversion test is related to its ability to detect infections that occurred during the interval

between two sampling dates. In prospective studies, biological sampling (serum or saliva) can be combined with symptoms diaries to produce information on the association of certain infections with specific types of symptoms and/or the association of exposures with infections or interventions (designed to reduce exposure) with a lack of symptoms [74].

Prior studies have used pathogen-specific antibody markers and demonstrated their ability to identify waterborne infections that were more widespread than previously appreciated. In the massive Cryptosporidium outbreak in Milwaukee in April 1993, a retrospective analysis was conducted with banked serum specimens from children that had routine lead level surveillance in blood from March to May of that year and showed a seroprevalence increase from 15-17% to 82-87% for levels of IgG antibody against the immunodominant Triton-17 and 27-kDa *C. parvum* antigens [75]. This demonstrated that the outbreak had affected a greater proportion of the population with infection when accounting for both symptomatic and asymptomatic infections than the previous estimate of 26% that only surveyed the population using the cryptosporidiosis case definition (watery diarrhea) [76]. Teunis et al. applied these approaches in the European Union to estimate seroconversion rates for *Campylobacter* infections and found that

they were several orders of magnitude higher than the notification rates, reflecting both detection deficits in the surveillance and the reality that these enteric infections often remain asymptomatic[77]. Frost et al. used serum antibodies to *Cryptosporidium* from a population in Hungary to determine that those using groundwater had significantly lower serological responses than those using conventionally filtered and disinfected surface water and found that riverbank filtration may be an effective alternative treatment to reduce *Cryptosporidium* exposures and infections for individuals using surface water sources [78]. Tollestrup et al. focused on non-outbreak settings where a low probability of outbreak detection should be expected and found a significant association for residents in the River Valley of New Mexico using onsite wastewater systems combined with private wells to have a strong response to the 27-kDa *Cryptosporidium* antigen [73]. And lastly, in the first postal population-based survey that used saliva, Morris-Cunnington et al. used approximately 5,500 self-collected oral fluid samples along with a questionnaire of demographic and social information to successfully demonstrate that antibody prevalence data along with risk factor data can be used assess the population-based immunity to common viral infections in England and Wales [47].

Such application of immunological biomarkers in epidemiologic studies also can improve knowledge of the temporal patterns of antibody responses, which can be used to extrapolate incidence estimates based on cross-sectional data on pathogen-specific antibody responses in the population [77, 79, 80]. Others have expanded this approach using parametric statistical models [65, 81-83] to determine incidence of infection based on pathogen-specific antibody results from a single cross-sectional sampling time. The person-to-person variability in antibody responses to a specific pathogen and limited data on temporal patterns of antibody responses in various populations affects the precision of such estimates. A pattern of antibody responses may also be affected by the number of prior infections and the time interval from the previous infection. This may further limit the applicability of the available antibody pattern data to populations with comparable epidemiological characteristics or to research questions focused on intra-individual variability in antibody responses over time.

In low-income communities where there is less developed drinking water and wastewater infrastructure and individuals may experience repeated exposures to multiple waterborne pathogens, the application of immunological biomarkers can be used as a monitoring and evaluation tool

for infrastructure and point-of-use interventions. The multiplex immunoassay methodology targeting salivary IgG and IgA responses to potentially waterborne pathogens[50] can be applied as a minimally invasive and objective exposure and outcome screening tool to assess the efficacy of interventions designed to reduce pathogen exposure and/or AGI illness within a specified population. Such multiplex pathogen antibody screening tools could improve the objectivity of water, sanitation, hygiene, and health programs and interventions. Integration of these biomarkers into monitoring activities for the Sustainable Development Goals recently adopted at the 2015 UN Summit (https://sustainabledevelopment.un.org/topics), could improve the evidence base for Goal 6 which is to "by 2030, achieve access to adequate and equitable sanitation and hygiene for all and end open defecation" (Target 6.2)[5].

Challenges and perspectives for future work

Pathogen-specific antibody assays represent a promising tool for understanding the relative contribution of waterborne versus other pathways to infectious disease burden in population-based settings. However, assays based on invasive serum specimens may fail to capture a majority of cases in

[5] https://sustainabledevelopment.un.org/topics/waterandsanitation

population-based field studies. Because they can be self-administered and returned by mail, salivary antibody assays may increase participation in surveys of potentially waterborne infections in populations that are difficult to reach, including children, pregnant women, and individuals living in remote, resource-limited settings. Saliva collection can also be self-administered and returned by mail to reach a larger proportion of the general population. This may facilitate a more fine-scale, spatio-temporal study of the ecology and natural history of waterborne disease, including elucidation of optimal points of intervention to prevent waterborne pathogen transmission.

While such minimally invasive pathogen-specific salivary antibody biomarkers are promising, challenges remain in their broad application to diverse pathogen exposures and infections. Not all pathogens elicit a robust systemic or salivary antibody response. Additionally, a majority of waterborne infections may be asymptomatic and not result in adverse health effects. Therefore, the incidence of infections estimated from cross-sectional antibody data may not be representative of disease burden but only reflect recent or historical exposure to a pathogen [84]. Nevertheless, cross-sectional antibody response data can provide an improved estimate of human

exposure to certain pathogens and can be used as an epidemiological tool to estimate the contribution of waterborne versus other pathways to the total infection pressure. However, the underlying infection and immune response to the pathogen must be considered in the interpretation of cross-sectional seroprevalence estimates and depends on whether the infection results in lifetime immunity following one exposure or the infection is acute and immunity wanes following exposure.

The detection of cytokines in serum and saliva also presents an opportunity to measure the onset of waterborne infections. However, cytokines are not capable of identifying a specific causative agent, rather they are more generic biomarkers of infection. The hallmark for a viral infection begins with a wave of cytokine production [85] and their presence can be employed as a marker of infection (Table 2). Cytokine levels in serum of individuals infected with norovirus that were shown to be significantly increased included IFN-gamma, IL-6, IL-8, IL-12p70, MCP-1 and TNF-alpha two days following exposure [86]. Evidence has shown that the elevation of cytokines in a newborn's salivary gland epithelium promotes secretory immunity [87]. Proinflammatory cytokines can upregulate the polymeric Ig receptor (pIgR), including IL-17, which is particularly abundant at mucosal

sites [88]. The extracellular part of pIgR is essential for resistance against proteolytic degradation of the secretory component of secretory IgA found in saliva and gut mucosa [89]. A challenge in using cytokines in saliva is to determine if there is a serum-saliva association, for which there is currently limited evidence [90]. Although elevated levels of IL-6, which has a major role in the regulation of inflammatory bowel diseases, was found to be elevated in both the saliva and serum of patients when compared to reference persons [91]. There could also be specific hyper-inflammatory physiological states (systemic infection/sepsis, burns, etc.) when more of the variance in salivary levels of cytokines could be due to systemic circulating cytokine levels [90]. An area for future study is identifying if a specific waterborne pathogen generates a unique or predictive cytokine profile that is observable in both saliva and serum.

Conclusion

The ability to estimate waterborne infections via measurements of host immunological response at the population-level is improving as technological and analytical advancements are made. Diagnostic advancements are enabling a paradigm shift in how waterborne infections can be measured, not just in clinical settings or outbreak settings but also

more widely as tools for population-based screening of incidence and prevalence. The measurement of salivary antibody responses to specific pathogens as biomarkers of waterborne infection hold great potential to expand surveillance to reach larger numbers of people in diverse population-based settings. Future work lies in the development of sensitive and specific multiplexed serum and salivary immunoassays to measure exposures to, and infections with, specific waterborne pathogens.

Compliance with Ethics Guidelines

Conflict of Interest Douglas A. Granger is the founder and chief scientific and strategy advisor at Salimetrics LLC and SalivaBio LLC and these relationships are managed by the policies of the committees on conflict of interest at the Johns Hopkins University School of Medicine and the Office of Research Adherence and Integrity at Arizona State University. All other authors declare no conflict of interest.

Human and Animal Rights and Informed Consent This is a review article which does not report new results of human or animal subjects performed by the authors.

Figure 1. The Iceberg Concept of Waterborne Infection Surveillance

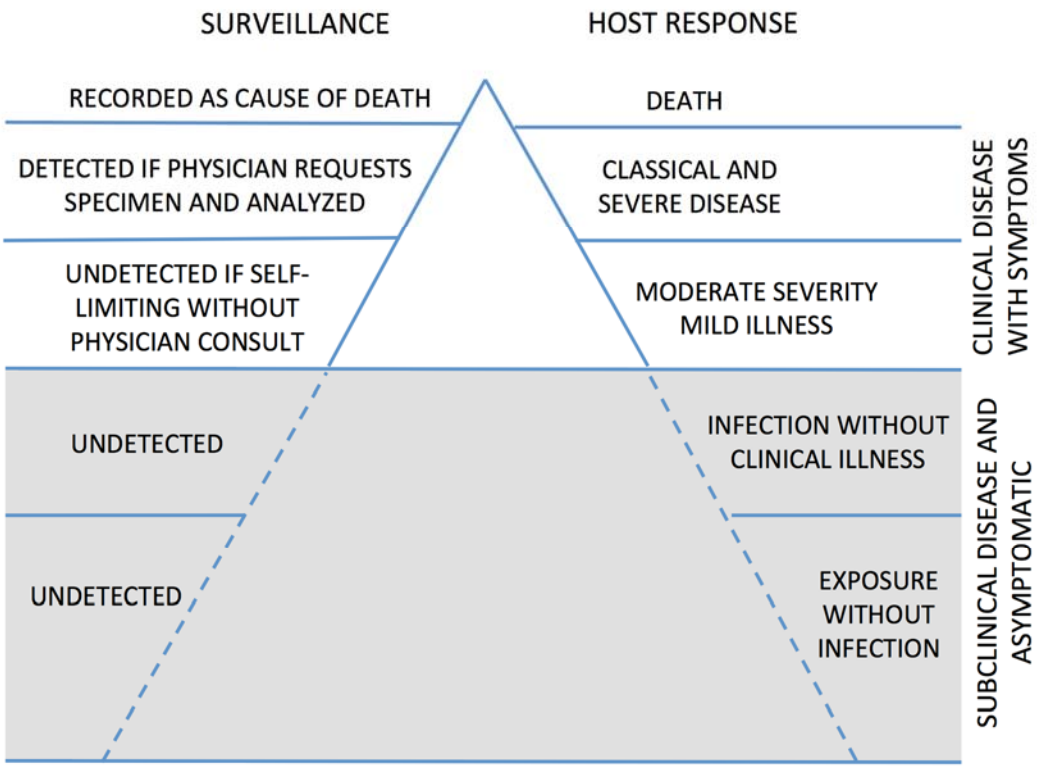

Figure 2. Trajectories of antibody titers during infection from a waterborne pathogen

