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Enteropathogen infections in areas with poor access to water, sanitation, and hygiene: environmental drivers, co-infections, and potential interventions

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Abstract

Enteropathogen infections in areas with poor access to water, sanitation, and hygiene: environmental drivers, co-infections, and potential interventions

By Anna N. Chard

Enteric disease — including diarrheal illness and soil-transmitted helminth (STH) infections — is common in low-resource settings and primarily driven by inadequate access to water, sanitation, and hygiene (WASH). To better understand the potential for environmental improvements to mitigate enteric infection, we designed and conducted a series of studies in rural Lao People's Democratic Republic to measure the prevalence of enteropathogens, elucidate how enteropathogens interact with each other, and quantify the role of WASH in schools as a potential environmental mediator of infection in school children.

We conducted a longitudinal cluster-randomized controlled trial (RCT) to evaluate the impact of a comprehensive WASH in schools (WinS) evaluation on pupil absence and health among 100 randomly selected primary schools (50 intervention and 50 control). Within this study, we conducted a cross-sectional sub-study to examine the underlying drivers of enteropathogen infections and co-infection among households in the RCT school-hosting communities. We utilized a household survey to measure demographics and WASH access, and collected stool samples from three household members (child <5, school-aged child, and their parent). Stool samples (n=891) were analyzed for 25 enteropathogens using a quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay.

Enteropathogen infection was nearly universal; 98.3% of participants had at least one infection (mean=4.3 infections, standard deviation=2.0). Associations between household- and village-level WASH transmission pathways and infection were heterogenous across taxa and specific pathogens. STH infection was associated with lower odds of concurrent viral infections (odds ratio [OR]: 0.48, 95% confidence interval [CI]: 0.28, 0.83), but higher odds of concurrent bacterial infections (OR: 1.81, 95% CI: 1.06, 3.07) and concurrent protozoal infections (OR: 1.50, 95% CI: 0.95, 2.37). In the parent trial, we found no impact of the WinS intervention on any primary (pupil absence) or secondary (enrollment, dropout, grade progression, diarrhea, respiratory infection, conjunctivitis, STH) impacts. Results highlight the challenges and complexities of mitigating enteric disease due to a diverse range of pathogens, multiple transmission routes, within-host interactions, and human-environment interactions.

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Introduction

Diarrheal disease — caused by a variety of pathogens, including bacteria, viruses, and parasites — is a leading cause of morbidity and mortality worldwide [1, 2]. Soil-transmitted helminth (STH) infections are one of the most ubiquitous human infections, affecting over one billion people [3, 4]. Repeated enteric infections, even asymptomatic infections, can result in chronic and harmful sequelae such as environmental enteropathy, malnutrition, and growth stunting [5-11]. The burden of these enteric infections is greatest among the poor and is primarily driven by inadequate access to water, sanitation, and hygiene (WASH) [12-14].

Enteric pathogens are excreted in feces and travel through the environment, where they can infect another human host via multiple fecal-oral pathways (fluids, fields, flies, fingers, fomites, and food) as traditionally depicted by the F-diagram [15]. Interventions to prevent fecal contamination of one's environment — such as safe disposal of feces, handwashing, and consumption of microbiologically safe water — may interrupt these transmission pathways and reduce risk of infection [16, 17].

Improvements in WASH are considered to be the greatest global public health achievements in the last 150 years [18, 19]. However, there is mixed evidence of the ability of these environmental interventions to mitigate enteric disease based on findings from the public health "gold standard" of randomized controlled trials. Evaluations of interventions that improve water, sanitation, and hygiene access have revealed 33%, 25%, and 30% reductions in risk of childhood diarrhea, respectively [20], as well as reductions in neglected tropical diseases (NTDs) such as STH, trachoma, and schistosomiasis [14, 21-23]. Yet, results from several large-scale household-based

WASH impact evaluations have failed to demonstrate a significant reduction in diarrhea [24-28] or STH infection [24, 27].

One challenge is that enteric disease may be caused by over 40 enteric pathogens (bacteria, viruses, protozoa, and helminths), all with different etiologies and dominant transmission pathways [29-33]. Studies utilizing recently developed methods such as multiplex molecular diagnostic assays are now identifying these pathogens and expanding the current understanding of the prevalence and distribution of specific enteric pathogen infections and co-infections among children in many low-income countries [32-40]. However, few of these etiological surveys have elucidated how factors such as environmental conditions and socio-demographics impact the burden and etiology of enteric infections. A better understanding of the dominant pathogenic causes of disease, their transmission pathways, and populations most at risk could help guide the targeting of WASH interventions to improve their effectiveness.

This dissertation research is a part of the Water, Sanitation, and Hygiene for Health and Education in Laotian Primary Schools (WASH HELPS) study, a longitudinal cluster-randomized controlled trial (RCT) designed to evaluate the impact of UNICEF Lao People's Democratic Republic (Lao PDR) WASH in Schools (WinS) project. Although the purpose of this RCT was to explore the role of WinS in improving school absence, diarrhea, respiratory infection, and STH infection, examining the underlying drivers of infection and co-infection helps explain and contextualize results of the WinS RCT. Therefore, the aim of this dissertation was to better understand the potential for environmental improvements to mitigate enteric disease in low-resource settings. We developed a study design and analysis plan to measure the prevalence of enteropathogens,

elucidate how enteropathogens interact with the environment and each other, and quantify the role of WASH in schools as a potential environmental mediator of infection.

Chapter 1 examines the leading pathogenic causes of enteric infection in Saravane Province, Lao PDR, a rural and poor area of the country with minimal access to improved water and sanitation. There are several unknown factors about enteropathogen transmission in low-income settings that Chapter 1 addresses. First, school-aged children and adults are important actors in some disease transmission cycles, but are often neglected in surveys of enteric illness [41, 42]. We examine enteropathogen prevalence across three age groups — children under five years, primary school-aged children (approximately 5-12 years), and adults — from the same household, and evaluate differences in infection by age group. Second, many WASH trials are designed to prevent exposure to *human* feces and are implemented at the household level. Therefore, the roles of animal feces, a substantial risk factor for enteric infection [43-45], and community WASH access, which may offer herd protection against diarrhea and other WASH-related conditions [46-54], are often overlooked. Chapter 1 addresses these evidence gaps by quantifying the association between household- and community-level WASH access and exposure to animal feces on enteropathogen infection. Additionally, data collected from multiple individuals from the same household and multiple households from the same village further elucidates how household- and village-level clustering is associated with enteropathogens.

Chapter 2 examines drivers of enteric disease by exploring associations between STH infection and non-helminthic enteropathogen infections (viruses, bacteria, protozoa). Given shared risk factors for infection, the geographic and demographic distribution of STH largely coincides with that of acute diarrhea. Enteric pathogen co-infections and multi-parasitism are common, and are

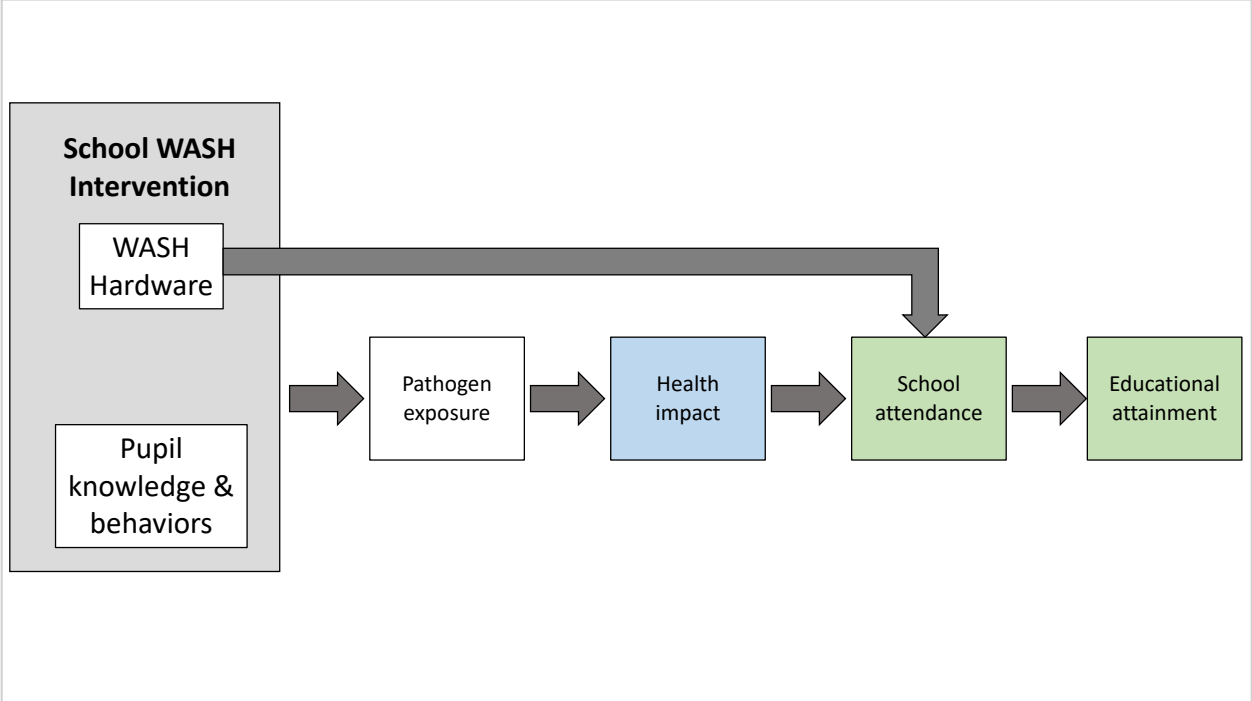
often considered the rule rather than the exception among populations living in socially and economically marginalized communities, rural areas, and tropical or subtropical climate zones [55].

STH are powerful immunomodulators. They can alter susceptibility to secondary microbial infections through direct modulation of host immunity [56, 57], resulting in a range of neutral, facilitative, or antagonistic interactions that may subsequently impact health outcomes [58, 59]. However, interactions between STH and microparasites within the human host and the impacts of these interactions on human health are poorly understood due to the diversity of co-infecting species and their numerous possible interactions [60, 61]. Although many humans are typically infected with multiple pathogens [62], most studies of co-infection are limited to measuring interactions between pairs of parasites [61]. To better understand STH/enteric pathogen interactions and identify trends in pathogen interaction within human hosts, Chapter 2 examines co-infection between the five most prevalent STH species and 20 microparasites (six viruses, nine bacteria, and five protozoa).

Chapter 3 explores the role of the *public domain* in enteric disease transmission [63]. One important public domain is schools, where young children often spend much of their days. WinS programs are justified as part of political and development agendas often as a means to improve children's health and boost educational attendance and achievement [64-66]. WinS programs also support feeding programs and preventive chemotherapy (PC) to reduce reinfection with STH [67]. However, evidence for the impact of WinS interventions on pupil absence and health is mixed [68-74].

Figure I.1 shows a simplified theory of change for the relationship between WinS, health, and educational attainment. Briefly, 1) provision of sanitation facilities, safe drinking water, and handwashing facilities (hardware) combined with health education and behavior change messaging will reduce pathogen exposure; 2) reduced pathogen exposure will lead to reduced illness; 3) reduced illness will lead to higher school attendance; and 4) higher school attendance will lead to greater educational attainment. WASH hardware may also directly impact school attendance by, for example, providing a safe space for female students to manage their menstruation.

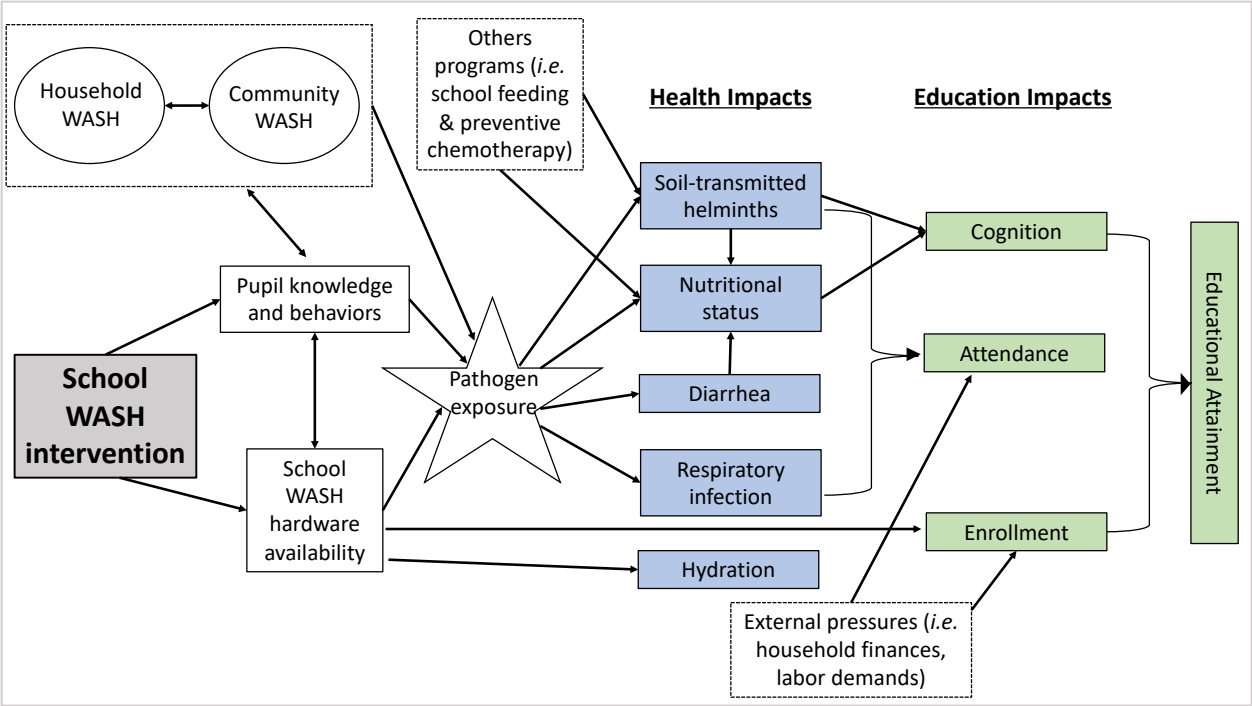
Figure I.1. *Simplified WinS Theory of Change*



However, this simple framework reflects only superficial steps, which may partially explain the mixed impacts of previous WinS evaluations [68-74]. Each step in the theory of change is affected by many more variables than the one immediately preceding it. For example, pathogen exposure

also occurs outside of schools (in the household and other public settings), poor WASH access is not the only cause of pupil illness, and illness is not the only cause of school absence. **Figure I.2** presents a more robust and realistic framework for how WinS improvements achieve impact.

Figure I.2. Expanded WinS Theory of Change



Chapter 3 presents results from the WASH HELPS study, a longitudinal cluster RCT to evaluate the impact of UNICEF Lao PDR's WinS project on school attendance, diarrhea, respiratory infection, and STH. In addition to the primary, intention to treat (ITT) analysis, which given the suboptimal fidelity and adherence of the intervention (see Appendix 1) may underestimate treatment effects [75], Chapter 3 includes a secondary analysis to quantify the impact of the project as implemented by UNICEF and adhered to by schools and pupils. Together with Chapters 1 and 2, assessing these factors along the WinS theory of change provides a better understanding of not only *if* but *why* and *how* the WinS intervention succeeded or not.

Dissertation Aims

Aim 1: Determine conditions associated with enteropathogen infection in rural Lao PDR.

Aim 1.1. Compare the odds of enteropathogen infection between children <5, school-aged children, and adults.

Aim 1.2. Identify differences in associations between taxa- and species-level enteropathogen infection and WASH transmission pathways, including household- and community-level WASH access and exposure to animal feces.

Aim 1.3. Evaluate the extent to which an individual's odds of enteric infection is explained by household- and village-level clustering.

Aim 2: Evaluate how STH infection modifies the odds of concurrent enteric (non-helminthic) pathogen infection.

Aim 3: Evaluate the impact of a comprehensive WinS intervention on pupil school absence, reported diarrhea, reported symptoms of respiratory infection, and STH infection.

Aim 3.1. Examine the effect of intervention fidelity and adherence on project impacts.

Study Setting

Lao PDR is located in Southeast Asia and is classified by the United Nations as a least developed country [76]. Saravane Province, where this research took place, is the poorest province in the country, with nearly half of the population living in poverty [77]. In 2015, 80% percent of Laotians had access to an improved water source, while 73% of population had access to improved

sanitation, with estimates lower in rural areas [78]. Among our study population, 47% of households had improved water access and 23% had improved sanitation. The prevalence of open defecation in Lao PDR is among the highest in the region [78]. Poor WASH access in Lao PDR is responsible for 3 million disease episodes, 6,000 premature deaths, and USD $193 million in economic losses, annually [79]. Of all countries in Southeast Asia, Lao PDR has the highest probability of child and adolescent (aged 0-14) death in the region, driven in large part by diarrheal illness [2].

In 2008, less than one-third of primary schools in Lao PDR had WASH facilities. In 2013, UNICEF began a four-year WinS improvement program in Lao PDR. Through this program, UNICEF delivered WASH facilities to 400 schools nationwide. Schools in Saravane Province, where the WASH HELPS study took place, benefitted from a comprehensive WinS intervention, including both hardware (an improved water source; a toilet block consisting of 3 toilet compartments designated for disabled, boy, and girl students; and handwashing facilities consisting of two sinks with taps connected to the water supply) and software components (water filters for classrooms; group handwashing facilities, hygiene education and behavior change promotion).

Study Design

The WASH HELPS study was designed to evaluate the impact of the UNICEF WinS program on pupil education and health outcomes (Aim 3). We conducted a 3-year longitudinal cluster RCT among 100 randomly selected schools lacking functional WASH facilities (50 intervention, 50 control) in Saravane Province. The WASH HELPS study design, sampling, and data collection methods are described in detail in Appendix 1.

Briefly, data were collected longitudinally from September 2014 through March 2017. Trained enumerators visited study schools every six to eight weeks during the school year (September until May). The main outcome of interest was absence, as measured by roll-call. Secondary outcomes were a one-week recall of absence, diarrhea, and symptoms of respiratory infection as reported by pupils during pupil interviews among a cohort of 40 pupils randomly selected from grades 3-5 and followed for the duration of the study. Each year, stool samples were collected from up to 50 pupils per school prior to distribution of preventative chemotherapy (PC), according to the WHO protocol for school-based STH surveillance [80]. Stool samples were tested for *Ascaris lumbricoides*, *Trichuris trichuria*, and hookworm (*Ancyclostoma duodenale* and *Necatur americanus*) using the Kato Katz technique [81].

To evaluate the roles of household- and community-level WASH access in enteric infection (Aim 1) and better understand enteric pathogen co-infection (Aim 2), we conducted a cross-sectional household survey and collected stool samples from other household members (parents and siblings <5 years old). A subset of 50 villages (25 intervention and 25 comparison) with schools participating in the WASH HELPS study were selected using stratified random sampling based on district and WASH HELPS study intervention status. In each village, we randomly selected 25 households that had a child attending the primary school participating in the WASH HELPS study and a child <5 years old living in the household. At each household, the female head of household was surveyed on household demographics, asset and animal ownership, recent illness among household members, and WASH access and behaviors. Structured observations of WASH facilities were made when available.

In conjunction with the household survey, we collected stool samples from the pupil, the pupil's parent/caregiver (preference was given to female parent/caregiver), and the pupil's sibling <5 years old (if multiple siblings, preference was given to youngest sibling). All samples were tested upon collection for STH using the Kato Katz method [81]. For households that returned all three subjects' stool samples on the same day (*n*=891 samples/297 households) an aliquot of the stool sample was taken for subsequent enteric pathogen analysis by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. Samples were analyzed for 25 enteropathogens (five STH, six viruses, nine bacteria, and five protozoa) using a custom TaqMan Array Card (TAC) (Thermo Fisher, Carlsblad, CA, USA). The qPCR data combined with the household survey data were used to address Aims One and Two.

Additional Research

Included in the Appendix of this dissertation are three manuscripts, written and published during my tenure as a PhD Student and Candidate, which further explore the role of WinS as a modality to improve child health:

Appendix 1: *Design, Intervention Fidelity, and Behavioral Outcomes of a School-Based Water, Sanitation, and Hygiene Cluster-Randomized Trial in Laos*

This paper presents the design, baseline results, and intermediate (fidelity and adherence) results of the WASH HELPS study. Similar to previous WinS impact evaluations in Mali and Kenya [82, 83], we report high quality of project delivery such as provision of a functional water supply, toilets, and handwashing facilities. However, there was sub-optimal fidelity to project outputs such as soap provision, water availability, and promoting group hygiene activities and adherence to key behaviors such as handwashing with soap following toilet use. These results justified our decision

to conduct a secondary analysis of the effect of intervention fidelity and adherence on intervention impacts (Aim 3.1).

Appendix 2: *The impact of school water, sanitation, and hygiene improvements on infectious disease using serum antibody detection*

This study was nested within a longitudinal impact evaluation of the Dubai Cares Water, Sanitation, and Hygiene in Schools Initiative in Mali (DCIM WASH) project, a comprehensive school-based WASH intervention [74]. We explored the feasibility of using dried blood spots (DBS) as an alternative to self-reported diarrhea, which is subject to bias, or to PCR analysis of stool samples, which can be logistically challenging to collect in the field. This study was novel in the use of blood antibody data to assess the impact of a WASH intervention, as well as the application of factor analysis and linear latent models to analyze antibody data. We found that evidence of person-to-person and food/water-transmitted enteric disease was lower among students attending intervention schools, which is consistent with the results from the parent trial that showed reductions in pupil-reported diarrhea [74]. This paper supports the theory of change that WinS may improve pupil health, and adds to the heterogenous evidence base for the impact of WinS on pupil diarrhea.

Appendix 3: *The impact of water consumption on hydration and cognition among schoolchildren: Methods and results from a crossover trial in rural Mali*

The availability of water during the school day is essential for supporting personal hygiene, sanitation, and maintaining a clean school environment. Dehydration is associated with reduced cognitive performance among adults [84-87], but few studies have investigated the relationship

between dehydration and cognition in children. Linking drinking water availability to cognitive skills among children in water-scarce areas could provide novel evidence to support the theory of action whereby WinS improves educational attainment. We established the proof of principle that water provision increased hydration, but found no evidence that improvements in hydration status led to improvements in cognitive performance. However, results may have been masked by a strong practice effect and the power to detect significant differences was limited. This study demonstrated the feasibility of collecting biometric measurements of hydration status and testing cognitive abilities in schools in resource-poor settings, which can be applied to future WinS research.

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Chapter 1. Environmental and spatial determinants of enteric pathogen infection in rural Lao People's Democratic Republic: A cross-sectional study[1]

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Abstract

Recent large-scale water, sanitation, and hygiene (WASH) trials have found limited impact on health outcomes. The aims of this study were to estimate the prevalence of enteropathogens among children <5, school-aged children, and adults, to quantify variations in association between taxa- and pathogen-level enteropathogen infection and WASH transmission pathways, and to estimate associations between household- and village-level clusters and enteropathogen infection. We conducted a cross-sectional survey in 50 villages in Saravane Province, Lao People's Democratic Republic. We collected fecal samples from 891 children <5, school-aged children, and adults living in 297 randomly selected households, and collected survey and observational data on household demographics, WASH access, and animal ownership. Fecal samples were analyzed for 25 enteropathogens using a quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay. We observed a high prevalence of enteropathogen infection across age groups (98.3%). Using logistic regression, we found that few household- or village-level WASH transmission pathways were significantly associated with odds of enteropathogen infection. An improved sanitation facility was associated with higher odds of viral infection (odds ratio (OR): 2.79, 95% confidence interval (CI): 1.26, 6.18) and a basic handwashing facility was associated with lower odds of viral infection (OR: 0.44, 95% CI: 0.24, 0.82). Animal ownership was associated with greater odds of protozoa infection (OR: 3.59, 95% CI: 1.53, 8.46). A basic handwashing facility (OR: 0.54, 95% CI:0.35, 0.85) and community sanitation coverage (OR: 0.82, 95% CI 0.73, 0.93) were associated with lower odds of soil-transmitted helminth (STH) infection. Village-level Median Odds Ratios (MOR) were higher than household-level MOR for viruses, protozoa, and STH, indicating that village-level clusters were more relevant to understanding odds of infection than were household-level clusters. Results suggest that WASH

access, as expected, is associated with lower enteric illness, but WASH access as currently defined does not reveal a measurably protective association with infection for many etiologies. Focusing resources to achieve high uptake of a single, community-wide intervention targeted at specific pathogens may be more effective than a comprehensive intervention at a household level.

Introduction

The health risks associated with inadequate access to water, sanitation, and hygiene (WASH) are well documented [1-3]. Interventions to improve water, sanitation, and hygiene have revealed 33%, 25%, and 30% reductions in risk of childhood diarrhea, respectively [4]. Improved WASH is associated with reductions in neglected tropical diseases (NTDs) such as soil-transmitted helminths (STHs), trachoma, and schistosomiasis [5-8]. Yet several large-scale household-based WASH impact evaluations have failed to demonstrate a significant reduction in diarrhea [9-13] or STH infection [9, 12].

Enteric infections and diarrheal diseases may be caused by over 40 pathogens (bacteria, viruses, protozoa, and helminths) shed in both human and animal feces, many of which can persist in environmental reservoirs, with different etiologies and dominant transmission pathways [14-18]. Consumption of fecally contaminated food and water and interaction with fecally contaminated environments (i.e., soil and surface water) is a critical transmission pathway across all taxa (defined here as virus, bacteria, protozoa, or STH) [19, 20]. Person-to-person transmission is a key transmission pathway for enteroviruses [21, 22] and some bacteria (e.g. *Shigella*) [23], while some STH (e.g. hookworm and *S. stercoralis*) are transmitted transdermally [24].

One hypothesis for these inconclusive findings is that interventions are not sufficiently targeting the relevant transmission pathways for the most prevalent pathogens. For example, the WASH

Benefits Kenya trial promoted water chlorination [11], which is ineffective against *Cryptosporidium* spp, one of the leading causes of moderate-to-severe diarrhea among young children in a nearby area [25]. Another hypothesis is that many WASH trials are designed to prevent exposure to human feces and do not adequately address exposure to animal feces [26-29]. Animal feces present a substantial risk to human health, as animals may be the leading driver of pathogen diversity in the environment [19], many enteropathogens that cause moderate to severe diarrhea are of animal origin, and approximately one-third of deaths due to diarrhea among children <5 years are attributed to pathogens that can be found in animal feces [30].

A further consideration of these null results is that most WASH interventions are implemented at the household, rather than the community-level [9-11, 31]. Indeed, households are important loci of WASH-related disease transmission, since domestic activities and behaviors can result in the sharing of infective sites, thus leading to similar risks of infection among household members [32, 33]. However, evidence suggests that sanitation can provide community-level, or herd protection on health outcomes such as diarrhea, trachoma, nutritional status, and infant mortality [34-42]. Open defecation or inadequately managed sanitation resulting in environmental contamination of fecal sludge can increase direct and indirect contact with fecal contamination through soil, surface water, and feces in public settings [19, 43], which can lead to ingestion of enteric pathogens [44], even among households with toilets [39, 45].

Here we describe the prevalence and distribution of WASH-related enteric pathogens in the context of household and community WASH access. There are several unknowns about enteropathogen transmission that this study addresses. First, with the exception of some STHs (e.g., hookworm) [24], rates of enteric infection are thought to be highest among young children

[46]. Enteric pathogens are responsible for 1.7 billion episodes of diarrhea per year among children <5 [47], and diarrhea is one of the leading causes of death in children in this age group [48]. As such, most WASH interventions and etiological surveys of enteropathogen infection focus on children <5 years old [9-11, 25, 31, 49]. However, intestinal infectious disease is the second leading cause of death among children aged 5-9 worldwide [50], and there are more than 2.8 billion episodes of diarrhea per year among children >5, adolescents, and adults [51]. Yet, there is a gap in evidence on the enteric pathogen prevalence and etiology among older age groups [51, 52], who are an important component of the pathogen transmission cycle. Additionally, distinguishing village-level from household-level environmental effects has been challenging in previous studies. By collecting enteropathogen data from multiple individuals within the same household and multiple households within the same village we can better elucidate how household- and village-level clustering is associated with enteropathogens.

We conducted a cross-sectional analysis of fecal samples collected from children <5, school-aged children, and adults residing in rural Lao People's Democratic Republic (Lao PDR). The aims of this study were to 1) identify the pathogens associated with enteric infection and whether they differed by age group; 2) identify differences in associations between taxa- and pathogen-level enteric infection and WASH transmission pathways, including household- and community-level WASH access and exposure to animal feces; and 3) estimate the association between household- and village-level clustering and odds of enteropathogen infection.

Methods

Setting

This cross-sectional study was nested within the WASH HELPS study, a longitudinal cluster-randomized trial evaluating a comprehensive school-based water, sanitation, and hygiene (WASH) intervention in 100 schools in Saravane Province, Laos. Detailed methods of the parent study are described elsewhere [53]. The WASH HELPS study is registered at clinicaltrials.gov (NCT02342860).

Ethics

This study was approved by Emory University's Institutional Review Board (IRB0076404) and the Lao Ministry of Health's National Institute of Public Health National Ethics Committee (No. 043 NIOPH/NECHR). Adult participants provided informed verbal consent for the household survey and stool collection for themselves and their children prior to any data collection.

Study design

Methods are described in detail elsewhere [54]. Briefly, we used stratified random sampling to select 50 of the 100 school-hosting villages participating in the WASH HELPS study. In each village, we randomly selected 25 households meeting two eligibility criteria: 1) having a child attending the primary school participating in the WASH HELPS study, and 2) having a child <5 years old living in the household. We conducted a household survey to collect information on household demographics, asset and animal ownership, recent illness among household members, and WASH access and behaviors. We also conducted structured observations of WASH facilities when present.

During the household survey, we distributed three pre-labeled, resealable plastic bags, each containing a plastic spoon to collect stool samples from the pupil, the pupil's parent/caregiver (preference was given to female parent/caregiver), and the pupil's sibling <5 years old (if multiple siblings, preference was given to youngest sibling). Participants were instructed to collect the first stool on the following morning. Stool samples were collected in the morning and transported with a cold chain to the field laboratory within two hours of collection. A second return visit was made the following day if households did not return all three participants' stool samples on the first day. All data were collected between February-April 2017 (dry season), prior to annual school-based chemotherapy for STH. The time frame corresponded with the final round of data collection and conclusion of the WASH HELPS study [53].

For this sub-study, a subset of 297 households (*n*=891 samples) were selected for additional enteropathogen analysis via quantitative reverse transcription polymerase chain reaction (qRT-PCR) using stratified random sampling based on district and village size and WASH HELPS intervention status. Subjects were eligible for inclusion only if all three subjects in the household (adult, school-aged child, and child <5 years old) returned their stool sample on the same day. Including multiple subjects from the same household allowed us to quantify household-level clustering of infection and distinguish village-level effects from household-level effects.

Laboratory analysis

Laboratory procedures have been described in detail elsewhere [54]. Briefly, in the field laboratory we aliquoted 200 mg of stool into a DNA/RNA Shield Collection and Lysis Tube (Zymo Research, Irvine, CA, USA). One field control was processed each day using DNA/RNA-free water to evaluate the possibility of false positives from contamination in the field laboratory during

sampling. Samples were kept frozen at -20°C until transported to a laboratory at Emory University, where they were subsequently stored at -80°C until further processing. Total nucleic acid was extracted from samples using the ZymoBIOMICS DNA/RNA Mini Kit (Zymo Research, Irvine, CA, USA), according to manufacturer instructions. One extraction blank was included per batch to exclude the possibility of false positives from contamination during extraction. Extractions were analyzed via quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis using a custom TaqMan Array Card (TAC) (Thermo Fisher, Carlsblad, CA, USA) with compartmentalized, probe-based qPCR assays for 25 enteropathogens [55, 56]. TAC primer and probe sequences are listed in **Table S1** (see Additional file 1). TAC preparation was based on the protocol described by Liu et al., with the exception of including 0.3 μM BSA to reduce inhibition of nucleic acid amplification [55].

Two researchers manually read TAC data; a third researcher resolved conflicting results. Multicomponent plots were inspected for increases in fluorescence for the FAM-based gene-specific probe to validate true amplification of the complete gene target. Samples were considered positive only when the corresponding field and extraction blanks were negative, otherwise the data were considered invalid [56].

Measures

In the primary analysis, the outcome variables were presence/absence of any viral infection, bacterial infection, protozoal infection, or STH infection. In the secondary analysis, the outcome variables were presence/absence of each individual pathogen.

The exposure variables included household-level improved drinking water source (reported), improved sanitation facility (observed), and basic handwashing facility (observed), all classified according to WHO/UNICEF Joint Monitoring Programme standards [57]; animal ownership, which was reported as owning any cows, goats, sheep, poultry (chickens or ducks), or pigs; and village-level prevalence of an improved drinking water source ("improved drinking water coverage"), an improved sanitation facility ("improved sanitation coverage"), and a basic handwashing facility ("basic handwashing facility coverage"). Village-level WASH prevalence was calculated by aggregating household-level WASH access variables at the village-level (cluster), excluding each individual's own household in order to better represent indirect exposure and to avoid forced correlation between household- and village-level covariates [34]. Village-level WASH prevalence was re-scaled with cut-points at each 10^{th} percentile to aid interpretability.

To examine the odds of enteric infection across age groups, we categorized each subject as a child <5 years old (CU5); school-aged child (SAC), defined as a child enrolled in primary school (class 1-5); or adult. Socioeconomic status was determined through a series of questions and observations about household construction materials (roof, floor, and walls), ownership of a mobile phone, and presence of electricity. These variables were chosen based on those used in the Demographic and Health Surveys for measures of wealth in Lao PDR [58], and we used principal component analysis methods to derive one single wealth metric from all of the wealth assets combined [59]. The number of household members was defined as all people currently living in the household full time at the time of the survey.