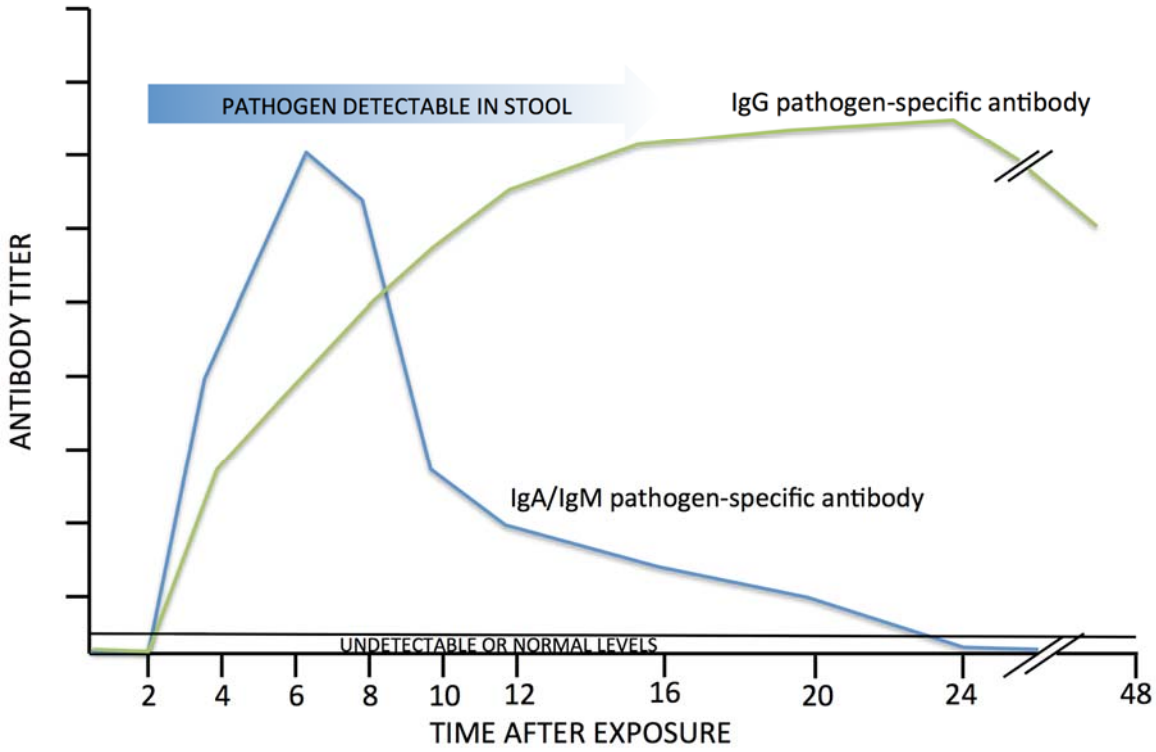

Table 1. Data sources that provide estimates of the most common waterborne pathogens attributable to the burden of waterborne infections.

Region	Date source	Top waterborne pathogens identified
United States	CDC Morbidity Mortality Weekly Report (MMWR) Surveillance for Waterborne Disease Outbreaks Associated with Drinking Water, 2011-2012 [16]	Norovirus, and Shiga toxin-producing *E. coli*
	CDC MMWR for Outbreaks of Illness Associated with Recreational Water, 2011–2012 [24]	*Cryptosporidium* spp.
Developing countries	The Global Enteric Multicenter Study (GEMS) [17]	Rotavirus, *Cryptosporidium* spp., *Shigella*, *Giardia* spp.,[6] *Vibrio cholerae,*[7] *Campylobacter* spp[2]
	The Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development Project (MAL-ED) [18]	*Giardia* spp.[8]
	Ishii et al (2015)[19] and Hoofnagle et al (2012) [20]	Hepatitis A and E virus[9]

[6] In univariate analyses *Giardia* was identified significantly more frequently in controls than in patients with moderate-to-severe diarrhoea aged 12–59 months in ten of the 14 age-site strata {Kotloff, 2013 #193}.
[7] Important in selected sites in GEMS study[17].
[8] *Giardia* spp. was in the top five pathogens for highest prevalence in diarrheal and non-diarrheal stools for both 0-11 month and 12-24 month age groups[18].
[9] Hepatitis A and E viruses are the most common causes of feces-transmitted acute viral hepatitis worldwide.

Table 2. Immunologic biomarkers for waterborne pathogens with highest attributable global acute gastrointestinal disease burden

PATHOGEN OF INTEREST	SPECIMEN	IMMUNOLOGIC BIOMARKER RESPONSE	REFERENCE
Cryptosporidium spp.	Serum	IgG antibody	Priest, J. W., et al. (2001) [92]; [58]; Crump, J. A., et al. (2007) [93]; Sarkar, R., et al. (2012) [94]; Becker, D. J., et al. (2015) [95]; Checkley, W., et al. (2015) [96]
	Saliva	IgG and IgA antibody	Cozon, G., et al. (1994) [97]; Moss, D. M., et al. (2004) [67]; Egorov, A. I., et al. (2010) [98]; Griffin, S. M., et al. (2011) [50];
Campylobacter	Serum	IgG, IgM and IgA antibodies	Ang, C. W., et al. (2011); [84]; Teunis, P. F., et al. (2012) [79]; Rokosz-Chudziak, N. and W. Rastawicki (2014) [99].
	Stool	Cytokines (IL-1β, IL-6, IL-8, TNF-α, and IFN-®), IgA antibodies	Tribble, D. R., et al. (2010) [100]; Islam, D., et al. (2014) [101];
	Saliva	IgG and IgA antibodies (responses to acid-glycine extracts of *C. jejuni* strain 81116 and an aflagellate mutant, and a whole-cell R2 sonicate)	Cawthraw, S. A., et al. (2002) [102]
Giardia intestinalis	Serum	IgG and IgA antibodies	Crump, J. A., et al. (2007) [93]; Jiménez, J. C., et al. (2009) [103]; Priest, J. W., et al. (2010) [64]; Moss, D. M., et al. (2014) [66]
	Saliva	sIgA, IgA and IgG antibody (responses against *G. duodenalis*)	Rodriguez, O. L., et al. (2004) [104]; El-Gebaly, N. S., et al. (2012) [105]
Hepatitis A virus	Serum	IgM and IgG antibodies	Vitral, C. L., et al. (2014) [11]; Hundekar, S., et al. (2015) [106]
	Saliva	IgM and IgG antibodies	Laufer, D. S., et al. (1995) [107]; Ochnio, J. J., et

			al. (1997)[108]; Morris-Cunnington, M. C., et al. (2004) [47]; Tourinho, R. S., et al. (2015) [109]
Hepatitis E virus	Serum	IgG and IgM antibody, cytokines (IL-5, IL-6, IL-8, IL-10, IL-2, IFN-γ, TNF-α, TGF-β1, IL-1β)	Adjei, A. A., et al. (2009) [110]; Pas, S. D., et al. (2013) [111]; Wu, W. C., et al. (2014) [36]; Kumar, A., et al. (2014) [112]; Gu, G., et al. (2015) [113]; Cong, W., et al. (2015) [35]; Heaney, C. D., et al. (2015) [114, 115]
Norovirus	Serum	IgG and IgA antibodies, cytokines (IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and IL-12, IFN-γ, TNF-α)	Erdman, D. D., et al. (1989) [62]; Monroe, S. S., et al. (1993) [37]; Moe, C. L., et al. (2004) [38]; Lindesmith, L., et al. (2005) [56]; Crump, J. A., et al. (2007) [93]; Newman, K. L., et al. (2015) [86]
	Stool	IgA antibody	Iritani, N., et al. (2007) [116]; Ramani, S., et al. (2015) [117]
	Saliva	IgA and IgG antibodies	Moe, C. L., et al. (2004) [38]; Lindesmith, L., et al. (2003) [57]; Lindesmith, L., et al. (2005) [56]; Griffin, S. M., et al. (2011) [50]; Griffin, S. M., et al. (2015)[51]
Rotavirus	Serum	IgM, IgA and IgG antibodies, cytokines (IFN-γ, TNF-α, IL-8, and IL-10)	Grimwood, K., et al. (1988) [118]; Azim, T., et al. (2003) [119]; Xu, J., et al. (2005) [120]; Premkumar, P., et al. (2014) [121]; Sindhu, K. N., et al. (2014) [122]; Moon, S. S., et al. (2015) [123]
	Stool	IgM, IgA and IgG antibodies	Stals, F., et al. (1984) [124]; Grimwood, K., et al. (1988) [118]; Azim, T., et al. (2003) [119]
	Saliva	IgM, IgA and IgG antibodies	Stals, F., et al. (1984) [124]; Grimwood, K., et al. (1988) [118]; [125]; Friedman, M. G., et al. (1996) [126];
Shiga toxin-producing *Escherichia coli*	Serum	IgG antibodies against 51 O serogroup strains, B subunit of Stx2 and Stx1	Ludwig, K., et al. (2001) [127]; Kulkarni, H., et al. (2002) [128]; Fernández-Brando, R. J., et al. (2011) [129]; Guirro, M., et al. (2014) [130]
	Saliva	IgM and IgA antibodies	Ludwig, K., et al. (2002) [131]; Chart, H., et al. (2003) [132]