E. coli pathotypes were classified according to the following gene targets: EAEC (*aatA* and/or *aaiC*), EHEC (*eae* with *stx1* and/or *stx2*, and without *bfpA*), typical EPEC (*bfpA* with or without

eae), atypical EPEC (*eae* without *bfpA*, *stx1*, or *stx2*), ETEC (*eltB* for heat-labile toxin [LT] and *estA* with or without *eltB* for heat-stable toxin [ST]) [25].

Statistical analysis

We calculated odds ratios (ORs) and 95% confidence intervals (CIs) for each primary and secondary outcome using logistic regression models, with random intercepts at the village and household levels to account for clustering. All analyses were evaluated for statistical significance at α=0.05. All data were analyzed using Stata Statistical Software: Release 15 (StataCorpLP, College Station, TX, USA).

To estimate the association between village- and household-level clustering and odds of infection, we calculated the median odds ratio (MOR) of the random intercepts. The MOR translates area-level variance to the OR scale, and can be interpreted as the median increased odds of infection that one would have by moving to another area (village or household) with higher odds of infection [60]. In other words, the MOR represents the extent to which an individual's odds of infection are determined by its village or household, after adjusting for other measured covariates [60, 61].

We also examined the intraclass correlation coefficient (ICC), which estimates the proportion of observed variation in the outcome due to clustering. Because we used logistic regression, we employed the latent variable method, which converts both the individual- and area-level components of the variance to the logistic scale prior to computing the ICC [60]. ICC scores range from 0 to 1; a low value indicates that villages/households are relatively independent and suggests that village/household level factors are not relevant to understanding differences in the outcome,

whereas a value closer to 1 indicates that village/household-level factors are strongly associated with the outcome [62].

Results

We collected a total of 2,269 fecal samples from the same number of participants. Of these, all three subjects in the household (adult, SAC, and CU5) returned their stool sample on the same day in 297 households (*n*=891 subjects) and thus were eligible for inclusion in the study. Samples from 890 participants were included in the analysis (1 sample was excluded due to insufficient amount for nucleic acid extraction). We suspected contamination by one or more target pathogens of 66 samples in the field (EPEC=1, rotavirus=11, *Shigella*/EIEC=21, STEC *stx2*=33, EAEC=40, *C. difficile*=1, *A. lumbricoides*=1) and 78 samples in the laboratory (rotavirus=64, astrovirus=3, *C. jejuni/C. coli*=8); these samples were excluded from taxa- and pathogen-specific analyses.

Description of study population, WASH access, and pathogen prevalence

Household and community-level WASH factors are described in **Table 1.1**. All adult participants were female, 150 (50.5%) of CU5 were female, and 143 (48.2%) of SAC were female.

Table 1.1. Description of study population, Saravane Province, Lao PDR, 2017

	Total (*n*=297 households)
Household-level characteristics	
Household population size, mean (SD)	7.3 (3.1)
Improved toilet, n(%)	67 (22.6%)
Improved drinking water source, n(%)	140 (47.2%)
Basic handwashing facility, n(%)	100 (33.7%)
Animal ownership, n(%)	282 (94.9%)
Village-level characteristics	
Improved sanitation coverage, mean % (SD)	22.6% (30.6%)
Improved drinking water source coverage, mean % (SD)	47.1% (40.1%)
Basic handwashing facility coverage, mean % (SD)	33.7% (22.8%)

SD= standard deviation

Pathogen prevalence by age group is described in **Table 1.2**. One or more enteropathogens were identified in 875 (98.3%) of the subjects. The mean (SD) number of enteropathogen infections per person was 4.3 (2.0), with little variation by age group. Bacterial infections were the most prevalent, with 85.2% of subjects having at least one bacterial infection, followed by protozoal infections (74.9% of subjects), STH infections (69.3% of subjects), and viral infections (34.6% of subjects). The most common enteropathogens detected were Giardia (70.9%), hookworm (48.4%), EAEC (47.8%), ETEC (36.9%), and EPEC (35.2%).

Concordance of pathogen infection among CU5, SAC, and adults living in the same household ("household triad") is shown in **Figure 1.1**. The highest concordance among the complete household triad was observed for *Giardia* (all three members of the household triad had a *Giardia* infection in 40.5% of households), followed by hookworm (24.3%), EAEC (18.5%) and Rotavirus (11.9%).

Table 1.2. Prevalence of enteropathogens, stratified by age group and ordered from most to least prevalent, Saravane Province, Laos, 2017

	Child <5 (n=297)	**SAC*** (n=297)	**Adult** (n=296)	**Total** (n=890)
Any enteropathogen	294 (99.0%)	292 (98.3%)	289 (97.6%)	875 (98.3%)
Mean (SD) enteropathogens	4.2 (1.9)	4.2 (2.1)	4.4 (2.1)	4.3 (2.0)
Any bacteria[1]	241 (86.1%)	230 (82.7%)	239 (86.9%)	710 (85.2%)
Any protozoa	249 (83.8%)	231 (77.8%)	187 (63.2%)	667 (74.9%)
Any STH[1*]	163 (55.1%)	197 (66.3%)	208 (70.3%)	568 (63.9%)
Any virus[1]	104 (37.6%)	90 (32.7%)	92 (33.5%)	286 (34.6%)
Giardia intestinalis	238 (80.1%)	221 (74.4%)	172 (58.1%)	631 (70.9%)
Hookworm	110 (37.0%)	158 (53.2%)	163 (55.1%)	431 (48.4%)
EAEC[1*]	128 (44.9%)	129 (45.1%)	149 (53.4%)	406 (47.8%)
ETEC*	107 (36.0%)	89 (30.0%)	132 (44.6%)	328 (36.9%)
EPEC[1*]	109 (37.7%)	103 (35.9%)	89 (31.8%)	301 (35.2%)
Aeromonas spp.	64 (21.5%)	80 (26.9%)	111 (37.5%)	255 (28.7%)
Rotavirus[1]	72 (26.0%)	67 (24.4%)	72 (26.2%)	211 (25.5%)
Campylobacter jejuni[1]	82 (27.8%)	71 (24.2%)	43 (14.6%)	196 (22.2%)
Strongyloides stercoralis	43 (14.5%)	58 (19.5%)	84 (28.4%)	185 (20.8%)
Shigella/EIEC[1*]	47 (16.4%)	48 (16.4%)	54 (18.7%)	149 (17.1%)
Trichuris trichiura	49 (16.5%)	55 (18.5%)	42 (14.2%)	146 (16.4%)
Cryptosporidium spp.	56 (18.9%)	42 (14.1%)	42 (14.2%)	140 (15.7%)
EHEC*	23 (7.7%)	35 (11.8%)	49 (16.6%)	107 (12.0%)
Ascaris lumbricoides[1]	29 (9.8%)	28 (9.4%)	24 (8.1%)	81 (9.1%)
Norovirus GII	24 (8.1%)	21 (7.1%)	24 (8.1%)	69 (7.8%)
Salmonella enterica	10 (3.4%)	9 (3.0%)	23 (7.8%)	42 (4.7%)
Astrovirus	11 (3.7%)	8 (2.7%)	4 (1.4%)	23 (2.6%)
Sapovirus	12 (4.0%)	4 (1.3%)	1 (0.3%)	17 (1.9%)
Clostridium difficile[1]	4 (1.3%)	3 (1.0%)	3 (1.0%)	10 (1.1%)
Norovirus GI	1 (0.3%)	4 (1.3%)	3 (1.0%)	8 (0.9%)
Adenovirus 4041	0 (0.0%)	4 (1.3%)	2 (0.7%)	6 (0.7%)
Cryptosporidium hominus	0 (0.0%)	1 (0.3%)	0 (0.0%)	1 (0.1%)
Entamoeba histolytica	1 (0.3%)	0 (0.0%)	0 (0.0%)	1 (0.1%)
Cryptosporidium parvum	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

*SAC=school-aged child, STH=soil-transmitted helminth, EAEC=enteroaggregative *Escherichia coli*, EHEC=enterohemorrhagic *E. coli*, EPEC=enteropathogenic *E. coli*, ETEC=enterotoxigenic *E. coli,* EIEC=enteroinvasive *E. coli*

[1]number of samples missing due to suspected field or laboratory contamination: virus=63, bacteria=57, STH=1, EAEC=40, EPEC=34, rotavirus=63, *C. jejuni/C. coli*=8, *Shigella*/EIEC=11, *A. lumbricoides*=1, *C. difficile*=1

Associations between age and odds of enteropathogen infection

Odds of enteropathogen infection differed by age group for some but not all taxa (**Table 1.3**). Odds of viral and bacterial infections did not significantly differ across age groups. Odds of protozoal infection decreased with age; compared to adults, protozoal infection was more likely among CU5 (OR=3.12, 95% CI=1.92, 5.07) and SAC (OR=1.94, 95% CI=1.22, 3.07). Odds of STH infection increased with age; compared to adults, STH infection was less likely among CU5 (OR=0.40, 95% CI=0.25, 0.64), but there was no difference between SAC and adults.

Similarly, pathogen-specific odds of infection differed by age group for some but not all pathogens (**Figure 1.2**). CU5 had higher odds of *Giardia*, *C. jejuni*, and sapovirus infection, but lower odds of ETEC, *Aeromonas*, EHEC, *Salmonella*, hookworm, and *S. stercoralis* infection, compared to adults. School-aged children had higher odds of *Giardia*, but lower odds of ETEC, *Aeromonas*, *Salmonella*, and *S. stercoralis* infection, compared to adults.

Table 1.3. Adjusted odds ratios and 95% confidence intervals of associations between demographic and WASH covariates and viral, bacterial, protozoal, and soil-transmitted helminth (STH) enteric infections, Saravane Province, Laos, 2017

	Virus[1] (*n*=827)	**Bacteria[2] (*n*=833)**	**Protozoa[3] (*n*=890)**	**STH[4] (*n*=889)**
Child <5 years (ref: adult)	1.15 (0.69, 1.91)	0.97 (0.53, 1.76)	**3.12 (1.92, 5.07)**	**0.40 (0.25, 0.64)**
School-aged child (ref: adult)	0.82 (0.49, 1.37)	0.72 (0.40, 1.30)	**1.94 (1.22, 3.07)**	0.80 (0.50, 1.28)
Female (ref: male)	0.75 (0.46, 1.22)	1.12 (0.66, 1.90)	0.66 (0.41, 1.05)	1.07 (0.70, 1.62)
Socioeconomic status	0.88 (0.75, 1.04)	1.01 (0.86, 1.18)	1.06 (0.94, 1.20)	**0.85 (0.76, 0.96)**
Household population size	0.94 (0.86, 1.04)	0.95 (0.88, 1.03)	1.02 (0.95, 1.10)	1.03 (0.96, 1.10)
Improved toilet	**2.79 (1.26, 6.18)**	0.46 (0.21, 1.01)	0.85 (0.46, 1.57)	0.71 (0.40, 1.25)
Improved drinking water source	1.48 (0.76, 2.88)	0.64 (0.33, 1.25)	0.88 (0.52, 1.49)	1.01 (0.60, 1.70)
Basic handwashing facility	**0.44 (0.24, 0.82)**	0.95 (0.54, 1.70)	0.76 (0.48, 1.21)	0.54 (0.35, 0.85)
Household animal ownership	1.32 (0.37, 4.74)	1.34 (0.46, 3.89)	**3.59 (1.53, 8.46)**	2.18 (0.87, 5.45)
Improved toilet coverage[5]	1.01 (0.85, 1.21)	1.08 (0.94, 1.24)	0.96 (0.86, 1.08)	**0.82 (0.73, 0.93)**
Improved drinking water coverage[5]	0.97 (0.85, 1.11)	1.00 (0.90, 1.11)	0.93 (0.84, 1.01)	1.00 (0.91, 1.11)
Basic handwashing facility coverage[5]	0.94 (0.75, 1.18)	1.00 (0.85, 1.18)	0.95 (0.82, 1.10)	0.98 (0.84, 1.14)
Median Odds Ratio-Village[6]	**3.89 (2.64, 6.69)**	**1.97 (1.48, 3.25)**	**2.07 (1.59, 3.10)**	**2.46 (1.89, 3.56)**
Median Odds Ratio-Household[6]	**3.10 (2.23, 4.94)**	**2.27 (1.58, 4.27)**	**1.96 (1.45, 3.39)**	**1.73 (1.28, 3.29)**
ICC- Village	0.30	0.11	0.13	0.20
ICC- Household	0.21	0.16	0.11	0.07

All models include random intercepts at the village and household levels to account for clustering.

[1] Virus includes one or more of the following pathogens: astrovirus, adenovirus, norovirus GI, norovirus GII, rotavirus, or sapovirus. [2] Bacteria includes one or more of the following pathogens: *Aeromonas*, *C. difficile*, *C. jejuni*, EAEC, EHEC, EPEC (typical or atypical), LT- or ST-ETEC, *Shigella* spp./EIEC, or *Salmonella.* [3] Protozoa includes one or more of the following pathogens: non-hominus and non-parvum *Cryptosporidium* spp., *C. hominus*, *C. parvum*, *E. histolytica*, and *G. intestinalis.* [4] Soil-transmitted helminths (STH) includes one or more of the following helminths: hookworm (*N. americanus* and/or *A. duodenale*), *A. lumbricoides*, *T. trichiura*, or *S. stercoralis.*

[5]WASH covariate coverage is interpreted as the change in odds of infection per 10% increase in WASH covariate coverage at the village level

[6]Median odds ratio is interpreted as the median increased odds of infection that one would have if moving to another area (household or village) with higher odds of infection, after accounting for other covariates in the model.

Viruses

Improved sanitation in the household was associated with higher odds of viral infection (OR=2.79, 95% CI=1.26, 6.18). A basic handwashing facility in the household was associated with lower odds of viral infection (OR=0.44, 95% CI=0.24, 0.82). Animal ownership was not associated with viral infection. The MOR-V for viral infection (MOR= 3.89, 95% CI= 2.64, 6.69) was higher than the MOR-HH (MOR= 3.10, 95% CI=2.23, 4.94). After adjusting for model covariates, 30% of remaining residual variation in odds of viral infection (ICC=0.30) was due to clustering at the village-level, and 21% was due to household-level clustering (ICC=0.21).

Trends among individual viral pathogens were largely consistent with the taxa-level trend. With one exception, in which household improved sanitation was associated with higher odds of rotavirus, household WASH and community WASH coverage covariates were not statistically associated with infection. Generally, point estimates for a household-level improved drinking water source and sanitation coverage trended towards a protective association with individual viral pathogens, while animal ownership trended towards higher infection odds. Point estimates for household sanitation, household handwashing facilities, improved drinking water coverage, and basic handwashing facility coverage were mixed.

Bacteria

No WASH covariates were statistically associated with bacterial infection, though point estimates for household WASH access trended towards a protective association, while point estimates for community sanitation coverage and household animal ownership trended towards higher infection odds. The MOR-HH for bacterial infection (OR=2.27, 95% CI=1.58, 4.27) was higher than the MOR-V (OR=1.97, 95% CI=1.48, 3.25). After adjusting for model covariates, household-level

clustering explained 16% (ICC=0.16) of the remaining residual variance in odds of bacterial infection while village-level clustering explained 11% (ICC=0.11).

Trends among individual bacterial pathogens were consistent with the taxa-level trend. Point estimates for household WASH access generally trended towards a protective association; point estimate trends for village-level WASH coverage were mixed. Animal ownership trended towards higher infection odds for six of the eight enterobacteria in the analysis but was statistically associated only for ETEC.

Protozoa

Household and community WASH covariates were not statistically associated with protozoal infection, though all point estimates trended towards a protective association. Animal ownership was associated with higher odds of protozoal infection (OR=3.59, 95% CI=1.53, 8.46). The MOR-V (MOR=2.07, 95% CI=1.59, 3.10) was similar to the MOR-HH (MOR=1.96, 95% CI=1.45, 3.39), and both were of greater relevance to odds of protozoal infection than were WASH covariates, but not age or animal ownership. After adjusting for model covariates, village-level clustering explained 13% of remaining residual variance in odds of protozoal infection (ICC=0.13) while household-level clustering explained 11% (ICC=0.11).

Trends among individual protozoal pathogens were consistent with the taxa-level trend. Point estimates for household and village WASH access trended towards a protective association with *Giardia* and *Cryptosporidium* spp. infection. Animal ownership was associated with higher odds of both *Giardia* and *Cryptopsoridium spp.* infection, but was statistically associated only for *Giardia.*

STH

The presence of a basic handwashing facility in the household (OR=0.54, 95% CI=0.35, 0.85) and increasing improved sanitation coverage (OR=0.14, 95% CI=0.04, 0.50) were associated with lower odds of STH infection. Although not statistically associated with STH infection, point estimates for household-level improved toilet and basic handwashing facility coverage trended towards a protective association, while point estimates for household and village-level improved drinking water sources and animal ownership trended towards higher infection odds. The MOR-V for STH infection (MOR=2.46, 95% CI=1.89, 3.56) was higher than the MOR-HH (MOR=1.73, 95% CI=1.28, 3.29). Village-level clustering explained 20% of the remaining residual variance in odds of STH infection (ICC=0.20) while household-level clustering explained 7% (ICC=0.07).