Shigella	Serum	IgA, IgM and IgG subtypes to *S. sonnei* O-antigen, IgA and IgG antibodies to *S. flexneri* 2a lipopolysaccharide, total IgA antibody-secreting cells (ASC) and anti-LPS IgA ASC, cytokines (IFN-γ, TNF-α, TNF-β, IL-4, IL-6, TGF-β)	Van De Verg, L. L., et al. (1996) [133]; Raqib, R., et al. (1997) [134]; Rasolofo-Razanamparany, V., et al. (2001) [135]; Levine, M. M., et al. (2007) [136]; Muhsen, K., et al. (2014)[137]; Thompson, C. N., et al. (2014) [138]
	Stool	Cytokines (TNF-α, IL-6)	Azim, T., et al. (1995) [139]
	Saliva	IgA antibody	Schultsz, C., et al. (1992) [140];
Vibrio cholerae	Serum	IgA and IgG antibodies, IgG, IgM, and IgA ASC	Chowdhury, F., et al. (2008) [141]; Johnson, R. A., et al. (2012) [142]; Fujii, Y., et al. (2014) [143]; Khan, A. I., et al. (2015) [144]
	Stool	IgA antibody	Qadri, F., et al. (2003) [145]
	Saliva	IgA antibody	Jertborn, M., et al. (1986) [146]

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Chapter 5

Secretory IgA in the saliva of children is associated with household sanitation conditions and fecal contamination in the Peruvian Amazon

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Abstract

We assessed the relationship between fecal contamination of the household environment and secretory immunoglobulin-A (SIgA) in the saliva of children between three and four years old. We compared the SIgA marker between 69 children in a peri-urban community of Iquitos Peru with a history of numerous enteric infections and limited access to water and

sanitation infrastructure. Fecal contamination was assessed both by questionnaire for the household water, sanitation and hygiene characteristics along with quantification of *E.coli* on floors, tables and drinking water. Adjusted for potential confounders, children in households with pit latrines versus those with flush toilets to a septic had reduced SIgA (-0.17 $\log_{10}$ SIgA ug/mL (95% CI: -0.24, -0.10) and -0.11 $\log_{10}$ SIgA/TP (95% CI: -0.21, -0.008) and children from homes with greater *E.coli* contamination in their drinking water had higher levels of SIgA (comparing the third highest quartile to the lowest quartile with +0.11 log10 SIgA ug/mL [95% CI: 0.03, 0.19]). These results demonstrate the ability for salivary SIgA to differentiate between households using different sanitation options within a community. They also validate the proof-of-concept for using salivary SIgA as an objective outcome in field-based studies and justify further investigation in studies with larger sample sizes to detect differences in pre and post intervention settings.

Introduction

Children who grow up in extreme poverty without safe water and and adequate sanitation often suffer from repeated enteric infections and diarrhea due to high fecal contamination in their household environments. When

these pathogens are introduced early in life, during the critical period of gut development under 24 months of age, they may damage the absorptive capacity of the intestine contributing to malnutrition and result in long-term growth deficits.[1] Enteric infections can also compromise the intestinal barrier and increase intestinal inflammation leading to the condition of environmental enteropathy (EE),[2] though the mechanisms involved with immune response are poorly understood.[3] Therefore, there is a need to understand how fecal contamination in the environment impacts the mucosal immune system as it contributes to EE in children.

Research that associates water, sanitation and hygiene conditions of children's living environments to their growth outcomes has increased substantially in recent years[4-8] and necessitates investigation into how fecal contamination can impact the underlying biological mechanisms.[9] Limited studies have examined the impact of unsanitary environmental conditions on gut barrier function, absorptive capacity of the small intestine and intestinal inflammation.[7, 10, 11]. There has been even less work done to understand the mucosal immune system response of children in environments without safe water and adequate sanitation. Research has shown that recurrent enteric infections during infancy and other factors indicating increased microbial

pressure were associated with high levels of secretory immunoglobulin A (SIgA) in saliva.[12] And results from a study of children in a slum of São Paulo speculated to have EE suggested that environmental factors influenced the early development of the SIgA system.[13]

Salivary SIgA has potential to be a robust marker for microbial exposure in an EE cohort where children are undernourished. The SIgA response in salvia has been shown to illustrate the response in the gut to antigenic exposure.[14] In a study that measured SIgA abundance and affinity in well-nourished and malnourished groups of children from São Paulo, no differences were observed suggesting that in this respect their immune system was not impaired.[15] On the other hand, differences in SIgA in saliva have been observed in Pakistani infants who were heavily exposed to *Escherichia coli* from birth where their antibody levels increased significantly by 2 and 3 weeks of age[16] compared to less exposed Swedish infants where such levels for both for total SIgA and SIgA antibodies to *E. coli* O antigens were not reached until 1 year of age[17] and the differences between the two groups is possibly explained by the differences in the antigenic exposure.[18] Interestingly, the same salivary SIgA response was not observed in the serum IgA antibody response which remained low in both

groups. This illustrates that antigen exposure on the mucosal system may result first in production of mucosal antibodies.[16]

The aim of this study was to investigate the associations of salivary SIgA in children enrolled in an EE cohort to the fecal contamination in their household environments. We hypothesized that children living in households with conditions more likely to foster enteric pathogens would have higher concentrations of SIgA in their saliva. Children enrolled in the MAL-ED[10] cohort study in Iquitos, Peru between the ages of 3 and 4 years old were sampled for saliva. The varying water, sanitation and hygiene conditions in their home were characterized at the same time their floors, tables and drinking water in each home were sampled to quantify the number of *E. coli* bacteria.

Methods

Study community

This study took place in three peri-urban communities of Iquitos, Peru (3°47'S, 73°20'W) located next to the Nanay River, Santa Clara de Nanay,

[10] Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development

Santo Tomas, and La Union. The tropical conditions of the Amazon river system are highly favorable to pathogens to persist in the environment. There is an average temperature of 25.8 degrees Celsius[19] and rainfall is frequent and occurs throughout the year.[19] Diarrheal incidence is high in children 12-23 months of age when compared to the literature in the last decade[20] with 4.38 episodes per child-year.[21] Stunting prevalence in the study community is also remarkably high with 46.3 percent of children under 5 years old classified as stunted (height for age z-score < -2)[19] when compared to the rates in Africa and Asia where 35.6 percent and 26.8 percent of children under 5 years old are stunted, respectively.[22] In a prior cohort study in Santa Clara frequent causes of bacterial diarrhea were *Shigella*, *Campylobacter*, and *enterotoxigenic E. coli*.[21] Norovirus is also thought to be a significant cause of diarrhea and has been found in 21.3 percent of diarrheal stool samples and 3 percent of non-diarrheal samples.[23] *Giardia lamblia* had a higher presence in asymptomatic stool samples with 21.3 percent compared to symptomatic diarrheal samples with 18.9 percent. The communities lack centralized sewerage infrastructure and are prone to frequent fecal contamination from onsite storage of human feces in either pit latrines or septic systems[21] that can overflow during flooding from the

Nanay River or when fecal matter is not hygienically emptied, transported and/or treated.

Water, sanitation and hygiene household characterization

Households were characterized for their household water, sanitation and hygiene (WASH) conditions at the beginning of the study in May and June of 2015 and at the end during August and September of 2015. During each household visit, a household questionnaire was administered in Spanish based on the Demographic and Health Surveys[24] and was a shortened version of the standardized questionnaire used during the MAL-ED study. The questionnaire assessed the type of sanitation facility used by household members and whether or not this facility was shared, the household's primary water source, mode of water treatment, time it takes to fetch water, hygiene behavior and crowding. Information was also collected on socio-economic factors such as housing construction materials, length of tenancy, electricity access, maternal education, and monthly income. Given the propensity for households to keep free-ranging or corralled chickens in this community, participants also were interviewed in a separate survey regarding the presence of chickens in the home to evaluate the influence of chicken feces on bacterial contamination in the household.

Household E. coli sampling and evaluation

Household floors, tables and drinking water were sampled at the beginning and end of the study in May to June and August to September of 2015. The floors and tables were sample for *E. coli* bacteria using a modified dry electrostatic cloth method based on one designed for household settings.[25] The highly trafficked floor areas were sampled near the entrance and in the kitchen area where cooking activities were performed to represent the likely encounters that children have with fecal contamination on floors. Different floor material types (e.g. dirt, wood, cement) were recorded at both locations at the time of sampling to determine if the varying surface types influenced the presence of *E. coli* bacteria. A sample of drinking water was collected during the sampling at the end of the study by requesting a glass of water from the main interviewee in the same manner they would fetch one for themselves. Prior to field collection, sterile packets of dry electrostatic cloths (Swiffer™; Proctor & Gamble, Cincinnati, OH) were prepared and autoclaved as previously described (Chapter 3, Methods Section). For each collection on floors and tables an adapted protocol from Davis et al. (2012) was used where a prepared cloth was passed over a 30 cm by 30 cm surface with medium pressure to maximize the amount of pick-up from the surface

[25]. The cloth was then placed into a sterile 700 mL Whirlpak bag (Nasco, Fork Atkinson, WI) and 5 mL of sterilized Milli-Q ultra-pure water to guard against microbial desiccation during transport. All surface and water samples were stored in a cooler on ice at 4°C during field collection and transported to the laboratory. Samples were processed within six hours of collection and enumerated following USEPA Method 1604[26] using m-coliblue24 commercial media (HACH, Loveland, U.S.A.). Results were reported in colony forming units (CFU) per 900 cm^2 sampled for floors and tables and CFU per 100 mL of water.

Saliva collection and analysis

Children under 48 months of age who were enrolled in the MAL-ED cohort were eligible for the study. The children were visited weekly for saliva collection over a three month period from June through August 2015. During the final two weeks of the study repeat samples were collected from children 2 to 3 days following the protocol sample for that week and used for validation. Along with each saliva sample, a form was completed by the field worker that recorded information about the sample including potential confounding factors (time since last waking, time of day, eating 20 minutes prior to the sample, if a mouthwash was performed, oral health of the child

and if the child was experiencing an episode of diarrhea). Saliva collection was performed with the Oracol device manufactured by Malvern Medical Developments (Worcester, UK), which has been shown to yield the highest quality oral fluid in terms of total and specific antibody concentrations.[27] Saliva samples were taken by field workers at the child's home in the presence of a caretaker by wiping the sponge swab around the gum margin for about a minute[28] until the sponge was visibly saturated with oral fluid. After collection of oral fluid, devices were brought on ice to a field laboratory and assigned a unique sample identifier before transportation to the laboratory. The oral fluid was extracted and stored in labelled screw cap tubes and immediately frozen at -80 degrees C until analyzed. It has been shown that long-term storage does not have an affect on antibody affinity[17] nor does long-term storage and multiple freeze-thaw cycles alter the molecular weight of IgA.[29]. For the detection of SIgA commercial enzyme immunoassay kits (Salimetrics LLC, State College, PA) were used that capture the full range of salivary SIgA levels and use 25 uL of saliva per test with minimal incubation times. The kits were kept stable at the recommended temperature of 2-8 degrees C. Final concentrations of SIgA are reported in μg/mL.

Stool collection and analysis

Under the MAL-ED protocol stool samples were collected on a monthly basis and children were followed twice weekly for active surveillance for diarrheal disease and illness. Prior to stool testing all samples were stored at −70°C. Stool samples collected from May to August 2015 of the children with saliva samples were analyzed for the presence or absence of both *Campylobacter* and norovirus infections. Enzyme immunoassay was used for detection of *Campylobacter* spp (ProSpecT, Remel, Lenexa, KS, USA) and PCR was used to test stool samples for norovirus of both genotypes I (GI) and II (GII).

Statistical analysis

The relationships between the concentration of salivary SIgA and each WASH variable or household fecal contamination sample were analyzed using generalized estimating equations and used to fit a linear regression model for each child and account for non-independence of saliva testing within each participant. The outcome variables were log-10 transformed to meet the normality assumptions of linear regression. In all analyses, SIgA was adjusted for time since waking, saliva volume and time in weeks of study adjusted while SIgA divided by TP was adjusted for time since waking

and time in weeks of study. Independent variables were analyzed as categorical or ordinal if they were continuous in nature. A hygiene index variable score was calculated from four questions as a cumulative score and categorized into three levels indicating how often they practiced the hygienic behaviors: i) always, ii) most of the time, and iii) sometimes. For the multivariate WASH analysis we selected a final parsimonious set of independent variables based on considerations of sample size and the minimization of the Akaike Information Criterion (AIC).[30] Beta coefficients and 95% confidence intervals were estimated and represent the log10-unit difference in SIgA or SIgA/TP when comparing a category of each independent variable (i.e. type of toilet facility used, floor type, etc) to its reference category. R-squared and Adjusted R-squared (in the case of low intraclass correlation) are presented to determine model fit. Data were visualized using R software version 3.0.3 (R-FSC, Vienna, Austria) and all statistical analyses were conducted using Stata version 12.1 (College Station, TX).

Ethics

The study protocol and questionnaires were approved by the institutional review boards from Johns Hopkins Bloomberg School of Public Health

(Baltimore, MD) and Asociación Benéfica Proyectos de Informática, Salud, Medicina, y Agricultura (A.B. PRISMA), Iquitos, Peru. All participants gave written consent prior to saliva collection and household sampling.

Results

A total of 69 children were enrolled upon 1 June 2015 who were less than 48 months of age. They ranged in age from 39 months to 48 months old with 11 of these children aging out of the cohort before the end of August. During saliva collection, 3.2 percent of the samples collected were from children reported to be undergoing a diarrheal episode. Given enrollment, there were 972 expected saliva samples with 907 samples collected for analysis, resulting in 6.7 percent of missing data. The coefficient of variation (CV) between the samples taken during the last two weeks of the study, 2 to 3 days from the protocol sample was 13.8 percent compared to the CV of samples taken weeks apart, which was 22.6 percent.

WASH variables associated with SIgA

The study population had various type of sanitation access, including 42.3% had access to a pit latrine with no flush, 37.2% had access to a pour flush toilet to a septic tank, and 9.7% did not have access to a toilet facility or

used the field or a bucket for a toilet. For water source, 68.6% used community hand pumps and 11.5% used the public taps with 48.6% doing nothing to treat their water, 22.0% using chlorine to treat their water, and 14.1% allowing their water to stand and settle before drinking it. For the hygiene index score 64.2% of the study population always practiced all of the hygienic behaviors, 21.9% practiced them most of the time, and 14.0% sometimes practiced the hygiene behaviors. The household floors of the study population were composed of 61.4% with dirt floors, 32.6% with cement floors and 6.0% with woods floors.

The unadjusted analyses (Table 1) found that for those using pit latrines compared to those with access to a pour flush toilet to a septic tank, there was -0.12 $\log_{10}$ SIgA ug/mL (95% CI: -0.19, -0.04) and -0.09 $\log_{10}$ SIgA/TP (95% CI: -0.18, 0.001). For those that used the public tap as their drinking water source compared to those that used the community hand pump, there was was +0.11 $\log_{10}$ SIgA ug/mL (95% CI: -0.03, 0.24) and +0.18 $\log_{10}$ SIgA/TP (95% CI: 0.10, 0.26). There was no significant statistical difference found for the comparisons between categories in either the hygiene index score variable or the floor types.

In the multivariate risk factor analysis (Table 2) the comparison between those using pit latrines to those with access to a pour flush toilet to a septic tank became highly significant and increased in effect size compared to the unadjusted analysis where there was -0.17 $\log_{10}$ SIgA ug/mL (95% CI: -0.24, -0.10) and -0.11 $\log_{10}$ SIgA/TP (95% CI: -0.21, -0.008). The concentration of SIgA/TP for those using the public tap as their drinking water source was no longer significant compared to those using the community hand pump. When comparing the SIgA of children in households in the highest quartile of the number of household members to those in the lowest quartile, there was +0.12 $\log_{10}$ SIgA ug/mL (95% CI: 0.04, 0.20). For children in households with longer tenancy, there were reduced levels of SIgA for all categories when comparing them to the lowest category of having lived in the house for less than a year (e.g. a household with more than twenty years tenancy in the same home had -0.13 $\log_{10}$ SIgA ug/mL (95% CI: -0.25, -0.02).

E. coli contamination associated with SIgA

There was a total of 117 household visits to sample the floors, tables, and drinking water in the homes of 69 children for whom saliva was also sampled. Of these homes, 48 were visited in the beginning and end of the

study period, and 21 were visited at either the beginning or end. For the homes that had one visit to sample household contamination, the data for those households during the period that were not sampled were considered missing resulting in 15.2% missing data for the household contamination sample.

Of the floor areas sampled, the levels of $\log_{10}$ E. coli CFU/900 cm^2 on kitchen floors were found to have the greatest association with the levels of SIgA in the children's saliva in these homes though not statistically significant (Table 3). The highest quartile of $\log_{10}$ E. coli CFU compared to the lowest quartile had -0.08 $\log_{10}$ SIgA ug/mL (95% CI: -0.18, 0.01) and -0.12 $\log_{10}$ SIgA/TP (95% CI: -0.25, 0.007) (Table 3). For the glass of drinking water, the $\log_{10}$ E. coli CFU per 100mL of water was found to be statistically significant when comparing the third highest quartile to the lowest quartile with +0.11 $\log_{10}$ SIgA ug/mL (95% CI: 0.03, 0.19) (Table 3).

SIgA associated with stool pathogen presence

A total of 317 stool samples were collected from the 69 children enrolled and analyzed for the presence or absence of *Campylobacter* spp and norovirus GI and GII for the period of May to August 2015. There were 27

stools that tested positive for norovirus GI, 29 stools positive for norovirus GII and 2 stools that were positive for both norovirus GI and GII. There were 134 stools that tested positive for *Campylobacter* spp with 32 children testing positive for *Campylobacter* spp two or more times during the study period.

As displayed in Figure 1, there was a negative association between the concentrations of salivary SIgA and the number of pathogens detected in a stool sample four weeks later with -0.27 pathogens detected in stool (95% CI: -0.53, -0.02) for every unit increase in $\log_{10}$ SIgA (Table 4a). There was also a decreased risk for a norovirus GII positive stool detection with increased concentrations of salivary SIgA three and four weeks prior (Table 4a).

Discussion

This study found evidence for an association between household contamination and the concentrations of salivary SIgA in children between the ages of three and four years old. The type of household toilet facility, number of people living in the home, and number of years tenancy of the household were all significantly associated with salivary SIgA in the

multivariate analysis. The elevated concentrations of salivary SIgA in this peri-urban community in Iquitos Peru is confirmed by comparison to other populations that are hypothesized to have less household contamination. For example, the mean $\log_{10}$ SIgA in Iquitos was 1.79 ug/mL (95% CI: 1.71,1.88) versus 1.60 ug/mL (95% CI: 1.51, 1.70) in an age-matched reference group of the children from rural North Carolina (data not published).