Trends among individual STH were largely consistent with the taxa-level trend. Although no statistical associations were found, household and village-level WASH access trended towards a protective association, with the exception of a household improved drinking water source, which trended towards higher infection odds. Animal ownership trended towards higher infection odds for all STH species except *A. lumbricoides*.

Discussion

In this study, we examined the leading pathogenic causes of enteric infections and household- and village-level risk factors for those infections across differently aged study subjects living in the same households in rural Lao PDR. We detected a high prevalence of enteropathogens among our study population, with 98.3% of subjects harboring at least one enteropathogen infection. Few household or village-level WASH covariates we assessed were statistically associated with odds of infection at the taxa- or individual pathogen-level, though WASH access generally trended

towards lower odds of infection. Our results point to animal ownership as a possible risk factor for enteric infections, which may outweigh the potential benefit to increased socio-economic and nutritional status they may confer.

Our multilevel analysis approach, which allowed us to estimate the residual variation between villages and households, highlighted the importance of contextual factors beyond WASH access that influence one's susceptibility to enteric infection. Recent evidence has demonstrated a substantial risk of enteric infection from the public domain by quantifying a diversity of enteropathogens in surface water, community water sources, and soil, including children's play sites [19, 63]. Additionally, children's exposure to enteric pathogens in their neighborhood may have spatial dimensions; the more area they have contact with in their neighborhood, the greater their risk of multi-pathogen exposure and pathogen dose [44]. We found that the village-level MOR was higher than the household-level MOR for all taxa except bacteria, meaning that the individual probability of infection not explained by the current set of covariates was influenced more by village-level factors than by household-level factors. Our results suggest that community-level interventions may be more effective than household-level interventions, particularly in places where enteroviruses, protozoa, and STH are the predominant etiologies of enteric illness.

At the taxa-level, we observed higher odds of infection among CU5 compared to other age groups only for protozoa, an association driven largely by *Giardia*, which is one of the first enteric pathogens to infect children [64]. Of the 18 pathogens included in our pathogen-specific analysis, CU5 had higher odds of infection compared to adults only for three pathogens: *Giardia*, *C. jejuni/C. coli*, and sapovirus. We found no significant difference in odds of infection across age groups for half of the 18 pathogens in our analysis, including rotavirus, which is the leading cause

of acute gastroenteritis in infants and young children in developing countries [65], the leading cause of death due to diarrhea among children <5 [48], and is typically considered a childhood illness [66]. Concordance of pathogen infection among the household triad differed across pathogens and ranged from 0.0% (sapovirus, *C. difficile*, and norovirus GI) to 40.5% (*Giardia*), though these patterns are partially driven by underlying pathogen prevalence. Household- and village-level clustering, as measured by the MOR, indicated substantial area-level variations relevant to understanding individual odds of infection. Together, these results suggest that the role of other household and village members in disease transmission should not be overlooked. More efforts to target older children, adolescents, and adults in etiological surveys of enteric illness and WASH interventions is warranted. Future research could further examine intra-household transmission patterns of enteropathogens.

Our measure of improved sanitation was largely representative of whether households reported using a toilet at all; an unimproved toilet was observed in only 5% of households and 75% of households reported open defecation by at least one household member. We observed that household-level improved sanitation was associated with 2.75 times *higher* odds of viral infection, while a household-level basic handwashing facility was associated with 56% lower odds of viral infection. Compared to bacteria and protozoa, viruses have a lower infectious dose and a higher rate of shedding, sometimes long after resolution of symptoms. As a result, viral pathogens spread easily from person to person and via fomites [21, 22, 65]. Evidence suggests that improvements in sanitation alone are not sufficient to prevent enterovirus transmission [67], especially rotavirus which is highly infectious and extremely persistent in the environment [68]. Our results are consistent with research from India which reported an increased risk (though not significantly) of previous viral infection among urban households with toilets [69]. Additionally, a study among

schools in rural Kenya reported higher hand contamination among schools that were provided improved toilets, but where inadequate hand hygiene was observed [70]. Hygiene of both hands and surfaces are critical to interrupting enterovirus transmission [67]. Our results substantiate evidence that without concurrent changes in hygiene, it is unlikely that sanitation alone will reduce incidence of enterovirus infections [66].

Our results are consistent with the established transmission pathways for STH via ingestion of eggs and contact with fecally contaminated food and soil [64]. Thus, handwashing and food hygiene to prevent egg ingestion, and sanitation to eliminate the environmental reservoir for STH and to prevent dermal contact with eggs (e.g. hookworm and *S. stercoralis*) are key interventions for control [71, 72]. We observed that a household basic handwashing facility was associated with 46% lower odds of STH infection. Having a toilet at the household level trended towards lower odds of STH, but was not statistically significant. However, each 10% increase in community sanitation coverage was associated with 18% lower odds of STH infection. Current STH control strategies focus predominately on preventative chemotherapy (PC) of SACs [73], but re-infection frequently occurs quickly following treatment [74]. Thus, long-term control requires eliminating the environmental reservoir for STH through improvements in WASH, particularly sanitation [5]. Furthermore, household latrines will not prevent hookworm infection if open defecation still persists by some members of the community [33]. Our results support the limited evidence that both PC and WASH are necessary for sustained control or elimination of STH, as long as sanitation reaches a high level of uptake [75]. To our knowledge, there is no evidence on community thresholds of sanitation associated with STH, as has been done for diarrhea, trachoma, nutritional status, and infant mortality [34-42]. Such evidence would be of great benefit to the WASH and NTD sectors to influence policy on STH programming and coordination between sectors [76].

Our results support conclusions from recent reviews that exposure to animal feces is a risk factor for enteropathogen infection, and consequently on diarrhea, NTDs, and nutritional outcomes [28, 29, 77]. Many enteric bacteria, protozoa, and some STH can be transmitted by animal feces [28]. Zoonotic transmission of enteroviruses is rare, with the exception of rotavirus and Hepatitis E [28, 65]. Consistent with these pathways, we found that animal ownership was associated with higher odds of protozoal infection, and trended towards higher odds of bacterial and STH infection and lower odds of viral infection. In our pathogen-specific analysis we observed that animal ownership was associated with higher odds of *Giardia* and ETEC, and trended towards higher odds of infection with an additional 10 of the 18 pathogens in the species-specific analysis. These trends were largely consistent with the existing evidence base on zoonotic transmission of enteropathogens, with some exceptions. Giardia, *C. jejuni*/*C. coli*, EHEC (a subset of Shiga toxin-producing *E. coli* (STEC)), *Salmonella*, *Cryptosporidium* spp., and *S. stercoralis*, all have animal hosts and are capable of zoonotic transmission [78-81]. However, animal ownership trended towards higher odds of infection for some pathogens that are not considered zoonotic, likely due to limited variation in the measure of animal ownership (95% of households reported owning at least 1 animal). For example, STEC is considered the only zoonotic *E. coli* pathotype; ETEC and EAEC have been isolated in animals but are not transmissible to humans, likely because adhesin factors are species-specific [81, 82]. *Shigella* has been isolated in non-human primates, but humans are the only significant reservoir [79], and animal *T. trichiura* species do not infect humans [79]. Additionally, while the hookworm species included in our TAC (*N. americanus* and *A. duodenale*), are not transmitted zoonotically, *A. ceylanicum* is transmitted to humans by dogs and cats [79, 83], and is commonly found in Southeast Asia, including Lao PDR [84, 85]. Therefore, this specific

trend may be influenced by an underlying, but unmeasured association with *A. ceylanicum*, though additional research is warranted.

Although domestic animals are associated with increased pathogen diversity in the public domain [19], animal feces is often not taken into consideration in the design of household or community WASH interventions, which may partially explain the lack of effect observed in recent randomized trials [28, 29]. In their review on animal feces and health outcomes, Penakalapati et. al identified only seven intervention studies that specifically targeted the primary barrier of exposure to animal feces [29]. Thus, even if the Sustainable Development Goals for universal access to safe water, coverage of safely managed sanitation, and handwashing with soap are obtained, then both direct and indirect exposure to human feces will be eliminated, but exposure to animal feces will remain [29], and risk for enteric disease transmission will persist. More research on WASH-related exposure pathways for animal feces and on interventions to interrupt these exposure pathways is needed.

Strengths and limitations

This study has several strengths. First, measuring enteropathogen prevalence among adults, children, and infants residing in the same household allowed us to quantify associations between village- and household-level clustering and enteropathogen infection. Second, diarrhea is considered a disease of importance only for young children, despite evidence that morbidity is also high among older children, adolescents, and adults [51]. Our study is one of the few enteropathogen surveys to include older children and adults, and we observed high levels of enteropathogen infection across age groups. Third, participating villages and households were randomly selected. Fourth, we detected and quantified enteropathogens using qPCR, which

provides a higher sensitivity (98%) and specificity (100%) than conventional methods. Further, the multi-target detection capacity allowed us to examine 25 infectious pathogens [55], including a number of pathogens for which prevalence data in Lao PDR and the Southeast Asian region is scarce. For example, Strongyloidiasis is considered one of most neglected STHs among the NTDs [86], and there is limited evidence on the prevalence of *S. stercoralis* in Lao PDR [87]. We observed an overall *S. stercoralis* prevalence of 20.8%, the second highest among the STH. Additionally, we observed a substantial prevalence of Aeromonas (28.7%), which is common in soil but has also been linked to a number of intestinal and extraintestinal infections [88, 89], and has been implicated in outbreaks of diarrhea [89]. However, Aeromonas is often overlooked as an etiological agent of diarrhea [89]. Given that Aeromonas was the fourth most common bacteria isolated from our study population, future research on the etiology of diarrhea of should consider Aeromonas in their spectrum of causative pathogens.

Our study is subject to limitations. First, we do not have reliable diarrhea data. Detection of enteric pathogens in stool via molecular assays such as TAC can indicate asymptomatic or symptomatic infection, shedding due to recent exposure, or pathogen carriage due to gut colonization. Nonetheless, the detection of pathogens in stool indicates a person's exposure to the pathogen, regardless of symptoms, and even subclinical infections may lead to detrimental long term sequalae such as environmental enteropathy, malnutrition, and growth stunting [90-92]. Additionally, fecal waste from individuals with asymptomatic infections still represents an exposure risk to others [49]. Second, we identified random laboratory contamination in 144 samples. If contamination was suspected, the observation was dropped from the relevant taxa- or pathogen-specific model. We ran a sensitivity analysis between models where all contaminated observations were dropped, regardless of taxa or pathogen, and models where only relevant

contaminated taxa/pathogen were dropped, and identified no significant differences between the models. Third, we were unable to measure direct exposure to animal feces so we relied on animal ownership as a proxy, as has been done in the majority of previous studies on animal feces exposure [29, 77]. Additionally, our measures of improved/unimproved WASH access, as defined by the WHO/UNICEF Joint Monitoring Programme [57], may not be valid proxies of the conditions that result in enteric pathogens in the environment, and they may not sufficiently account for other possible exposure routes such as flies, food contamination due to factors such as animal slaughtering practices, or stored drinking water.

Conclusions

We observed that household- and village-level WASH access was generally associated with lower odds of enteric infection, but few WASH covariates were statistically associated with enteric infection at either the taxa- or individual pathogen-level. Transmission pathways varied by enteropathogen taxa, underscoring the challenges of addressing both acute and chronic infections using many of the existing WASH intervention approaches. Our results suggest that WASH access, as expected, is associated with lower enteric illness, but WASH access as currently defined does not reveal a measurably protective association with infection for many etiologies. Given previous research establishing that comprehensive WASH programs do not provide additive benefits over single interventions for health outcomes [2, 26, 27, 31] or environmental fecal contamination [93], our results suggest that focusing resources to achieve high uptake of a single, community-wide intervention targeted at specific pathogens may be more effective than a comprehensive intervention to a smaller population at a household level.

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Figures

Figure 1.1. Prevalence of enteropathogens within the household triad and infection concordance.

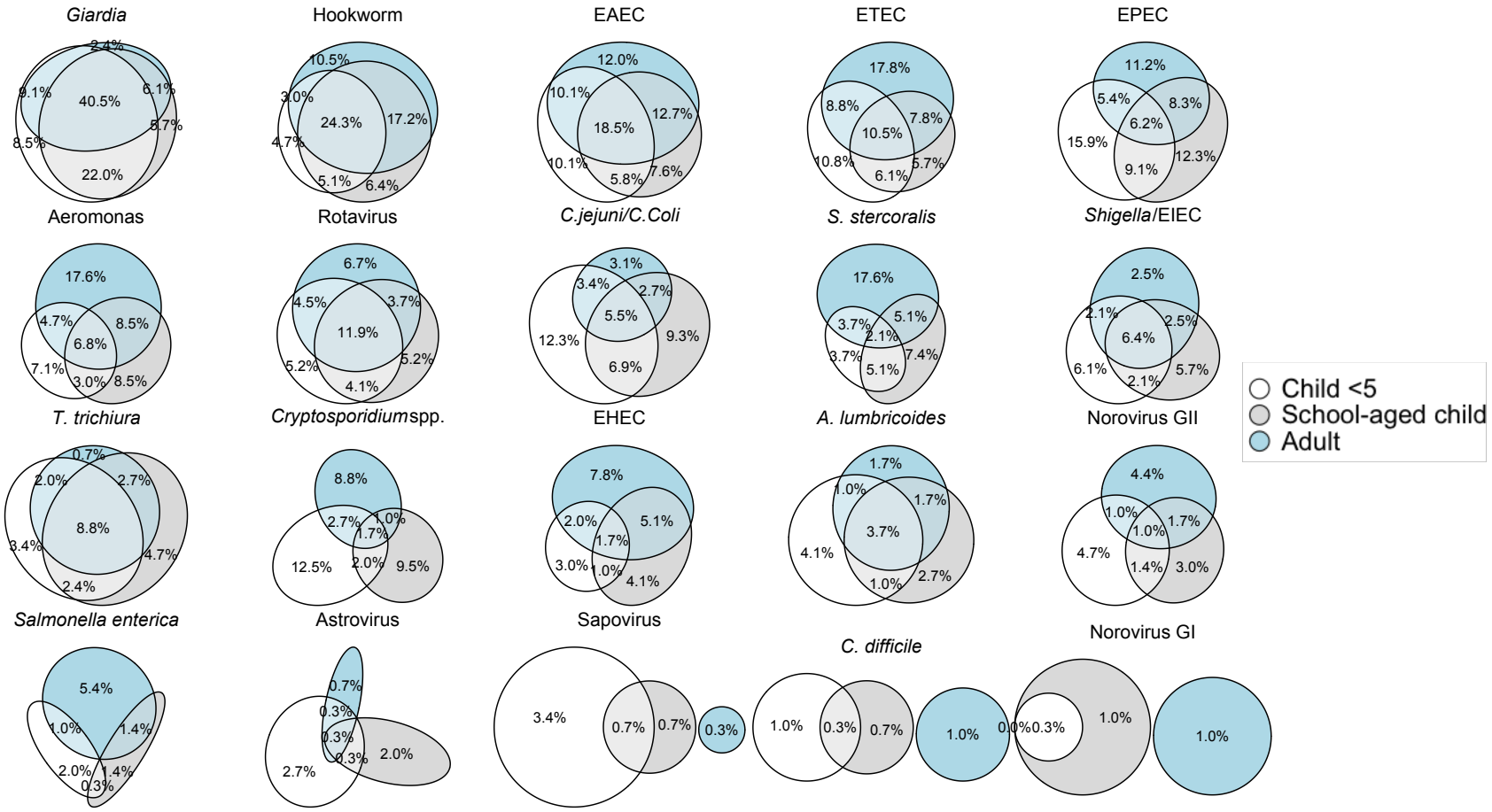
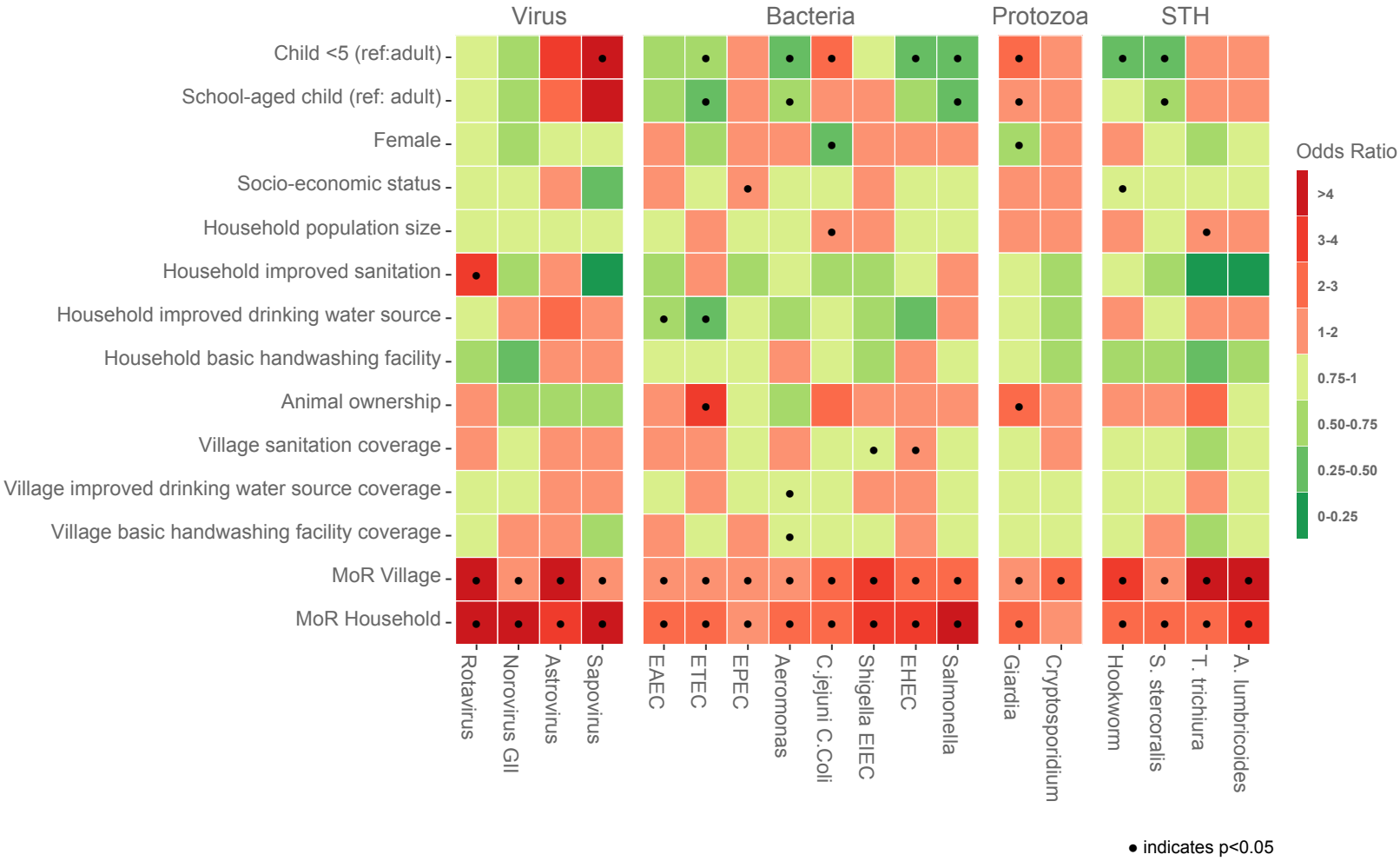