The finding of significantly lower salivary SIgA in children from homes with pit latrines compared to those with a flush toilet to a septic supports may indicate impaired mucosal immunity in the mucosal surfaces that occurs when there is enhanced susceptibility to enteric infections and are often more frequent and severe in protein-calorie malnutrition.[31] The increased enteric infections and therefore, impaired mucosal immunity as indicated by lower SIgA in the saliva may be attributable to the increased contamination of households by pit latrines. This finding is in line with the finding from Chapter 3 of this dissertation that found increased contamination on household floors from an unimproved sanitation facility, such as a pit latrine.

The utility of salivary SIgA to measure the overall exposure to fecal contamination in the household is further supported by the increased SIgA detected in the saliva of children living in homes with the greatest number of household members compared to the lowest quartile. This finding is confirmed by a study in Swedish children that found that infants with older siblings were associated with higher SIgA levels.[12] This study also found that having a history of more than three infections in infancy (another environmental factor associated with a high microbial load stimulating the immune system) was associated with higher SIgA levels, thereby having a protective effect on late-onset wheezing. These findings are also similar to this study, in that those with higher SIgA had a lower likelihood of detection of a pathogen in their stool sample four weeks later.

This study had several important limitations. Most notable was the lack of heterogeneity across the different WASH characteristics. This is best demonstrated by the drinking water source variable where the majority of the population used the community hand pumps. The sample sizes were small for the other less protected water sources thereby preventing decisive statistical results. At the same time, there was a significant increase in salivary SIgA for the children in homes with higher levels of *E.coli* in

drinking water which suggests that drinking water may be an important modulator of SIgA in saliva. We also did not control for the number of respiratory infections that may potentially confounding the relationships observed.

The strengths of this study include an intensive longitudinal follow-up study design that collected weekly SIgA measurements in a field-based setting. The age requirements for enrollment into the study had a tight window to ensure that the age-dependence of salivary SIgA did not bias results. Additionally, a baseline and end line community questionnaire was administered to ensure that any changes in the WASH characteristics were accurately represented thereby reflecting any changes observed in salivary SIgA.

Conclusion

Our study provides new evidence for the use of salivary SIgA as a potential marker for fecal contamination in the household environment. Household use of a pit latrine resulted in significantly lower levels of salivary SIgA compared to those using a pour flush toilet to a septic tank, potentially indicating impaired mucosal immunity from repeated enteric infections. The

ability to differentiate salivary SIgA levels within a community validates this proof-of-concept for using salivary SIgA as an objective outcome in field-based studies and these findings justify its use within in a larger sample size where differences are detected in pre and post intervention settings.

Table 1. WASH Household Risk-Factor Analysis for Salivary IgA marker using generalized estimating equations with robust variance estimation to account for correlations due clustering at the child level.

	Log10 sIgA[11]	R^2	Log10 sIgA/TP[12]	R^2
SANITATION				
Type of toilet facility that households usually use (N=67, n=736):				
Flush toilet to septic tank (n=272)	REF		REF	
No facility/bush/field or bucket toilet (n=71)	0.02 (-0.12, 0.17)	0.215	-0.004 (-0.14, 0.13)	0.102
Pit latrine without flush (n=317)	**-0.12 (-0.19, -0.04)****		**-0.09 (-0.18, 0.001)***	
Flush toilet to piped sewer system (n=37)	**-0.14 (-0.24, -0.05)****		**-0.16 (-0.29, -0.02)***	
Ventilated improved pit latrine (n=17)	-0.11 (-0.38, 0.17)		**-0.14 (-0.21, -0.07) †**	
Flush toilet to somewhere else (n=22)	-0.10 (-0.24, 0.05)		0.02 (-0.26, 0.29)	
Shared Sanitation Facility (N=64, n=681):				
Unshared (n=504)	REF	0.190	REF	0.089
Shared (n=177)	0.05 (-0.04, 0.14)		0.03 (-0.09, 0.15)	
WATER				
Drinking water source (N=67, n=736):				
Community hand pump (n=507)	REF		REF	
Household piped connection (n=7)	-0.04 (-0.09, 0.02)		0.01 (-0.05, 0.08)	
Public tap (n=83)	0.11 (-0.03, 0.24)		**0.18 (0.10, 0.26) † †**	
Protected well (n=7)	**0.26 (0.20, 0.31) † †**	0.207	**0.10 (0.04, 0.16)****	0.117
Unprotected well (n=7)	**0.16 (0.11, 0.21) † †**		**0.31 (0.24, 0.36) † †**	
Surface water (n=21)	**-0.11 (-0.21, -0.01)***		**-0.23 (-0.35, -0.11) † †**	
Other (n=104)	0.01 (-0.06, 0.09)		-0.02 (-0.14, 0.10)	
Time to fetch water in minutes (N=66, n=713):				
Q1 (n=354)	REF		REF	
Q2 (n=0)	--	0.199	--	0.098
Q3 (n=242)	-0.04 (-0.12, 0.04)		-0.03 (-0.12, 0.06)	
Q4 (n=117)	-0.02 (-0.10, 0.06)		0.01 (-0.10, 0.13)	

[11] Adjusted for time since waking, saliva volume and time in weeks of study
[12] Adjusted for time since waking and time in weeks of study

Methods to treat water (N=67, n=729):				
Do nothing (n=354)	REF	0.202	REF	0.106
Let is stand and settle (n=106)	-0.06 (-0.14, 0.03)		**-0.12 (-0.24, -0.01)***	
Chlorine (n=161)	-0.008 (-0.10, 0.08)		0.03 (-0.09, 0.15)	
Boiling (n=50)	0.13 (-0.05, 0.32)		**0.14 (0.003, 0.27)***	
Other (n=58)	0.01 (-0.08, 0.10)		-0.03 (-0.15, 0.08)	
CHICKENS				
Presence of chickens in HH (N=69, n=842):				
No (n=628)	REF	0.179	REF	0.079
Yes (n=214)	0.02 (-0.05, 0.09)		0.02 (-0.07, 0.12)	
Chickens are corralled (N=38, n=211):				
No (n=171)	REF	0.305	REF	0.138
Yes (n=40)	-0.01 (-0.17, 0.14)		0.01 (-0.18, 0.21)	
Number of chickens in household (N=64, n=501):				
Q1 (n=190)	REF	0.171	REF	0.079
Q2 (n=153)	-0.05 (-0.16, 0.05)		-0.01 (-0.13, 0.10)	
Q3 (n=45)	0.04 (-0.06, 0.15)		**0.13 (0.03, 0.23)****	
Q4 (n=113)	-0.02 (-0.12, 0.08)		0.04 (-0.10 0.19)	
HYGIENE				
Hygiene Score (N=67, n=729):				
Always (n=470)	REF	0.195	REF	0.092
Most of the time (n=158)	0.02 (-0.06, 0.10)		-0.03 (-0.11, 0.05)	
Sometimes (n=101)	-0.01 (-0.12, 0.10)		0.006 (-0.15, 0.16)	
SOCIO-ECONOMIC STATUS				
Monthly income per capita (in USD) (N=67, n=716):				
Q1 (n=222)	REF	0.203	REF	0.096
Q2 (n=135)	**0.08 (0.007, 0.16)***		0.05 (-0.05, 0.15)	
Q3 (n=256)	0.01 (-0.08, 0.10)		0.01 (-0.09, 0.12)	
Q4 (n=103)	0.04 (-0.07, 0.14)		0.05 (-0.06, 0.17)	
Maternal Education (years) (N=66, n=724):				
Q1 (n=184)	REF	0.201	REF	0.109
Q2 (n=231)	-0.06 (-0.18, 0.05)		-0.08 (-0.21, 0.05)	
Q3 (n=171)	-0.01 (-0.13, 0.10)		0.006 (-0.13, 0.14)	
Q4 (n=138)	0.02 (-0.09, 0.14)		0.07 (-0.06, 0.21)	
HOUSEHOLD CHARACTERISTICS				

Floor type (N=66, n=728):				
Cement (n=237)	REF	0.204	REF	0.204
Wood (n=42)	-0.08 (-0.18, 0.02)		-0.06 (-0.23, 0.11)	
Dirt (n=449)	-0.06 (-0.12, 0.006)		0.01 (-0.07, 0.10)	
Crowding (# of rooms in HH/# people sleeping in HH (N=67, n=723):				
Q1 (n=195)	REF	0.204	REF	0.095
Q2 (n=214)	-0.06 (-0.15, 0.03)		-0.05 (-0.15, 0.05)	
Q3 (n=139)	-0.01 (-0.13, 0.11)		-0.008 (-0.15, 0.13)	
Q4 (n=175)	0.05 (-0.03, 0.12)		0.02 (-0.10, 0.14)	
Number of household members (N=67, n=723):				
Q1 (n=253)	REF	0.199	REF	0.100
Q2 (n=160)	-0.01 (-0.11, 0.08)		-0.10 (-0.23, 0.03)	
Q3 (n=195)	-0.06 (-0.16, 0.04)		-0.09 (-0.20, 0.02)	
Q4 (n=115)	-0.001 (-0.08, 0.07)		-0.03 (-0.13, 0.07)	
Electricity connection (N=66, n=735):				
Yes (n=668)	REF	0.196	REF	0.093
No (n=67)	0.03 (-0.06, 0.13)		-0.06 (-0.21, 0.08)	
Wall type (N=67, n=728):				
Concrete (n=194)	REF	0.200	REF	0.091
Wood (n=536)	-0.06 (-0.12, 0.004)		-0.002 (-0.09, 0.08)	
Roof type (N=66, n=728):				
Metal (n=699)	REF	0.204	REF	0.104
Palm/thatch (n=29)	**0.22 (0.17, 0.27) † †**		**0.28 (0.12, 0.44) †**	
Tenancy in household (N=67, n=736):				
Less than a year (n=106)	REF	0.207	REF	0.104
Between one and five years (n=252)	**-0.11 (-0.20, -0.02)***		-0.03 (-0.18, 0.12)	
Between five and ten years (n=188)	**-0.15 (-0.25, -0.05)****		-0.10 (-0.25, 0.05)	
Between ten and twenty years (n=105)	**-0.12 (-0.23, -0.02)***		-0.07 (-0.21, 0.07)	
More than twenty years (n=85)	**-0.14 (-0.23, -0.06)****		**-0.19 (-0.34, -0.04)***	

* Significance at the p<0.05 level
** Significance at the p<0.01 level
†Significant difference at the p<0.001 level
††Significant difference at the p<0.0001 level

Table 2. Multivariate WASH Household Risk-Factor Analysis for Salivary IgA marker using generalized estimating equations with robust variance estimation to account for correlations due clustering at the child level.

	Log10 sIgA[13] (N=66, n=715)	Log10 sIgA/TP[14] (N=66, n=715)
R^2 (Adjusted R^2)	0.262 (0.219)	0.171 (0.123)
Type of toilet facility that households usually use		
Flush toilet to septic tank (n=271)	REF	REF
No facility/bush/field or bucket toilet (n=58)	-0.007 (-0.11, 0.09)	0.02 (-0.08, 0.11)
Pit latrine without flush (n=310)	**-0.17 (-0.24, -0.10) † †**	**-0.11 (-0.21, -0.008)***
Flush toilet to piped sewer system (n=37)	-0.10 (-0.23, 0.04)	-0.04 (-0.23, 0.15)
Ventilated improved pit latrine (n=17)	-0.18 (-0.42, 0.05)	**-0.15 (-0.28, -0.02)***
Flush toilet to somewhere else (n=22)	**-0.22 (-0.35, -0.09)****	-0.08 (-0.31, 0.15)
Drinking water source		
Community hand pump (n=492)	REF	REF
Household piped connection (n=7)	-0.12 (-0.27, 0.03)	-0.04 (-0.28, 0.20)
Public tap (n=77)	0.03 (-0.06, 0.13)	0.09 (-0.03, 0.21)
Protected well (n=7)	**0.26 (0.16, 0.36) † †**	**0.13 (0.01, 0.25)***
Unprotected well (n=7)	0.10 (-0.03, 0.24)	**0.32 (0.16, 0.47) † †**
Surface water (n=21)	**-0.16 (-0.29, -0.03)***	-0.14 (-0.35, 0.08)
Other (n=104)	0.01 (-0.06, 0.09)	-0.01 (-0.13, 0.11)
Floor type		
Cement (n=224)	REF	REF
Wood (n=42)	0.06 (-0.05, 0.17)	-0.009 (-0.13, 0.11)
Dirt (n=449)	0.01 (-0.06, 0.08)	0.02 (-0.07, 0.11)
Number of household members		
Q1 (n=252)	REF	REF
Q2 (n=160)	0.0007 (-0.08, 0.08)	-0.10 (-0.21, 0.008)

[13] Adjusted for time since waking, saliva volume and time in weeks of study
[14] Adjusted for time since waking and time in weeks of study

Q3 (n=188)	0.007 (-0.06, 0.08)	-0.07 (-0.17, 0.04)
Q4 (n=115)	**0.12 (0.04, 0.20)****	0.07 (-0.06, 0.20)
Wall type		
Concrete (n=192)	REF	REF
Wood (n=515)	**-0.09 (-0.18, 0.002)***	-0.08 (-0.16, 0.002)
Roof type		
Metal (n=678)	REF	REF
Palm/thatch (n=29)	**0.37 (0.30, 0.43) † †**	**0.43 (0.28, 0.57) † †**
Tenancy in household		
Less than a year (n=100)	REF	REF
Between one and five years (n=251)	**-0.12 (-0.22, -0.02)***	-0.003 (-0.15, 0.14)
Between five and ten years (n=174)	**-0.19 (-0.30, -0.08)****	-0.07 (-0.24, 0.09)
Between ten and twenty years (n=105)	**-0.18 (-0.29, -0.07)***	-0.03 (-0.19, 0.12)
More than twenty years (n=85)	**-0.13 (-0.25, -0.02)***	-0.16 (-0.34, 0.01)

* Significance at the p<0.05 level

** Significance at the p<0.01 level

†Significant difference at the p<0.001 level

† †Significant difference at the p<0.0001 level

Table 3. Associations of household contamination on floors, tables and drinking water with Salivary IgA as measured in the saliva of 4-year olds living in the households using generalized estimating equations with robust variance estimation to account for correlations due clustering at the child level.

	Log10 sIgA[15] (ug/mL)	R^2	Log10 sIgA/TP[16] (ug/mL)	R^2
Log_{10} *E. coli* on entrance floor (CFU per 900 cm^2) (N=67, n=736):				
Q1 (n=200)	REF	0.198	REF	0.092
Q2 (n=188)	-0.05 (-0.13, 0.03)		-0.03 (-0.11, 0.06)	
Q3 (n=164)	-0.02 (-0.11, 0.06)		0.02 (-0.09, 0.12)	
Q4 (n=184)	0.002 (-0.08, 0.09)		-0.004 (-0.12, 0.11)	
Log_{10} *E. coli* on kitchen floor (CFU per 900 cm^2) (N=67, n=736):				
Q1 (n=190)	REF	0.200	REF	0.099
Q2 (n=190)	-0.04 (-0.12, 0.04)		-0.03 (-0.13, 0.07)	
Q3 (n=188)	-0.03 (-0.13, 0.06)		-0.06 (-0.16, 0.05)	
Q4 (n=168)	-0.08 (-0.18, 0.01)		-0.12 (-0.25, 0.007)	
Log_{10} *E. coli* on main table (CFU per 900 cm^2) (N=67, n=730):				
Q1 (n=219)	REF	0.198	REF	0.098
Q2 (n=166)	-0.06 (-0.14, 0.03)		-0.09 (-0.20, 0.02)	
Q3 (n=173)	-0.05 (-0.15, 0.05)		-0.06 (-0.16, 0.04)	
Q4 (n=172)	-0.03 (-0.12, 0.06)		0.02 (-0.09, 0.13)	
Log_{10} *E. coli* in glass of drinking water (CFU per 100 mL) (N=55, n=372):				
Q1 (n=257)		0.180		0.098
Q2 (n=0)	REF		REF	
Q3 (n=26)	--		--	
Q4 (n=89)	**0.11 (0.03, 0.19)****		0.07 (-0.17, 0.31)	

[15] Adjusted for time since waking, saliva volume and time in weeks of study
[16] Adjusted for time since waking and time in weeks of study

	-0.01 (-0.14, 0.11)		0.01 (-0.12, 0.15)	

* Significance at the p<0.05 level
** Significance at the p<0.01 level

Figure 1. Relationship between the number of pathogens found in stool (Campylobacter spp, norovirus GI and GII) with concentrations of log10 SIgA in saliva four weeks prior

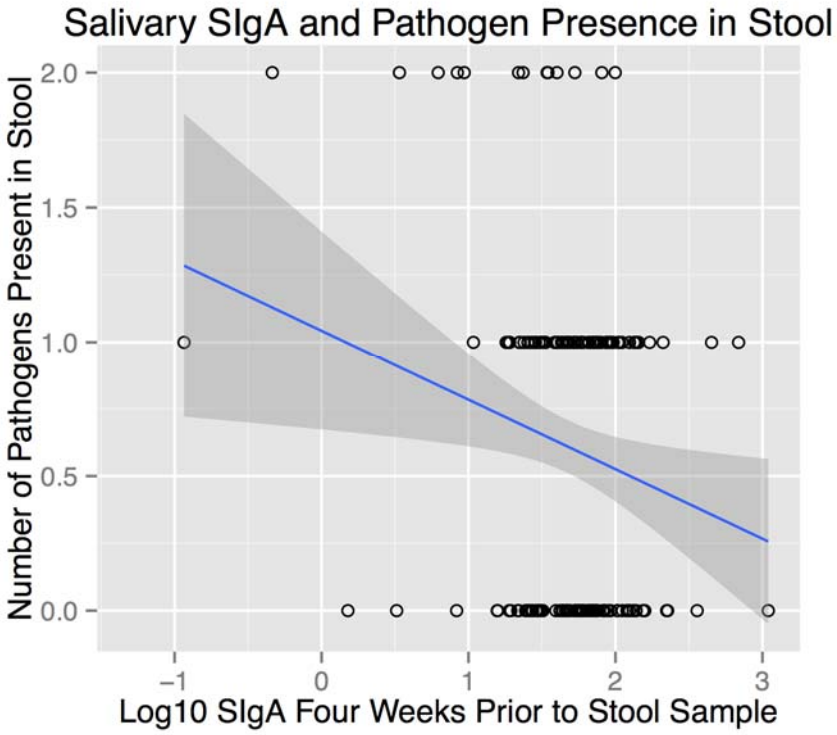

Table 4. Associations of Salivary IgA with the number of pathogens detected in stool (Norovirus GI, Norovirus GII, and Campylobacter) using GEE models adjusting for time in weeks of study.

	Norovirus GI + Norovirus GII + Campylobacter Number of pathogens per stool (0, 1, 2 (or 3)
Same week saliva (n=226):	
log_{10}SIgA	0.01 (-0.19, 0.22)
log_{10}(SIgA/TP)	-0.11 (-0.30, 0.08)
1 week prior saliva (n=201):	
log_{10}SIgA	-0.12 (-0.33, 0.10)
log_{10}(SIgA/TP)	-0.11 (-0.30, 0.08)
2 week prior saliva (n=178):	
log_{10}SIgA	0.02 (-0.29, 0.33)
log_{10}(SIgA/TP)	-0.11 (-0.34, 0.12)
3 week prior saliva (n=152)	
log_{10}SIgA	-0.002 (-0.22, 0.22)
log_{10}(SIgA/TP)	-0.09 (-0.28, 0.10)
4 week prior saliva (n=159):	
log_{10}SIgA	**-0.27 (-0.53, -0.02)**
log_{10}(SIgA/TP)	-0.18 (-0.39, 0.03)
5 week prior saliva (139):	
log_{10}SIgA	0.005 (-0.34, 0.35)
log_{10}(SIgA/TP)	-0.08 (-0.39, 0.22)
6 week prior saliva (114):	
log_{10}SIgA	-0.36 (-0.78, 0.06)
log_{10}(SIgA/TP)	0.03 (-0.41, 0.47)

Table 5. Associations of Salivary IgA with Norovirus GI, Norovirus GII, and Campylobacter detection in stool using GEE models adjusting for time in weeks of study.