Figure 1.2. Associations between enteropathogen infection and age, water, sanitation, and hygiene access, and village- and household-level cluster



Chapter 2. Associations between soil-transmitted helminthiasis and viral, bacterial, and protozoal enteroinfections: A cross-sectional study in rural Laos[2]

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[2] This chapter is a manuscript under review at *Parasites and Vectors*. The structure is consistent with journal requirements.

Abstract

Background: Humans are susceptible to over 1,400 pathogens. Co-infection by multiple pathogens is common, and can result in a range of neutral, facilitative, or antagonistic interactions within the host. Soil-transmitted helminths (STH) are powerful immunomodulators, but evidence of the effect of STH infection on the direction and magnitude of concurrent enteric microparasite infections is mixed.

Methods: We collected fecal samples from 891 randomly selected children and adults in rural Laos. Samples were analyzed for 5 STH species, 6 viruses, 9 bacteria, and 5 protozoa using a quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay. We utilized logistic regression, controlling for demographics and household water, sanitation, and hygiene access, to examine associations between STH infection and concurrent viral, bacterial, and protozoal infection.

Results: We found that STH infection was associated with lower odds of concurrent viral infection (odds ratio [OR]: 0.48, 95% confidence interval [CI]: 0.28, 0.83), but higher odds of concurrent bacterial infections (OR: 1.81, 95% CI: 1.06, 3.07) and concurrent protozoal infections (OR: 1.50, 95% CI: 0.95, 2.37). Trends were consistent across STH species.

Conclusions: Results suggest that associations between STH and concurrent microparasite co-infection may differ by microparasite taxa. The associations between STH and concurrent microparasite infection may reflect a reverse association due to the cross-sectional study design. Additional research is needed to elucidate the exact mechanism of the immunomodulatory effects of STH on concurrent enteric microparasite infection.

Keywords: soil-transmitted helminths (STH); microparasite; enteric disease; co-infection; water, sanitation, hygiene (WASH), qPCR

Introduction

Humans are susceptible to over 1,400 known parasite species, including viruses, bacteria, protozoa, helminths, and fungi [1]. Co-infection by multiple pathogens is common, and is often considered the rule rather than the exception among populations living in socially and economically marginalized communities, rural areas, and tropical or subtropical climate zones [2]. Co-infections result in a range of neutral, facilitative, or antagonistic interactions [3, 4]. These interactions have important implications for host susceptibility to infection, disease severity [3, 4], and treatment efficacy [5-7].

Soil-transmitted helminth (STH) infections are one of the most ubiquitous human infections, affecting over one billion people worldwide [8, 9]. It is estimated that STH co-infections occur in over 800 million people [10]. However, interactions between STH and microparasites (defined here as a virus, bacteria, or protozoa) within the human host and the impacts of these interactions on human health are poorly understood [11].

Helminths are powerful immunomodulators [12, 13] and can affect microparasite infections via at least two distinct immune mechanisms. First, helminths usually induce a type 2 (Th2) immune response, including elevations in cytokines such as interleukin 4 (IL-4), IL-5, and IL-13, as well as development of type 2 helper T cells [11, 14, 15]. Microparasites generally induce a type 1 (Th1) immune response, which elevates cytokines IL-12, IL-17, IL-23, interferon-γ (IFN-γ) and

tumor necrosis factor (TNF)-α [11, 14]. The Th2 cytokines downregulate the Th1 cytokines that enable hosts to fight microparasite infection, resulting in a dampened immune response [14]. Second, to protect themselves from host immunity, helminths, like microparasites, suppress both Th1 and Th2 responses by enhancing regulatory T cell (T_{reg}) activity, which causes the release of regulatory cytokines such as IL-10 and transforming growth factor (TGF)-β, and leads to reduced immune responses against microparasite infection [15]. Helminths may also interact with microparasites via shared resources [13, 16, 17] by, for example, reducing the surface area availability for microparasite attachment or by monopolizing a cell type necessary for microparasite replication [18]. Such disparate responses may lead to within-host interactions by altering host susceptibility to infection [11, 19], altering the virulence of co-infecting pathogens [11, 19], and affecting the host's ability to clear co-infecting pathogens [19, 20].

Understanding the impact of pathogen co-infection on human health is difficult due to the diversity of co-infecting species and their numerous possible interactions [16]. Even though many humans typically harbor multiple pathogens [15], most studies of co-infection measure interactions between pairs of parasites [16]. In this study, we examine co-infection between five STH species and 20 microparasites, including six viruses, nine bacteria, and five protozoa in human hosts. To identify trends in pathogen interaction, we evaluate interspecific associations between STH and enteric microparasite infection at the at the taxa level (e.g. viruses, bacteria, and protozoa).

Materials and Methods

Study Setting and Design

This cross-sectional study was nested within the Water, Sanitation, and Hygiene for Health and Education in Laotian Primary Schools (WASH HELPS) study, a longitudinal cluster-randomized trial evaluating a comprehensive school-based water, sanitation, and hygiene (WASH) intervention in 100 schools in Saravane Province, Lao People's Democratic Republic (Lao PDR; Laos). Detailed methods of the parent study are described elsewhere [21]. The WASH HELPS study is registered at clinicaltrials.gov (NCT02342860).

Of the 100 schools participating in the WASH HELPS study, 50 (25 intervention and 25 comparison) were selected using stratified random sampling based on district size and WASH HELPS study intervention status. In each school-hosting village (there is only one school per village), we randomly selected 25 households. Households were eligible for inclusion if they had a child attending the primary school participating in the WASH HELPS study, and that pupil had a sibling <5 years old living in the household. At each household, the female head of household was surveyed on household demographics, asset and animal ownership, recent illness among household members, and WASH access and behaviors. Structured observations of WASH facilities were made when available.

In conjunction with the household survey, we collected stool samples from the pupil, the pupil's parent/caregiver (preference was given to female parent/caregiver), and the pupil's sibling <5 years old (if there were multiple siblings, preference was given to youngest sibling). To collect the stool samples, the female parent/caregiver was given three pre-labeled, resealable plastic bags each containing a plastic spoon. Caregivers were given diapers to collect stool from infants, when applicable. Written and pictorial instructions for stool collection were printed on the plastic bag, and participants were also provided verbal instructions. Participants were instructed to collect their first morning stool, and were informed that the field team would return to the household the following morning to collect all samples. If households did not return all three stool samples on the first day, participants were reminded of the stool collection procedures, provided new bags and spoons if needed, and a second return visit was made the following day. Stool samples were transported with a cold chain to the field laboratory within two hours of collection.

Upon collection, all samples were tested for STH using the Kato Katz method [22]. For this sub-study, stool samples from a subset of 297 households were randomly selected for additional enteropathogen analysis via quantitative reverse transcription polymerase chain reaction (qRT-PCR). Households were eligible for inclusion in this sub-study only if all three subjects in the household (adult, school-aged child, and child <5 years old) returned their stool sample on the same day. Households were randomly selected, proportional to district size, village size, and

WASH HELPS intervention status, from households participating in the household survey and STH testing by Kato Katz.

All data were collected between February-April 2017 (dry season), prior to annual school-based preventative chemotherapy (PC) for STH. The time frame corresponded with the final round of data collection and conclusion of the WASH HELPS study [21].

Laboratory Analysis

Following analysis for STH via Kato Katz, 200 mg of stool was aliquoted into a DNA/RNA Shield Collection and Lysis Tube (Zymo Research, Irvine, CA, USA) containing a lysis buffer and bead beating system, and beaten for 20 minutes using a Disrupter Genie vortexer (Scientific Industries, Bohemia, NY, USA) [23]. One field control was processed each day using DNA/RNA-free water to evaluate the possibility of false positives from contamination in the field laboratory during sampling. Samples were kept frozen at -20°C until transported to a laboratory at Emory University, where they were subsequently stored at -80°C until extraction.

Total nucleic acid was extracted from samples using the ZymoBIOMICS DNA/RNA Mini Kit (Zymo Research, Irvine, CA, USA), according to manufacturer instructions. Samples were spiked with bacterophage MS2 (ZeptoMetrix, Buffalo, NY, USA), an external control, to monitor extraction and amplification efficiency [23]. One extraction blank was included per batch to

exclude the possibility of false positives from contamination during extraction. Extractions were stored at -80°C until transported on dry ice to the University of Iowa for qRT-PCR analysis.

We created a custom TaqMan Array Card (TAC) (Thermo Fisher, Carlsblad, CA, USA) with compartmentalized, probe-based qPCR assays for 25 enteropathogens, including: five STH (*Ancylostoma duodenale, Ascaris lumbricoides, Necator americanus, Strongyloides stercoralis*, and *Trichuris trichiura);* six viruses (astrovirus, adenovirus, norovirus GI, norovirus GII, rotavirus, sapovirus); nine bacteria (*Aeromonas spp.*, *Campylobacter jejuni*, *Clostridium difficile*, enteroaggregative *Escherichia coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), atypical or typical enteropathogenic *E. coli* (EPEC), heat-labile- (LT) or heat-stable (ST) enterotoxigenic *E. coli* (ETEC), *Salmonella enterica,* and *Shigella* spp./Enteroinvasive *E. coli* (EIEC)*;* and five protozoa (*Cryptosporidium* spp., *Cryptosporidium hominus*, *Cryptosporidium parvum*, *Entamoeba histolytica,* and *Giardia intestinalis*) [24, 25]. The TAC included probes for the MS2 external control, as well as an 18S rRNA internal control. The TAC primer and probe sequences are listed in **Table S1** (see Additional file 1).

TAC preparation was prepared based on the protocol described by Liu et al. [24]. Ag-Path-ID One-Step RT-PCR kit (Thermo Fisher, Waltham, MA) was used as the master mix reagent for the TAC analysis. Bovine serum albumin (BSA) was also applied into the TAC master-mix to prevent the possibility of PCR inhibition that may arise in nucleic acids extracted from stools [26, 27]. For

each sample, 40 µL of DNA/RNA extract of equal volumes of DNA and RNA was mixed with 50 µL of 2X RT-buffer, 4 µL of 25X AgPath enzyme, 5.4 µL of nucleic acid-free water, and 0.6 µL of 50 mg/mL BSA to a total volume of 100 µL. All TAC runs were completed in a ViiA7 instrument with QuantStudio 7 software (Thermo Fisher, Waltham, MA), and the cycling conditions were as follows: holding stages of 45°C for 20 minutes and 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute.

TAC data were manually read by two independent researchers. True amplification was validated by inspecting the multicomponent plot for increases in fluoresce for the FAM-based gene-specific probe. Conflicting results were resolved by a third independent researcher. Samples were considered positive only when the corresponding field and extraction blanks were negative, otherwise the data were considered invalid [25].

Measures

Adult participants reported the age and sex of themselves, their primary school-aged child, and their child under five years old. The following variables were reported by the female head of household: household ethnicity, in which households of non-Lao-Tai ethnicity were considered ethnic minorities; the number of household members, which was derived by listing and counting all people currently living in the household full time; animal ownership, which was defined as owning any cows, goats, sheep, poultry (chickens or ducks), or pigs; and the main source of

household drinking water, which was further classified as improved/unimproved according to the World Health Organization/United Nations International Children's Fund (WHO/UNICEF) Joint Monitoring Programme (JMP) standards [28].

The following variables were reported by enumerators using structured observation: the presence of a household toilet, which was further classified as improved/unimproved according to WHO/UNICEF JMP standards [28]; and the presence of a household basic handwashing facility, classified according to WHO/JMP standards as having soap and water [28]. Socioeconomic status was determined through a series of questions and observations about household construction materials (roof, floor, and walls), ownership of a mobile phone, and presence of electricity. These variables were chosen based on those used in the Demographic and Health Surveys for measures of wealth in Laos [29]. We used principal component analysis to derive one single wealth metric from all of the wealth assets combined [30].

E. coli pathotypes were classified according to the following gene targets: EAEC (*aatA* and/or *aaiC*), EHEC (*eae* with *stx1* and/or *stx2*, and without *bfpA*), typical EPEC (*bfpA* with or without *eae*), atypical EPEC (*eae* without *bfpA*, *stx1*, or *stx2*), ETEC (*eltB* for heat-labile toxin [LT] and *estA* with or without *eltB* for heat-stable toxin [ST]) [31]. The number of microparasite infections was derived by summing all positive pathogens (range: zero to 20). We chose to use the *ipaH* gene target to be consistent with approaches used in other recent enteric disease studies of under-five

children. However, *ipaH* occurs in *Shigella* spp. and EIEC, and does not validate the presence of the large virulence plasmid of other virulence genes that are unique to *Shigella* spp.

Statistical Analysis

All data were analyzed using Stata Statistical Software: Release 15 (StataCorpLP, College Station, TX, USA).

We estimated the odds of concurrent microparasite infection using three separate logistic regression models for viral, bacterial, and protozoal infection outcomes. For the primary analysis, the main exposure of interest was any STH infection, as determined by qRT-PCR detection. Secondary analyses examined specific STH species (i.e., hookworm, *A. lumbricoides*, *T. trichiura*, or *S. stercoralis*) as main predictors. We controlled for the presence of the non-outcome microparasite taxa (e.g., the model of the association between STH and viral infection also controlled for concurrent bacterial and protozoal infection), as well as the following covariates determined *a priori* based on biological plausibility of affecting odds of both outcomes and STH infection: age group (i.e., adult, school-aged child, child <5 years old), sex, socioeconomic status, ethnic minority status, household population size, improved household toilet, improved household drinking water source, basic household handwashing facility, household animal ownership, and whether the school in the village was a beneficiary of a UNICEF WASH in Schools intervention. Random intercepts were included at the village and household levels to account for clustering.

The associations between STH infection or STH species and the number of concurrent microparasite infections were determined using separate Poisson regression models and are reported as beta coefficients representing the change in number of microparasite infections among subjects with STH (or specific STH species) infection compared to those without. Models included random intercepts at the village and household levels, and included the same covariates as the logistic regression models.

All models were assessed for effect modification by age group. All analyses were evaluated for statistical significance at $p<0.05$.

Ethics

This study was approved by Emory University's Institutional Review Board (IRB0076404) and the Lao Ministry of Health's National Institute of Public Health National Ethics Committee (No. 043 NIOPH/NECHR). Adult participants provided informed verbal consent for the household survey and stool collection for themselves and their children prior to any data collection.