	Norovirus GI Odds Ratio (95% CI)	Norovirus GII Odds Ratio (95% CI)	Campylobacter Odds Ratio (95% CI)
Same week saliva (n=226):			
Cases (*n*)	*19*	*17*	*99*
N	167	134	226
log_{10}SIgA	0.74 (0.25, 2.25)	0.82 (0.22, 3.05)	1.14 (0.49, 2.65)
log_{10}(SIgA/TP)	0.85 (0.24, 2.93)	0.55 (0.19, 1.56)	0.73 (0.35, 1.52)
1 week prior saliva (n=201):			
Cases (*n*)	*16*	*11*	*87*
N	141	104	201
log_{10}SIgA	1.10 (0.18, 6.71)	0.44 (0.07, 2.87)	0.64 (0.30, 1.40)
log_{10}(SIgA/TP)	2.69 (0.41, 17.7)	0.38 (0.08, 1.92)	0.59 (0.30, 1.14)
2 week prior saliva (n=178):			
Cases (*n*)	*15*	*9*	*78*
N	135	62	176
log_{10}SIgA	1.37 (0.18, 10.59)	0.70 (0.02, 21.02)	0.96 (0.38, 2.45)
log_{10}(SIgA/TP)	1.70 (0.26, 11.34)	1.40 (0.16, 12.28)	0.50 (0.21, 1.14)
3 week prior saliva (n=152)			
Cases (*n*)	*15*	*9*	*67*
N	130	60	149
log_{10}SIgA	2.85 (0.70, 11.56)	**0.30 (0.11, 0.83)**	1.06 (0.54, 2.09)
log_{10}(SIgA/TP)	2.48 (0.42, 14.49)	**0.35 (0.14, 0.88)**	0.71 (0.38, 1.35)
4 week prior saliva (n=159):			
Cases (*n*)	*17*	*10*	*71*
N	126	77	159
log_{10}SIgA	0.51 (0.19, 1.39)	**0.32 (0.11, 0.94)**	0.61 (0.27, 1.37)
log_{10}(SIgA/TP)	0.75 (0.32, 1.76)	0.55 (0.23, 1.28)	0.64 (0.31, 1.30)
5 week prior saliva (140):			
Cases (*n*)	*16*	*10*	*59*
N	100	79	139
log_{10}SIgA	1.49 (0.36, 6.13)	0.92 (0.15, 5.52)	0.95 (0.31, 2.88)
log_{10}(SIgA/TP)	2.11 (0.57, 7.82)	1.13 (0.29, 4.43)	0.51 (0.19, 1.38)

6 week prior saliva (116):			
Cases (*n*)	*15*	*10*	*46*
N	107	77	113
log_{10}SIgA	0.92 (0.14, 5.85)	0.28 (0.03, 2.77)	0.25 (0.05, 1.20)
log_{10}(SIgA/TP)	1.76 (0.39, 7.96)	2.58 (0.26, 25.36)	0.56 (0.14, 2.35)

Table S1. Sample size distribution for number of pathogens per stool (0, 1, 2 (or 3).

	Number of pathogens per stool (0, 1, 2 (or 3)
Same week saliva (n=226):	
0	108
1	102
2 or 3	16
1 week prior saliva (n=201):	
0	100
1	89
2 or 3	12
2 week prior saliva (n=178):	
0	88
1	79
2 or 3	11
3 week prior saliva (n=152)	
0	74
1	66
2 or 3	12
4 week prior saliva (n=159):	
0	75
1	71
2 or 3	13
5 week prior saliva (139):	
0	68
1	58
2 or 3	13
6 week prior saliva (114):	
0	57
1	45
2 or 3	12

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Chapter 6

Conclusion

This dissertation set out to go beyond the paradigm of diarrheal disease as the main outcome of interest in water, sanitation and hygiene (WASH) interventions in developing countries. The research that was conducted investigated the hypothesis that fecal contamination in the household environment due to a lack of adequate WASH conditions contributes to the development of environmental enteropathy in peri-urban, flood-prone communities in Iquitos, Peru. The dissertation study was designed around the conceptual model outlined in Figure 1 below.

Figure 1. Expected impact of improved sanitation

The importance of focusing on multiple transmission routes to understand health outcomes in response to improvements in sanitation was described by

John Briscoe in 1984 when he argued, that “the effect of improvements in water quality should not be evaluated by the reduction in disease due to water supply improvements in isolation, but rather by the degree to which the improvements in water quality affect the health effects of other (simultaneous or subsequent) essential changes in environmental conditions or personal health practices”.[1]

The research conducted in Specific Aim 1 of this work was the first study, to our knowledge, to longitudinally analyze household water, sanitation and hygiene characteristics of children from birth to 24 months of age and relate these characteristics to the newly developed EE fecal markers of NEO, AAT and MPO as a way to more effectively measure the risk a child faces in developing EE and provide a mechanism to prioritize interventions to children in greatest need. This study found that children with less protected water sources, an interrupted water supply and decreasing volumes of water stored in their homes experienced more EE. In addition, children that lived in homes where fecal matter was stored near their homes had greater occurrence of EE compared to children from homes that had no toilet facility. This is an important study in the field of WASH intervention research because the most commonly used outcome that has been used to

understand the EE mechanism in cluster randomized control trials (RCTs) has been linear growth.[2-4] While linear growth is an important, objective outcome for child health, it is also an irreversible outcome that often is measured after the critical period of 0 to 24 months of age for gut development. The significant findings in this study, considering that this study was conducted in a non-intervention based setting in a community that was relatively homogeneous in terms of its levels of WASH services, gives promise to the use of these fecal biomarkers in larger RCTs to detect differences in child gut health while there is still time to alter EE outcomes.

The study conducted to address the Specific Aim 2 of this dissertation characterized the floors pathway, for which there is limited research, as a mechanism for pathogen transmission. Contamination of household floors with enteric pathogens is especially of concern for children less than 24 months of age, as this age group is particularly vulnerable to developing EE for those with inadequate sanitation and pervasive fecal contamination. Therefore, we conducted an exposure assessment of the floors of children enrolled in the MAL-ED study site in Iquitos Peru and found that households with unimproved sanitation versus improved sanitation had higher levels of $\log_{10}$ *E. coli* bacteria CFU per 900 cm^2 of surface area in

fully adjusted multivariate regressions models. In addition, households that had shared sanitation facilities (compared to those that did not share) and households that had a presence of chickens (versus those that did not) both had higher levels of $\log_{10}$ *E. coli* bacteria CFU per 900 cm^2 of surface area in fully adjusted multivariate regressions models. The concentrations of *E. coli* contamination that we found on the dirt floors of these Peruvian homes were approximately 5 to 80 times more contaminated than other studies that had conducted similar studies.[5, 6] This may reflect either or both possibilities that the dry electrostatic cloth sampling method used in our study had a higher efficiency for sampling bacteria from floor surfaces than other methods and that the dirt floors in these tropical households carried particularly high bacterial loads. It is evident that if an infant is to play on a dirt floor in a home with unimproved sanitation and engages in hand to mouth activity, there is a greater chance that they will ingest fecal pathogens than if they had improved sanitation in their living environment. This study also demonstrated that improved sanitation along with an improved floor type (either cement or wood) resulted in a greater reduction of $\log_{10}$ *E. coli* bacteria CFU per 900 cm^2 of surface area than when there was only an improvement in the sanitation facility. This provides evidence that WASH

interventions may have a greater impact on child gut health and linear growth if they are paired with flooring improvements in households.

The third aim of this dissertation investigated the utility of salivary immunoassays using secretory immunoglobulin A (SIgA) as a marker of microbial pressure to improve assessment of recent exposure to fecal pathogens in a community-based longitudinal study. A review was also conducted of the pathogen-specific antibody biomarkers for waterborne pathogens to assess the prevalence and incidence of infections from pathogens that are typically waterborne but can also be transmitted via other fecal-oral routes. This review discussed the use of antibody biomarkers that have been most widely used in serum but are now increasingly being analyzed in saliva to understand the incidence and prevalence of infections that are potentially waterborne. The utility of these single pathogen assay platforms have limited application in contexts of extreme poverty where population-based infections are from a wide range of enteric pathogens. However, the development of multiplex platforms for antibodies to multiple pathogens has potential for population-based applications in developing countries where there is background knowledge of the burden and etiology of enteric infection in children, such as in the Global Enteric Multicenter

Study.[7] For the purposes of our study, since such a multiplex platform had not yet been developed for the peri-urban communities of Iquitos Peru, the marker of salivary SIgA was used as a global marker for microbial exposure in an EE cohort. The household characteristic that had the greatest association with the levels of SIgA in children's saliva in the fully adjusted multivariate model was the type of toilet facility used by the household. Children from homes with greater *E. coli* contamination in their drinking water also had higher levels of SIgA in their saliva. These results provide evidence for using SIgA as a global marker for environmental microbial pressure and confirmed the ease of use and minimally invasive collection method of saliva in children such that it could plausibly be applied widely across a population in a longitudinal study design to improve exposure assessments to fecal contamination.