Results

We collected a total of 2,269 fecal samples from the same number of participants. Of these, 891 participants from 297 households were eligible for inclusion in this study because all three participants in the selected household (adult, school-aged child, and child <5 years old) returned their stool sample on the same day. Data from 746 participants were included in the analysis (n=1

excluded due to insufficient sample amount for nucleic acid extraction, *n*=144 excluded due to suspected field (*n*=66) or laboratory (*n*=78) contamination of one or more target pathogens). The study population is described in **Table 2.1**.

At least one STH was present in 61.3% of participants (**Table 2.2**); hookworm was the most prevalent STH infection (43.6%). Of the microparasites, bacterial infections were the most common (86.8%), followed by protozoal infections (72.8%), then viral infections (33.2%). Prevalence of individual microparasites are described in **Table 2.2**. EAEC was the most common bacterial infection (47.3%), *Giardia* was the most common protozoal infection (68.9%), and rotavirus was the most common viral infection (24.1%). Kato Katz results are presented in **Figure 2.S1 (**see Additional file 1).

Associations between STH infection and viral, bacterial, and protozoal infection are described in **Table 2.3**. Age was not a significant effect modifier for any primary or secondary outcomes so we present unstratified results. STH infections were associated with lower odds of concurrent viral infection; this trend was consistent across all STH species and was statistically significant for any STH infection (odds ratio [OR]= 0.48, 95% confidence interval [CI]= 0.28, 0.83) and *S. stercoralis* (OR=0.52, 95% CI=0.29, 0.95). STH infections were associated with higher odds of concurrent bacterial infection. This trend was statistically significant for any STH infection (OR=1.81, 95% CI=1.06, 3.07) and *T. trichiura* (OR=5.97, 95% CI=2.05, 17.40). STH infections were associated

with higher odds of concurrent protozoal infection; this trend was consistent across all STH species and was statistically significant for hookworm (OR=1.78, 95% CI=1.11, 2.84).

STH infections were associated with a higher number of total concurrent microparasite infections (**Table 2.3**). This trend was consistent across all STH species, and was statistically significant for any STH infection (change in number of microparasite infections among subjects with STH infection compared to those without [β]=0.11, 95% CI=0.01, 0.21) and *T. trichiura* (β=0.18, 95% CI=0.03, 0.33).

Discussion

Within-host interactions between helminths and microparasites can affect a range of factors, including whether a pathogen can establish itself in a host, rate of growth and replication within a host, rate of clearance from the host, and severity of disease [19]. Evidence supporting whether such co-infections result in beneficial, harmful, or neutral interactions is mixed [3, 4, 18], and the mechanisms by which helminths and microparasites interact are not clearly established [11, 18]. Most studies of co-infection have examined interactions between two species [16], often utilizing *in vitro* or animal models and/or employing helminths and microparasites that are not commonly found in humans [12, 32-37]. Our approach addresses the limitations of these previous studies by taking a macro approach to co-infection in humans. Rather than examining pairwise associations between pathogens, we enhance our generalizability by examining the associations between STH

and microparasite taxa. Additionally, we control for the presence of other pathogen taxa beyond those of immediate interest, which is more realistic for low-income settings where humans harbor multiple infections that may have antagonistic or synergistic interactive effects [2-4]. Our analysis revealed a clear trend in which STH infection was associated with reduced odds of concurrent viral infection and increased odds of concurrent bacterial infection. STH infection was also associated with increased odds of protozoal infection, although this association was statistically significant only for the most prevalent STH, hookworm.

Our results are consistent with previous research reporting that helminths impair host immunity to concurrent enteric bacterial infection [7, 37, 38]. Helminth infection causes intestinal barrier dysfunction and increased "leakiness" of the intestinal epithelium [37, 39], which is one mechanism by which STH infection may increase odds of concurrent bacterial infection. For many enterobacteria to infect a host, the pathogen must exit the intestinal lumen and cross the epithelial barrier to invade cells in the small and large intestine [37, 40]. Intestinal epithelial cells are critical for gut homeostasis because they form physical and chemical barriers that protect the intestinal epithelia from invading pathogens [40]. For example, the Ly6/Plaur domain-containing 8 (Lypd8) protein, which is physical barrier found in the uppermost epithelial layer of the large intestine, inhibits invasion of bacteria in *Escherichia*, *Proteus*, and *Helicobacter* genera in the colonic epithelia [41]. Antimicrobial peptides (AMPs) are chemical barriers found in the small intestine that include defensin proteins, which cause cell disruption and protect against pathogenic bacterial

invasions such as *S. typhimurium* [40, 42]. Therefore, the enhanced permeability of the intestinal barrier due to helminth infection may facilitate the penetration of bacterial endotoxins [39, 43]. Further, hosts rely on their innate immune system to respond to such attacks through activation of Toll-like receptors, secretion of chemoattractant molecules and cytokines, and recruitment of cells such as neutrophils, monocytes, dendritic cells, and lymphocytes [37]. However, helminths can modulate this innate immune response to bacterial enteropathogens by stimulating regulatory cytokines (such as IL-10), antagonizing proinflammatory factors that can lead to more severe intestinal inflammation (such as keratinocyte-derived chemokine and macrophage inflammatory protein 2), impeding clearance of pathogens, and reducing availability of pathogen-specific cytokines [11, 37, 43].

We also found that STH infection, specifically hookworm, was associated with increased odds of concurrent protozoal infection. Our results are consistent with previous research in Venezuela, which found that *Giardia* prevalence was significantly higher among children harboring an *A. lumbricoides* infection compared to those without [44]. We found that protozoal infections were driven largely by *Giardia*, as 94.7% of subjects with a protozoal infection had *Giardia*. One possible mechanism by which helminths may increase susceptibility to protozoa is through the proinflammatory cytokine IFN-γ [45, 46], which is antagonized by the cytokine IL-4 triggered by helminth infection [15]. Evidence suggests that IFN-γ is significantly higher among humans infected with *Giardia* and *E. histolytica*, suggesting this cytokine has a protective role in host

defense [46-48]. However, helminths suppress IFN-γ, which may impede the host from mounting an effective immune response [18]. Additionally, intestinal barrier dysfunction and increased permeability of the intestinal lumen caused by helminth infection may be exacerbated by protozoal infection, thus facilitating the translocation of antigens and inducing a pro-inflammatory response within the intestine [46]. It is also possible that increased odds of STH infection given protozoal infection is reflecting the inverse association; in other words, that protozoal infection increases the odds of STH infection. *Giardia* is one of the earliest infections that children succumb to [31, 49], and can result in chronic infection [49, 50]. Like helminths, *Giardia* immunomodulates the host immune system and causes gut dysfunction [51, 52]. Thus, it is possible that chronic *Giardia* infection early in life may have preceded and enhanced susceptibility to STH infection.

Helminths are generally thought to increase transmission, virulence, and progression of microparasite infection, and reduce recovery [4, 15, 17], as supported by our results for bacterial and protozoal infections. However, some exceptions have been established in the literature [35, 53-55], and helminths are being explored as a possible curative tool for immune-mediated conditions such as allergies, asthma, and ulcerative colitis [56-58]. We found that helminth infection was negatively associated with odds of concurrent viral infection, contradicting existing research indicating that helminths may limit both innate and adaptive immune responses to viral infection [36, 59]. However, it is possible that helminths are protective against viral infection because the Th2 immune response induced by helminth infection has anti-inflammatory and

wound-healing properties [11, 15]. In the current study, viral infections were driven largely by rotavirus (60.5% of subjects with a viral infection), followed by norovirus GII (22.2%). Rotavirus infection induces oxidative stress and inflammatory signaling; this pro-inflammatory signaling is necessary for virus replication, but is inhibited by anti-inflammatory treatment [60]. Norovirus infection also causes alterations of the gut mucosa, including mucosal inflammation [61]. When a microparasite such as rotavirus or norovirus induces inflammation-mediated damage, helminths may protect the host from damage by secreting IL-10 and TGF-β and decreasing the production of pro-inflammatory cytokines [62], which may be protective against the detrimental inflammatory Th1 response induced by viral microorganisms [11, 19, 35, 62].

Strengths and limitations

Strengths of this study include the random selection of participating villages and households. Also, pathogens in stool samples were detected and quantified using qPCR, which provides a higher sensitivity (98%) and specificity (100%) than conventional methods [24]. Further, the multi-target detection capacity of this method allowed us to examine 25 infectious pathogens [24], whereas most existing studies on pathogen co-infection have focused on pairs of agents [16]. Additionally, there is a dearth of clinical data on helminth co-infection, and most studies have relied on mouse models [35]. The predominant species involved in human STH and enteric microparasite infection is influenced by a range of factors, including age and WASH access [63, 64]. We examined human subjects from three distinct age groups- adults, school-aged children, and children under five years

old- and controlled for potential confounding WASH and demographic variables to provide a more externally valid picture of STH and microparasite co-infection.

Our study is subject to a number of limitations. First, our data are cross-sectional so we do not know whether the STH or microparasite infection occurred first. Second, the high sensitivity of the TAC and other molecular assays may lead to the detection of prolonged shedding by attenuated pathogens and we cannot distinguish between symptomatic and asymptomatic infections [65]. However, even asymptomatic infections may lead to interactions within the host as well as other sequalae such as environmental enteropathy, malnutrition, and growth stunting [66-68]. Third, evidence suggests that the outcomes of helminth-microparasite co-infection are context dependent and may depend on helminth infection intensity [19, 44]. Based on the Kato Katz results from these samples, helminth infections were predominately of low infection intensity, so we were unable to stratify by infection intensity to evaluate differences in co-infection by infection intensity. Fourth, we discarded 144 samples due to suspected contamination, which may have limited statistical power. Household toilet ownership and use of an improved water source were lower among participants whose samples were discarded. While these factors may be associated with the pathogen profile of the participants, contamination was a random event unassociated with the participants and would not confound the relationship between STH infection and odds of microparasite infection. Last, we did not measure cytokines, interferon, or other measures of

immune response so we are unable to elucidate exact mechanisms of helminth-microparasite interaction.

Conclusions

Associations between STH infection and concurrent microparasite infection differed by microparasite taxa. We found that helminth infection was negatively associated with concurrent viral infection, but positively associated with concurrent bacterial and protozoal infections, after controlling for shared risk factors for infection. These results suggest that interventions to control STH, such as increasing community sanitation coverage to eliminate the environmental reservoir for STH, combined with PC with anti-helminthic drugs [69, 70], could have a spillover impact on bacterial and protozoal infections. Increased integration and collaboration between WASH and STH sectors is warranted [70]. Additional research is needed to elucidate the exact mechanism of immunomodulatory effects of STH on concurrent enteric microparasite infection.

List of abbreviations

AMP: antimicrobial peptide; BSA: bovine serum albumin; CI: confidence interval; EAEC: enteroaggregative *E. coli*; EHEC: enterohemorrhagic *E. coli*; EIEC: enteroinvasive *E. coli*; EPEC: enteropathogenic *E. coli*; ETEC: enterotoxigenic *E. coli*; IFN: interferon; IL: interleukin; JMP: Joint Monitoring Programme; Lao PDR; Laos: Lao People's Democratic Republic; Lypd8: Ly6/Plaur domain-containing 8 (protein); OR: odds ratio; PC: preventative chemotherapy; qRT-

PCR: quantitative reverse transcription polymerase chain reaction; STH: soil-transmitted helminth; TAC: TaqMan Array Card; TGF: transforming growth factor; Th1: Type 1 (immune response); Th2: Type 2 (immune response); TNF: tumor necrosis factor; T_{reg}: regulatory T cell; WASH: water, sanitation, and hygiene; WASH HELPS: Water, Sanitation, and Hygiene for Health and Education in Laotian Primary Schools; WHO/UNICEF: World Health Organization/United Nations International Children's Fund

Declarations

Ethics approval and consent to participate: This study was approved by Emory University's Institutional Review Board (IRB0076404) and the Lao Ministry of Health's National Institute of Public Health National Ethics Committee (No. 043 NIOPH/NECHR). Adult participants provided informed verbal consent for the household survey and stool collection for themselves and their children prior to any data collection.

Consent for publication: Not applicable.

Availability of data and material: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Tables

Table 2.1. Description of study population, Saravane Province, Laos, 2017

	Total (*n*=746)	**Child <5 years (*n*=249)**	**School-aged child (*n*=247)**	**Adult (*n*=250)**
Female	496 (66.5%)	121 (48.6%)	125 (50.6%)	250 (100.0%)
Median (IQR[1]) age	9 (24.5)	4 (2.0)	9 (3.0)	32 (11)
Ethnic minority[2]	351 (47.1%)	119 (47.8%)	114 (46.2%)	118 (47.2%)
Household has improved toilet[3]	230 (30.1%)	75 (30.1%)	77 (31.2%)	78 (31.2%)
Household utilizes improved drinking water source[3]	355 (47.6%)	118 (47.4%)	117 (47.4%)	120 (48.0%)
Household has basic handwashing facility[3]	262 (35.1%)	87 (34.9%)	89 (36.0%)	86 (34.4%)
Median (IQR) number of people living in household	6.5 (4)	6 (4)	6 (4)	7 (4)
Household owns animals	713 (95.6%)	238 (95.6%)	236 (95.6%)	239 (95.6%)
Beneficiary of school WASH[2] intervention	374 (50.1%)	126 (50.6%)	122 (49.4%)	126 (50.4%)

[1] Defined as those not belonging to the Lao-Tai ethnic group

[2] Interquartile range (IQR); water, sanitation, and hygiene (WASH)

[3] Classified according to WHO/UNICEF Joint Monitoring Programme standards

Table 2.2. Prevalence of soil-transmitted helminth (STH), viral, bacterial, and protozoal infections, Saravane Province, Laos, 2017

	Total (*n*=746)	**Child <5 years** (*n*=249)	**School-aged child** (*n*=247)	**Adult** (*n*=250)
Any STH[1]	457 (61.3%)	133 (53.4%)	154 (62.4%)	170 (68.0%)
Hookworm	325 (43.6%)	81 (32.5%)	118 (47.8%)	126 (50.4%)
A. lumbricoides	61 (8.2%)	23 (9.2%)	19 (7.7%)	19 (7.6%)
T. trichiura	119 (16.0%)	40 (16.1%)	46 (18.6%)	33 (13.2%)
S. stercoralis	154 (20.6%)	38 (15.3%)	45 (18.2%)	71 (28.4%)
Any virus[2]	248 (33.2%)	90 (36.1%)	78 (31.6%)	80 (32.0%)
Astrovirus	18 (2.4%)	8 (3.2%)	6 (2.4%)	4 (2%)
Adenovirus	6 (0.8%)	0 (0.0%)	4 (1.6%)	2 (0.8%)
Norovirus GI	8 (1.2%)	1 (0.4%)	4 (1.6%)	3 (1.2%)
Norovirus GII	55 (7.4%)	21 (8.4%)	15 (6.1%)	19 (7.6%)
Rotavirus	180 (24.1%)	61 (24.5%)	58 (23.5%)	61 (24.4%)
Sapovirus	11 (1.5%)	8 (3.2%)	3 (1.2%)	0 (0.0%)
Any bacteria[3]	640 (86.8%)	216 (86.8%)	206 (83.4%)	218 (87.2%)
Aeromonas spp.	224 (30.0%)	54 (21.7%)	70 (28.3%)	100 (40.0%)
Clostridium difficile	8 (1.1%)	3 (1.2%)	3 (1.2%)	2 (0.8%)
Campylobacter jejuni	163 (21.9%)	69 (27.7%)	55 (22.3%)	39 (15.6%)
EAEC	353 (47.3%)	113 (45.4%)	109 (44.1%)	131 (52.4%)
EHEC	91 (12.2%)	21 (8.4%)	29 (11.7%)	41 (16.4%)
EPEC	262 (35.1%)	94 (37.8%0	91 (36.8%)	77 (30.8%)
Typical	60 (8.1%)	15 (6.0%)	20 (8.1%)	25 (10.0%)
Atypical	202 (27.1%)	79 (31.7%)	71 (28.7%)	52 (20.8%)
ETEC	278 (37.3%)	95 (38.2%)	70 (28.3%)	113 (45.2%)
LT-ETEC	78 (10.5%)	35 (14.1%)	13 (5.3%)	30 (12.0%)
ST-ETEC	200 (26.8%)	60 (24.1%)	57 (23.1%)	83 (33.2%)
Shigella spp./EIEC	117 (15.7%)	37 (14.9%)	36 (14.6%)	44 (17.6%)
Salmonella enterica	37 (5.0%)	8 (3.2%)	9 (3.6%)	20 (8.0%)
Any protozoa[4]	543 (72.8%)	203 (81.5%)	188 (76.1%)	152 (60.8%)
Cryptosporidium spp.	105 (14.1%)	41 (16.5%)	34 (13.8%)	30 (12.0%)
Cryptosporidium hominus	1 (0.1%)	0 (0.0%)	1 (0.4%)	0 (0.0%)
Cryptosporidium parvum	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Entamoeba histolytica	1 (0.1%)	1 (0.4%)	0 (0.0%)	0 (0.0%)
Giardia intestinalis	514 (68.9%)	195 (78.3%)	179 (72.5%)	140 (56.0%)
Mean (standard deviation) number of microparasites[5]	3.3 (1.7)	3.3 (1.5)	3.1 (1.7)	3.3 (1.8)

[1] Soil-transmitted helminth (STH) includes one or more of the following helminths: hookworm (*N. americanus* and/or *A. duodenale*), *A. lumbricoides*, *T. trichiura*, or *S. stercoralis*. [2] Virus includes one or more of the following pathogens: astrovirus, adenovirus, norovirus GI, norovirus GII, rotavirus, or sapovirus. [3] Bacteria includes one or more of the following pathogens: *Aeromonas*, *C. difficile*, *C. jejuni*, EAEC, EHEC, EPEC (typical or atypical), LT- or ST-ETEC, *Shigella* spp./Enteroinvasive *E. coli*, or *Salmonella.* [4] Protozoa includes one or more of the following pathogens: non-hominus and non-parvum *Cryptosporidium* spp., *C. hominus*, *C. parvum*, *E. histolytica*, and *G. intestinalis*
All data come from quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Table 2.3. Associations between STH infection and concurrent virus, bacteria, protozoa infection, and number of microparasite infections; Saravane Province, Laos, 2017 (n=746)

	Virus[1*]	**Bacteria[2*]**	**Protozoa[3*]**	**Number of microparasite infections[†]**
Any STH[4]	0.48 (0.28, 0.83)	1.81 (1.06, 3.07)	1.50 (0.95, 2.37)	0.11 (0.01, 0.21)
Hookworm[5]	0.70 (0.40, 1.21)	1.22 (0.70, 2.12)	1.78 (1.11, 2.84)	0.09 (0.00, 0.19)
A. lumbricoides	0.66 (0.23, 1.87)	1.02 (0.35, 2.96)	1.42 (0.59, 3.41)	0.01 (-0.16, 0.18)
T. trichiura	0.53 (0.22, 1.29)	5.97 (2.05, 17.4)	1.79 (0.84, 3.80)	0.18 (0.03, 0.33)
S. stercoralis	0.52 (0.29, 0.95)	1.32 (0.69, 2.53)	1.30 (0.78, 2.17)	0.08 (-0.02, 0.18)

[1] Virus includes one or more of the following pathogens: astrovirus, adenovirus, norovirus GI, norovirus GII, rotavirus, or sapovirus. [2] Bacteria includes one or more of the following pathogens: *Aeromonas*, *C. difficile*, *C. jejuni*, EAEC, EHEC, EPEC (typical or atypical), LT- or ST-ETEC, *Shigella* spp. enteroinvasive *E. coli*, or *Salmonella.* [3] Protozoa includes one or more of the following pathogens: non-hominus and non-parvum *Cryptosporidium* spp., *C. hominus*, *C. parvum*, *E. histolytica*, and *G. intestinalis.* [4] Any soil-transmitted helminth (STH) includes one or more of the following helminths: hookworm (*N. americanus* and/or *A. duodenale*), *A. lumbricoides*, *T. trichiura*, or *S. stercoralis.* [5] Hookworm includes *N. americanus* and/or *A. duodenale.*

[*] Results are adjusted odds ratios and 95% confidence intervals and are interpreted as the change in odds of virus/bacteria/protozoa infection among subjects with STH (or specific STH species) infection comparted to those without

[†] Results are beta coefficients and 95% confidence intervals and are interpreted as the change in number of microparasite infections among subjects with STH (or specific STH species) infection comparted to those without

All models control for population group, sex, socioeconomic status, ethnic minority status, household population size, presence of an improved toilet in household, use of an improved household drinking water source, presence of soap for handwashing at household, household animal ownership, and whether the village school was a beneficiary of the UNICEF WASH in Schools intervention, and include random intercepts at the village and household level

Supplementary Material

Table 2.S1. Primers and Probes for Custom TaqMan Array Card

Assay ID	Pathogen	Gene Target	Forward Primer	Reverse Primer	Probe Sequence	Ref.
APMFXDE	Adenovirus 40-41	Fiber Gene	AACTTTCTCTCTTAATAGACGCC	AGGGGGCTAGAAAACAAAA	CTGACACGGGCACTCT	[1]
APNKRXC	EAEC *aaiC*	EAEC *aaiC*	ATTGTCCTCAGGCATTTCAC	ACGACACCCCTGATAAACAA	TAGTGCATACTCATCATTTAAG	[2]
APPRKG9	EAEC *aatA*	EAEC *aatA*	CTGGCGAAAGACTGTATCAT	TTTTGCTTCATAAGCCGATAGA	TGGTTCTCATCTATTACAGACAGC	[2]
APRWE26	EPEC *eae*	EPEC *eae*	CATTGATCAGGATTTTTCTGGTGATA	CTCATGCGGAAATAGCCGTTA	ATACTGGCGAGACTATTTCAA	[2]
APTZ9M3	EPEC *bfpA*	EPEC *bfpA*	TGGTGCTTGCGCTTGCT	CGTTGCGCTCATTACTTCTG	CAGTCTGCGTCTGATTCCAA	[2]
APU627Z	ETEC LT	ETEC LT	TTCCCACCGGATCACCAA	CAACCTTGTGGTGCATGATGA	CTTGGAGAGAAGAACCCT	[2]
CCU002	ETEC STh STp	STh STp	GCTAAACCAGYAGRGTCTTCAAAA TGAATCACTTGACTCTTCAAAA	CCCGGTACARGCAGGATTACAACA TGAATCACTTGACTCTTCAAAA	TGGTCCTGAAAGCATGAA TGAACAACACATTTTACTGCT	[2]
CCU001L	STEC *stx1*	STEC *stx1*	ACTTCTCGACTGCAAAGACGTATG	ACAAATTATCCCCTGWGCCACTATC	CTCTGCAATAGGTACTCCA	[2]
APXGRDV	STEC *stx2*	STEC *stx2*	CCACATCGGTGTCTGTTATTAACC	GGTCAAAACGCGCCTGATAG	TTGCTGTGGATATACGAGG	[2]
APYMJXT	*C. jejuni C. Coli*	*cadF*	CTGCTAAACCATAGAAATAAAATTTCTCAC	CTTTGAAGGTAATTTAGATATGGATAATCG	CATTTTGACGATTTTTGGCTTGA	[2]
APZTEHP	*C. difficile*	*tcdB*	GGTATTACCTAATGCTCCAAATAG	TTTGTGCCATCATTTTCTAAGC	CCTGGTGTCCATCCTGTTTC	[2]
AP2W73M	*Salmonella enteritidis*	*ttr*	CTCACCAGGAGATTACAACATGG	AGCTCAGACCAAAAGTGACCATC	CACCGACGGCGAGACCGACTTT	[1]
AP322NJ	*Shigella* spp./EIEC	*ipaH*	CCTTTTCCGCGTTCCTTGA	CGGAATCCGGAGGTATTGC	CGCCTTTCCGATACCGTCTCTGCA	[2]
AP47V9G	*Cryptosporidium*	18s rRNA	GGGTTGTATTTATTAGATAAAGAACCA	AGGCCAATACCCTACCGTCT	TGACATATCATTCAAGTTTCTGAC	[2]
AP7DPUE	*C. hominus*	LIB13	TCCTTGAAATGAATATTTGTGACTCG	AAATGTGGTAGTTGCGGTTGAAA	CTTACTTCGTGGCGGCGT	[1]
AP9HJEC	*C. parvum*	LIB13	TCCTTGAAATGAATATTTGTGACTCG	TTAATGTGGTAGTTGCGGTTGAAC	TATCTCTTCGTAGCGGCGTA	[1]
APAAAR2	*E. histolytica*	18s rRNA	ATTGTCGTGGCATCCTAACTCA	GCGGACGGCTCATTATAACA	TCATTGAATGAATTGGCCATTT	[2]
APCE4CY	*Giardia*	18s rRNA	GACGGCTCAGGACAACGGTT	TTGCCAGCGGTGTCCG	CCCGCGGCGGTCCCTGCTAG	[2]
APDJXWW	*A. duodenale*	ITS2	GAATGACAGCAAACTCGTTGTTG	ATACTAGCCACTGCCGAAACGT	ATCGTTTACCGACTTTAG	[1]
APEPTGU	*A. lumbricoides*	ITS1	GCCACATAGTAAATTGCACACAAAT	GCCTTTCTAACAAGCCCAACAT	TTGGCGGACAATTGCATGCGAT	[1]
APFVK2R	*N. americanus*	ITS2	CTGTTTGTCGAACGGTACTTGC	ATAACAGCGTGCACATGTTGC	CTGTACTACGCATTGTATAC	[1]
APGZFMN	*S. stercoralis*	Dispersed repetitive sequence	TCCAGAAAAGTCTTCACTCTCCAG	TGCGTTAGAATTTAGATATTATTGTTGCT	TCAGCTCCAGTTGAACAACAGCCTCCAA	[1]
APH497K	*T. trichiura*	18s rRNA	TTGAAACGACTTGCTCATCAACTT	CTGATTCTCCGTTAACCGTTGTC	CGATGGTACGCTACGTGCTTACCATGG	[2]

APKA3TH	MS2	MS2g1	TGGCACTACCCCTCTCCGTATTCAC	GTACGGGCGACCCCACGATGAC	CACATCGATAGATCAAGGTGCCTAC AAGC	[2]
CCU001L	Rotavirus	NSP3	ACCATCTWCACRTRACCCTCTATGAG	GGTCACATAACGCCCCTATAGC	AGTTAAAAGCTAACACTGTCAAA	[2]
CCU001L	Aeromonas	Aerolysin	TYCGYTACCAGTGGGACAAG	CCRGCAAACTGGCTCTCG	CAGTTCCAGTCCCACCACTT	[1]
APMFXDF	Astrovirus	Capsid	CAGTTGCTTGCTGCGTTCA	CTTGCTAGCCATCACACTTCT	CACAGAAGAGCAACTCCATCGC	[2]
CCU001L	Norovirus GI	ORF 1-2	CGYTGGATGCGNTTYCATGA	CTTAGACGCCATCATCATTYAC	TGGACAGGAGATCGC	[1]
CCU001L	Norovirus GII	ORF 1-2	CARGARBCNATGTTYAGR TGGATGAG	TCGACGCCATCTTCATTCACA	TGGGAGGGCGATCGCAATCT	[2]
CCU002	Sapovirus	RdRP	GAYCAGGCTCTCGCYACCTAC TTTGAACAAGCTGTGGCATGCTAC	CCCTCCATYTCAAACACTA	CYTGGTTCATAGGTGGTRCAG CAGCTGGTACATTGGTGGCAC	[2]
APNKRXD	PhHV	gB	GGGCGAATCACAGATTGAATC	GCGGTTCCAAACGTACCAA	TATGTGTCCGCCACCATCT	[2]
APPRKHA	*Enterococcus faecalis*	*ent*	GAGAAATTCCAAACGAACTTG	CAGTGCTCTACCTCCATCATT	TGGTTCTCTCCGAAATAGCTTTAGG GCTA	[3]
APRWE27	EHEC *E. coli* 0157	*rdbE*	TTTCACACTTATTGGATGGTCTCAA	CGATGAGTTTATCTGCAAGGTGAT	CTCTCTTTCCTCTGCGGTCCT	[1]

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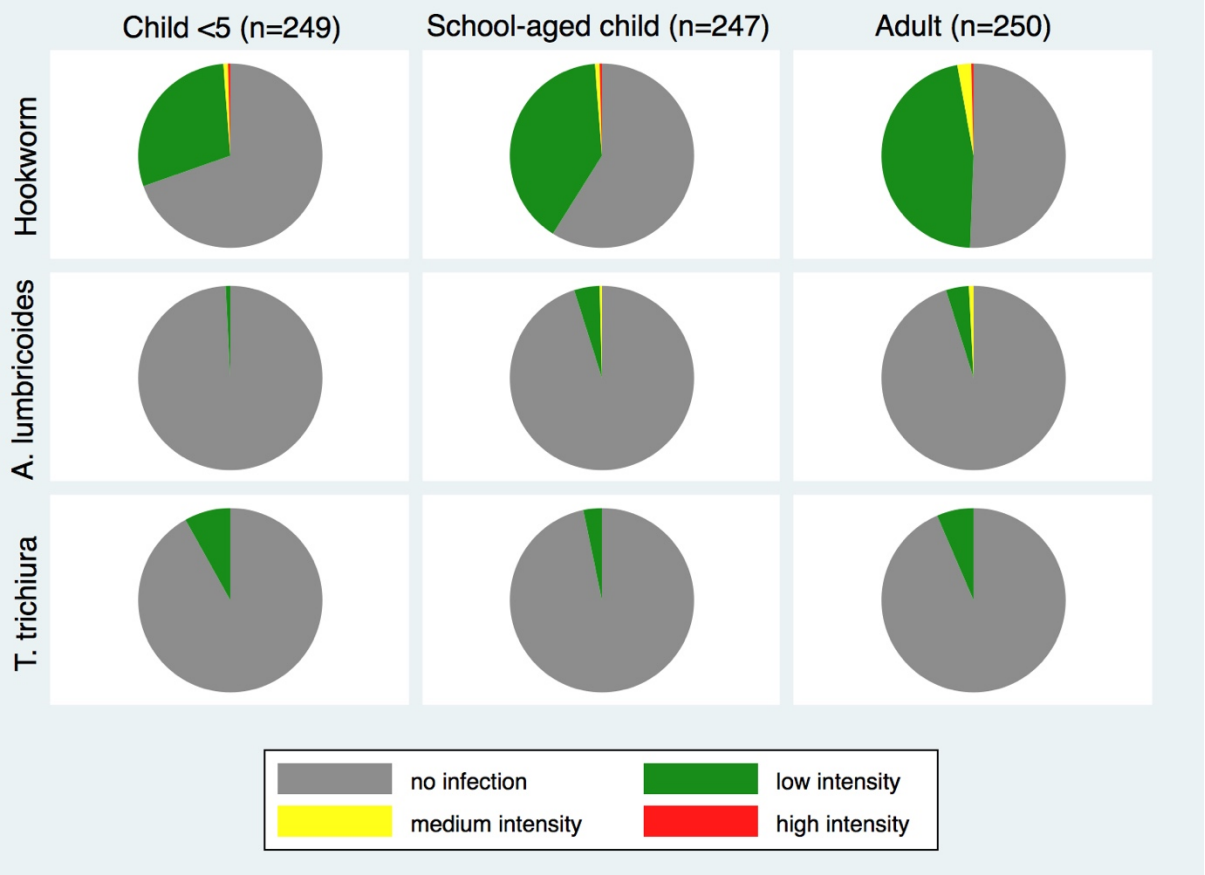
Figure 2.S1. Soil-transmitted helminth infection intensity according to Kato-Katz test, Saravane Province, Laos, 2017 (n=746)



Chapter 3. Impact of a school-based water, sanitation, and hygiene intervention on school absence, diarrhea, respiratory infection, and soil-transmitted helminths: Results from the WASH HELPS cluster-randomized trial[3]

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Abstract

Background: Water, sanitation, and hygiene (WASH) in schools is promoted by development agencies as a modality to improve school attendance by reducing illness. Despite biological plausibility, the few rigorous studies that have assessed the effect of WASH in schools (WinS) interventions on pupil health and school attendance have reported mixed impacts. We evaluated the impact of the Laos Basic Education, Water, Sanitation and Hygiene Programme— a comprehensive WinS project implemented by UNICEF People's Democratic Republic (Lao PDR) in 492 primary schools nationwide between 2013 and 2017— on pupil education and health.

Methods: From 2014-2017, we conducted a cluster-randomized trial among 100 randomly selected primary schools lacking functional WASH facilities in Saravane Province, Lao PDR. Schools were randomly assigned to either the intervention (n=50) or comparison (n=50) arm. Intervention schools received a school water supply, sanitation facilities, handwashing facilities, drinking water filters, and behavior change education and promotion. Comparison schools received the intervention after research activities ended. At unannounced visits every six to eight weeks, enumerators recorded pupils' roll-call absence, enrollment, attrition, progression to the next grade, and reported illness (diarrhea, respiratory infection, conjunctivitis), and conducted structured observations to measure intervention fidelity and adherence. Stool samples were collected annually prior to de-worming and analyzed for soil-transmitted helminth (STH) infection. In addition to our primary intention-to-treat analysis, we conducted secondary analyses to quantify the role of intervention fidelity and adherence on project impacts.

Results: We found no impact of the WinS intervention on any primary (pupil absence) or secondary (enrollment, dropout, grade progression, diarrhea, respiratory infection, conjunctivitis,

STH infection) impacts. Even among schools with the highest levels of fidelity and adherence, impact of the intervention on absence and health was minimal.

Conclusions: While WinS may create an important enabling environment, WinS interventions alone and as currently delivered may not be sufficient to independently impact pupil education and health. Our results are consistent with other recent evaluations of WinS projects showing limited or mixed effects of WinS.