A look toward 2030: Measuring and monitoring the Sustainable Development Goals

The Sustainable Development Goals (SDGs) recently adopted at the 2015 UN Summit have set ambitious targets to be reached by 2030 and Goal 6 states, "ensure availability and sustainable management of water and sanitation for all" (https://sustainabledevelopment.un.org/topics/waterandsanitation). The

SDGs follow on the Millennium Development Goals (MDGs) which in 2000 called for the world to halve, by 2015, the proportion of people without access to safe drinking water as well as the proportion of people who do not have access to basic sanitation. The goal for water has largely been met while the MDG for sanitation lagged behind the targeted 50% reduction. The new SDGs therefore mean that in the next decade there will be a large number of infrastructure investments and behavioral change programs that go into place to accomplish these goals. In order to monitor and measure the progress that these SDGs bring about it will be important to measure the impact on child health outcomes. To accomplish this, new and innovative tools and methodologies are needed that can be applied across populations both urban and rural.

This dissertation has laid out some examples of new tools and methodologies to help monitor and measure progress toward SDG 6. Most importantly, this thesis argues that diarrheal incidence health outcomes have reporting bias and measurement errors that justify a reappraisal of its use as the primary outcome to evaluate sanitation interventions. Here we argue for a paradigm shift towards evaluating a reduction in EE in the population in association with water and sanitation interventions and development

institutions have already begun to embrace the shift.[8] The fecal markers for EE could be used to measure improvements in gut health and salivary SIgA could be used as a tool to measure the overall reduction of exposure to fecal contamination. Meanwhile, to more directly measure reductions of fecal bacteria in the environment, the dry electrostatic cloth method developed during this research could be used to sample floors and surfaces in homes to detect the expected reductions in contamination that proper containment, disposal and treatment of fecal matter would bring to a community.

We look forward to a future where all communities will be able to meet their basic needs and have access to safe and sustainable water and sanitation. This goal may seem optimistic but we join with the authors of the SDGs to state that it is well within reach. This thesis aimed to contribute toward developing the tools necessary to ensure that this goal is met.

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EDUCATION

JOHNS HOPKINS UNIVERSITY, Bloomberg School of Public Health
PhD in Environmental Health Sciences
September 2012-May 2016
Advisor: Kellogg J. Schwab
Collaborators: Margaret N. Kosek and Christopher D. Heaney
National Science Foundation Fellow in Water, Climate and Health Integrative Graduate Education and Research Traineeship program
Project Title: "Beyond Diarrhea: Fecal-Oral Pathogen Transmission and Environmental Enteropathy in Iquitos, Peru"

STANFORD UNIVERSITY, Civil and Environmental Engineering
Master of Science
September 2008-June 2010
Focus: Water Studies Program Environmental Fluid Mechanics and Hydrology, Jenna Davis Research Group

UNIVERSITY OF VIRGINIA, Systems Engineering
Bachelor of Science
2000-2004
Thesis: "A Capacity-Factor Approach to Sustainable Municipal Sanitation Services in Lower-Income Communities"

PROFESSIONAL EXPERIENCE

The World Bank Group - South Asia Sustainable Development, Washington, DC
Water Resources Specialist
July 2010 – August 2012

Bangladesh Water Management Improvement Project

- Led component to strengthen water management organizations and engage local communities in the design, operation and routine maintenance of water infrastructure
- Field visits to supervise critical flood damage rehabilitation works
- Designed component for real-time flood monitoring information systems

Uttar Pradesh Water Sector Restructuring Project

- Led the Implementation Completion Report upon project closing to summarize the economic, financial, social, institutional, and environmental achievements of the project
- Participated in supervision missions to monitor and evaluate project procurement

Uttar Pradesh Water Sector Restructuring Project II

- Project preparation including identification, selection and management of consultants and subcontracting firms for field work and project components
- Awarded funds from the South-South Experience Exchange Trust Fund for "Strengthening Participatory Irrigation Water Management in Uttar Pradesh by Learning from the Water User Associations in the Yaqui Irrigation District in Mexico"
- Developed component to integrate SMS voice messaging system for Water Users' Associations regarding water availability

Bihar Flood Management Information System Project
- Managed UK Department for International Development grant for "Designing, Developing and Deploying an Embankment Database Module for Bagmati-Adhwara Basin"
- Led delegation of flood managers from the Bihar Water Resources Department to visit US agencies involved in flood management (US Army Corps of Engineers, the US Geologic Survey, and the National Oceanic and Atmospheric Administration) and learn how levees are managed and various flood forecasting and asset management tools are employed
- Training government officials in the Water Resources Department on flood modeling
- Managed bidding process for "Implementation of a Real Time Data Acquisition System for Bagmati-Adhwara basin"
- Drafted terms of reference consultants and reviewed bidding documents for project procurements

India Hydrology Project II
- Monitoring and evaluation of project implementation across 8 central water agencies and 13 states
- Drafted terms of reference consultants and reviewed bidding documents for project procurements

Karnataka Community Based Tank Management Project
- Project evaluation to measure results from all project components and determine sustainability of investments at the community level
- Drafted Implementation Completion Report

South Asia Water Initiative:
- Meta-data collection for regional glacier monitoring in the Himalayas
- Study design for groundwater modeling in the Ganges basin
- Editing for climate change in the Indus basin
- Workshop participant in 5th Abu Dhabi Dialogue to discuss transboundary water management with country representatives from Afghanistan, Bangladesh, Bhutan, China, India, Nepal, and Pakistan

Stanford University **September 2008 – June 2010**
Research Assistant
- Conducted data collection in Kenya for a multiple-use rural water study on the productive use of water which included designing survey instruments, training enumerators, and leading a field team in the Machakos county during the summer of 2009

- Researched the demand responsive model for rural water supply planning and the factors leading to cost recovery using data from communities in Bolivia, Peru and Ghana to understand which forms of community participation explain variation in hand pump sustainability

The World Bank Group - Latin America and the Caribbean Sustainable Development Water and Urban Unit, Washington, DC
Junior Professional Associate
July 2006 – August 2008

- Prepared lending and supervision documents for water, sanitation, urban upgrading, solid waste, tourism, and carbon finance projects
- Participated in mission visits for the preparation and negotiation of projects in Brazil, Colombia, the Dominican Republic, Ecuador, Peru, and Venezuela
- Monitoring and evaluation of project works for the Water and Sanitation Sector Reform project in Colombia and drafted mid-term review for project

The World Bank Group - Latin America and the Caribbean Sustainable Development Water and Urban Unit, Washington, DC
Short Term Consultant
October 2005 – June 2006

- Member of the Galapagos and Ecuadorian Coast Natural Resources Management Project preparation team and investigated the use of wetlands for wastewater treatment on the islands
- Built partnerships across organizations to promote sustainable tourism including Conservation International, UNESCO, the Nature Conservancy, the World Wildlife Fund, the Charles Darwin Foundation, USAID, La Junta de Jovenes en Galapagos, and La Fundación Natura

Washington Nationals Baseball, Washington DC 2005 – 2008
Ball girl

- Collected foul balls along baseline during regular season home games

MarketBridge Consulting, Washington DC June 2004 – Oct 2005
Business Analyst

- Executed sales and marketing strategy projects, created business plans, and analyzed data for Fortune 500 companies.

PUBLICATIONS

- Heaney CD, Exum NG, Dufour AP, Brenner KP, Haugland RA, Chern E, Schwab KJ, Love DC, Serre ML, Noble R, Wade TJ. Water quality, weather and environmental factors associated with fecal indicator organism density in beach sand at two

recreational marine beaches. Science of The Total Environment. 2014;497–498(0):440-7. doi: http://dx.doi.org/10.1016/j.scitotenv.2014.07.113

- Co-authored the World Bank publication "Managing municipal solid waste in Latin America and the Caribbean", October 2007

SELECTED HONORS & AWARDS

- Student Award from Department of Environmental Health Science Dr. C. W. Kruse Memorial Fund Scholarship (2015)
- Fisher Center Discovery Program grantee (2014), project title: "Salivary diagnostics for pathogens of clinical significance in childhood environmental enteropathy in Iquitos, Peru"
- Student Award from the Kazuyoshi Kawata fund in Sanitary Engineering and Science (2014)
- Acceptance to National Science Foundation Integrative Graduate Education and Research Traineeship for Water, Climate and Health – five years of funding to pursue doctoral studies at Johns Hopkins University (2012)
- Presidential Management Fellow 2010 with the US Army Corps of Engineers and National Oceanic and Atmospheric Administration (declined)
- John K. Vennard Civil and Environmental Engineering Scholarship (full scholarship to pursue graduate studies at Stanford)
- CASE Fellowship (funds outstanding graduate students and their faculty sponsors to research, write and teach case studies on the world's most critical social challenges, summer 2009)
- Virginia Engineering Foundation Grant, awarded for excellence in research for 2004 Capstone Project to pursue fieldwork in San Mateo Ixtatán, Guatemala (January 2004)

SELECTED SERVICE & TEACHING

- Lecturer, Urban Environments and Public Health: Water and Wastewater Treatment, Johns Hopkins University (2014)
- Teaching Assistant, Food and Waterborne Diseases, Johns Hopkins University (2014)
- Teaching Assistant, Issues for Water and Sanitation in Tropical Environmental Health, Johns Hopkins University (2014)
- President, Policy Internship Program Alumni Board of the University of Virginia (2006-2008)

SELECTED PRESENTATIONS

- Improving Infrastructure and Institutions in Bangladesh to Manage Multiple-Use Water Systems. Johns Hopkins Water Institute. Johns Hopkins University, June 2013, Baltimore, Maryland USA
- Creación de una Plataforma de Conocimiento Global para el Agua y Saneamiento en America Latina. Conferencia Latinoamericana de Saneamiento LATINOSAN 2007. World Bank Group, November 2007, Cali, Colombia