Keywords: water, sanitation, hygiene, schools, diarrhea, respiratory infection, soil-transmitted helminths, school absence

Introduction

School-aged children in low-income settings are at substantial risk for water, sanitation, and hygiene (WASH)-related infections such as pathogens causing diarrheal diseases, soil-transmitted helminths (STH), and trachoma [1-4]. Crowded, unsanitary conditions may facilitate the spread of pathogens, and increase pupils' risk for disease [5]. Improved access to WASH facilities combined with sufficient behavior change may not only prevent the spread of pathogens within the school domain but also lead to beneficial WASH habits at home and throughout the life course [5-8]. The limited data available indicate that only 69% of schools worldwide have access to sanitation facilities, while only 66% have access to water [9]. WASH in schools (WinS) targets and indicators have been included in the Sustainable Development Goals [10].

Despite the biological plausibility of WinS interventions to reduce illness and subsequently school absence, evidence of impact has been mixed. Some WinS efficacy studies, such as those assessing intensive handwashing programs in China and Egypt, reported reductions in absence and absence due to illness. However, with only 6- and 3-month follow up periods, respectively, and with soap being continuously supplied by the intervention or school administration, respectively, the long-term sustainability of handwashing behaviors linked to these impacts is unknown [11, 12].

Effectiveness trials of WinS projects have not replicated this success. A matched-control evaluation of a comprehensive WinS program in Mali revealed reductions in pupil-reported diarrhea, symptoms of respiratory infection, and absence due to diarrhea, but higher odds of absence overall among pupils enrolled in beneficiary schools. However, there were imbalances between the beneficiary and comparison groups at baseline, and the study was further limited by inconsistent fidelity to the intervention by implementing partners and participating schools [13].

A randomized controlled trial (RCT) of a WinS program in Kenya reported a 44% reduction in odds of *Ascaris lumbricoides* reinfection, but no overall impact on absence or diarrhea. Program impact differed by intervention arm (as individual and combined WASH interventions were employed) and subsets of the sample population [14-16]. Absence among girls in the hygiene promotion and water treatment arm reduced by 58% [16]. In water-scarce schools that received a comprehensive WASH intervention, including water supply improvements, risk of diarrhea among pupils reduced by 61% [15], while diarrhea among pupils' siblings under 5 years old reduced by 56% [17]. However, program impact may have been affected by incomplete and inconsistent intervention delivery (fidelity) and uptake and use by the target population (adherence) [15, 16]. A WinS intervention in Lao People's Democratic Republic (Lao PDR), Cambodia, and Indonesia had no impact on STH infection or being underweight, but reported evidence of improvement in dental cavities. Again, this evaluation was potentially limited by incomplete fidelity and adherence to the program, as well as a non-randomized design and contamination from concurrent programming in control schools [18].

Here, we present results from the Water, Sanitation, and Hygiene for Health and Education in Laotian Primary Schools (WASH HELPS) study, a cluster-RCT designed to measure the impact of a comprehensive WinS project – water supply, sanitation, handwashing, and behavior change - in Lao PDR on pupil absence, diarrhea, respiratory infection, and STH infection. Given past challenges in program fidelity and adherence to project outputs and behaviors [19], we also apply two analyses that have previously been used to evaluate the role of intervention fidelity and adherence on WinS project impacts [20, 21].

Methods

Study setting and intervention

The Laos Basic Education, Water, Sanitation and Hygiene Programme was implemented by UNICEF in 492 primary schools across thirteen provinces between 2013 and 2017. The WASH HELPS Study, a research component of the intervention, was conducted between September 2014 and May 2017 in Saravane Province, which was selected because it was the only province in which intervention activities had not yet occurred, thus allowing a randomized intervention trial.

The study setting, baseline results, intervention components, intervention outputs and outcomes, and their fidelity and adherence have been described in detail elsewhere [19]. Key outputs and outcomes of the project are listed in **Table 3.1**. Briefly, the comprehensive WinS project included provision of a school water supply, sanitation facilities, handwashing facilities (individual and group), drinking water filters, and behavior change education and promotion. The project was implemented in two phases; lessons learned from Group 1 schools (n=52; intervention started in 2014) were applied to improve the project for Group 2 schools (n=48; intervention started in 2015), leading to different levels of achievement at output and outcome levels between groups, as well as different durations of follow-up [19].

Study design, sampling, and data collection

We conducted a cluster-randomized, controlled trial among 100 primary schools (50 intervention, 50 comparison). Study design, sampling, and data collection methods have been previously published [19].

We used stratified random sampling to help ensure equal representation of control and intervention schools in each district, and that the number of schools selected in each district was proportional to the number of eligible schools in each district. We selected up to 40 pupils from grades 3-5 in each school using systematic stratified sampling, with grade and sex as the stratification variables. Pupils selected at baseline were followed throughout the entire study period; pupils who left the school due to abandonment or transfer were replaced at the beginning of the following academic year, maintaining equal grade and sex ratios when possible. Pupils who progressed from fifth to the sixth grade were replaced with pupils from grade three the following academic year. A total of 3,993 pupils were enrolled throughout the study period.

Data were collected over three or two school years (Group 1 and 2 schools, respectively) to measure uptake and sustainability of facilities and behavior change. To account for variabilities across time and season, data were collected throughout the school year, which consists of 33 weeks across two semesters (September-January and February-May), with five to six hours of instruction per day [22]. Trained enumerators visited study schools every six to eight weeks during the school year through March 2017, for a total of 11 (Group 1) or 7 (Group 2) visits per school. All visits were unannounced and during school hours. At each visit, enumerators conducted a roll call of all students enrolled in the school using sex- and grade-specific ledgers; interviewed the school directors; interviewed sampled pupils in grades 3–5; observed conditions and functionality of WinS hardware; and observed individual and group handwashing practices. Each year, stool samples were collected from up to 50 pupils per school prior to distribution of preventative chemotherapy as part of the National School Deworming Programme. Stool samples were tested for *Ascaris lumbricoides*, *Trichuris trichuria*, and hookworm (*Ancyclostoma duodenale* and *Necatur americanus*) using the Kato Katz technique [23].

Measures

Our primary impact of interest was pupil absence measured by school-wide roll-call at each visit. At the beginning of each data collection visit, enumerators visited each classroom with a roster of all students enrolled in the school, stratified by grade and sex. At each visit, enumerators confirmed with the head teacher whether there were any new students since the last visit or if any students had left the school. New students were added to the roster. Dropout was recorded for students who had dropped out since the last visit. Absences that were followed by a designation of dropout or transfer were removed from roll-call analysis.

Secondary educational impacts included enrollment, dropout, and progression. Enrollment was calculated at each visit by summing the count of pupils on the roll-call roster and subtracting those who had dropped out or transferred. In addition to student-level dropout recorded in the roll-call register, an aggregate school-level count of dropout was reported by the school at the end of each school year. Pupils who transferred to another school were not considered to have dropped out. Progression was school-reported at the end of each academic year as the count of students who passed the national exam and progressed to the next grade level. All secondary educational impacts were stratified by grade and sex.

Secondary health impacts included diarrhea, symptoms of respiratory infection, and conjunctivitis/non-vision related eye illness and were collected through pupil interviews. All health impacts were binary and self-reported with a one week recall period. Pupils were asked if they had had diarrhea using local terminology and were also asked how many times they had defecated each day; a pupil was considered to have had diarrhea if he or she had reported having diarrhea and had defecated three or more times in a 24-hour period [13, 15, 24]. Pupils were

considered to have symptoms of respiratory infection if they reported cough, runny nose, stuffy nose, or sore throat [13]. During the last visit we included negative-control questions about self-reported cuts/scrapes and toothache. These questions served as a measure of respondent bias, as there is no biological plausibility of an association between a WinS program and cuts/scrapes or toothache [25]. Data on STH infection were collected yearly. Any sample testing positive for the hookworms, *A. lumbricoides*, or *T. trichuria* considered positive for STH infection.

Intervention fidelity and adherence for this study has been described previously [19]. To measure fidelity- defined as how the intervention was delivered per the stated design- we created an index score in which one point was given for each of the 20 output criteria fulfilled (**Table 3.1**). For each visit, the minimum intervention fidelity score was zero and the maximum score was 20. To measure adherence- defined as achievement of behavioral outcomes promoted by the intervention- a similar index score was created. Although there were five behavioral outcomes of interest (**Table 3.1**), we excluded group compound cleaning from the index given that reported participation in group compound cleaning was nearly universal among both intervention and comparison schools at baseline (97.9%) [19], therefore the adherence score ranged from 0-4. A behavior was considered to be achieved when >75% of pupils reported or were observed to complete the behavior except for group handwashing, which was binary (either the school performed group handwashing or did not).

Analysis

Data were analyzed using Stata Statistical Software: Release 13 (StataCorp LP, College Station, TX) and SAS version 9.4 (SAS Institute, Cary, NC).

Intention to treat analysis. Our primary analysis was an intention-to-treat (ITT) analysis, which was used on all primary and secondary impacts. For binary impacts (roll-call absence, diarrhea, symptoms of respiratory infection, conjunctivitis/non-vision related eye illness, STH infection, toothache, cuts/scrapes), we estimated relative risk using a "modified Poisson" approach. This is a validated method to produce relative risk ratios for binary data using a multi-level mixed Poisson model with robust error variances [26], and was chosen for this analysis because Stata does not support the use of log-linear binomial regression when using mixed effects generalized linear models. Odds ratios were obtained when the modified Poisson model did not converge for a specific impact (e.g. toothache). Random intercepts at the school and pupil levels were included to account for clustering of pupils within schools and for repeated measures of pupils over time, respectively. For count impacts (enrollment, abandonment, progression), we estimated relative risk using Poisson regression models. As these data were aggregated at the school-level, we included a random intercept at the school level only.

All ITT models compared intervention schools to comparison schools as they were randomly allocated to intervention and comparison groups, without regard to project fidelity or adherence. Intervention and comparison schools were balanced on key indicators at baseline [19], therefore intervention schools were included in the analysis once UNICEF documented that full intervention implementation (e.g. both hardware and behavior change components) was complete. Since full implementation generally occurred at the same time in each district, comparison schools were also included once implementation occurred in their respective districts.

Models included several design variables, including the district and visit number, and controlled for the following fixed effects, determined a priori based on biological plausibility of affecting

impacts: pupil sex, pupil grade, school enrollment size, season (rainy or dry). The rice crop calendar (planting, growing, harvesting) was included as a fixed effect in the absence model because rice agriculture is the predominant economic activity in the province and the need to stay home and support the family was the leading cause of pupil-reported absence. Fully adjusted models were used to produce adjusted risk ratios (RR) for each of the associations of interest. These fixed effects, as well as whether the school was concurrently receiving aid from the World Food Program (WFP) school feeding program, were also assessed for effect modification. Covariates were determined to be effect modifiers if an interaction term between the covariate and intervention group was significant in the full model.

Intervention fidelity and adherence are important considerations when evaluating the impact of WASH programs. Assessing these factors along the causal 'theory of change' allows us to understand not only if but why and how that intervention succeeded or not in that context (i.e., was there theory failure [27]?). Further, assessment of the process can determine if the intervention followed the intervention protocol to activate that theory of change (i.e., was there intervention failure?). In contexts where fidelity and adherence to the intervention is imperfect, ITT results may underestimate the causal effect for the potential impact of changes to outputs or outcomes, resulting in null or mixed effects [28, 29]. Given the suboptimal fidelity and adherence of the intervention based on our monitoring data [19], we conducted a secondary analysis to quantify the impact of the project as implemented by UNICEF and adhered to by schools and pupils on the primary impact (roll-call absence) and select secondary impacts (diarrhea, respiratory infection, and STH prevalence). We explore two modeling frameworks that have been previously used to evaluate the role of fidelity and adherence to a school WASH intervention on project impacts: As-treated (AT) analysis and Structural Nested Models (SNMs) [20, 21]. Each framework operates

under different assumptions and differ in robustness and efficiency; as such, comparing estimates lends a more informed picture of project impact [30].

We conducted a sensitivity analysis to identify a meaningful threshold of fidelity and adherence. The scale of 20 outputs (fidelity) were categorized with cut-points at each 10th percentile and the scale of four outcomes (adherence) were unadjusted. We observed lower risk of absence among schools with 70-80% intervention fidelity and higher, but there was no clear evidence of a threshold for any other association (Figure 3.1). We thus selected a threshold of 75%, which is consistent with previous research on fidelity to WinS projects [20]. Only the SNM requires specifying a threshold of fidelity/adherence, however, we also applied the 75% threshold to the AT models for comparability between the two approaches.

As-treated analysis. The AT analysis groups subjects according to the treatment received and does not consider the treatment intended (as is the case with ITT analysis). Advantages to the AT approach are that it is analytically straightforward and easily supports our clustered and longitudinal study design. Disadvantages are that characteristics of schools with good fidelity or students who adhere to behaviors may be fundamentally different from those with poor fidelity/adherence, which can lead to confounding. This confounding may be remedied by controlling for the prognostic factors that led participants to choose to adhere, but only if those prognostic factors are known, which is often not the case [31].

For the AT analysis, we ran two separate models that were structurally identical to the ITT models. However, instead of using intervention status as the primary predictor, as in the ITT analysis, schools were grouped according to intervention fidelity (i.e. fulfilling ≥75% of outputs or not) and

adherence (i.e. fulfilling ≥75% of outcomes or not), respectively. AT models included the same covariates as the ITT models, with a priori identified fixed effects and random intercepts at the school and pupil levels. Only data collected after the implementor reported intervention delivery was complete were included. AT models were stratified by effect modifiers identified in the ITT analysis.

Structural nested model analysis. Second, we assessed the role of fidelity and adherence using SNMs, an instrumental variable approach. SNMs resolve the potential confounding issue presented by AT models because they do not break the randomization of intervention status [30]. Instead, SNMs create a counterfactual for each study participant in order to compare the risk of an impact among adherers against the risk of the impact had the same individual not adhered [20]. Unlike the ITT and AT models, to control for relevant covariates, a weighted distribution of population data is produced in order to remove the association between population-level confounders and randomization [20, 32]. While SNMs are advantageous because they account for unknown or unmeasured confounders, drawbacks are that they are more computationally intensive and rely on strong assumptions. SNM assumptions are described in detail elsewhere [20, 32]; briefly, they are as follows: (1) Exclusion restriction- randomization has no direct effect on the outcome; (2) Consistency- observed outcomes are possible under the fidelity/adherence level actually observed; (3) The potential outcomes used to estimate the SNM effects are independent of randomization; (4) No interaction- the model's causal effect is consistent across randomization groups.

Our code was derived from Garn et al [20] and adapted for a 2-arm trial. Because the SNM methodology we used does not accommodate repeated measures, we averaged time-varying pupil-level data (e.g. grade, absence, reported diarrhea, reported symptoms of respiratory infection) and

school-level data (output index, behavior index) across the data collection period. As such, binary variables such as absence, reported diarrhea, and reported symptoms of respiratory infection became a continuous variable between zero and one, in which zero indicated never being absent, reporting diarrhea, or reporting symptoms of respiratory infection, whereas one indicated always being absent, reporting diarrhea, or reporting symptoms of respiratory infection. Similar to the ITT and AT models described above, observations were included only after full implementation had been achieved. Models were adjusted using the same covariate variables as we used in the ITT and AT models. As with the AT models, achievement of ≥75% of outputs and ≥75% of outcomes were considered achieving fidelity and adherence, respectively. SNMs were stratified by effect modifiers identified in the ITT analysis [20].

For all analyses, results were considered statistically significant if the P-value was < 0.05.

Ethics

The WASH HELPS Study was approved by Emory University's Institutional Review Board (IRB0076404) and the Lao Ministry of Health's National Institute of Public Health National Ethics Committee (No. 043 NIOPH/NECHR). Both Institutional Review Boards approved consent *in loco parentis* (in the place of the parent) signed by the school director. Pupils who were selected for the pupil interview and/or stool collection provided informed verbal assent prior to any data collection. All consent/assent procedures occurred after randomization. The intervention was delivered to comparison schools in April 2017, after research activities ended. The study is registered at clinicaltrials.gov (NCT02342860).

Results

Baseline results and intervention fidelity and adherence

A total of 100 schools (n=50 intervention, n=50 comparison) were randomized, received the intervention, and included in the analysis (**Figure 3.2**). There were no significant differences in key pupil-level or school-level indicators between intervention and comparison groups at baseline, indicating that the cluster-randomization was successful in creating balanced groups [19]. Following full intervention implementation, intervention fidelity was 30.9% across all schools and visits and intervention adherence was 29.4%. Data on fidelity to specific project outputs and adherence to specific project behaviors across the evaluation period have been previously published [19].

Intention-to-treat analysis

We found no impact of the intervention on the primary impacts (roll-call absence) or secondary impacts (enrollment, progression, pupil-reported diarrhea, pupil-reported symptoms of respiratory infection, pupil-reported conjunctivitis, STH infection; **Table 3.2**).

There was some evidence of effect modification. Risk of diarrhea was higher in the rainy season compared to the dry season; when stratified by season, there was no significant impact of the intervention on diarrhea in either season (Dry season RR: 0.69, 95% CI: 0.44, 1.10; Rainy season RR: 1.14, 95% CI: 0.65, 1.99). Pupil sex, pupil grade, school enrollment size, receiving support from the WFP school feeding program, and the rice crop calendar (absence model only) did not modify the effect of any primary or secondary impacts.

We found no difference in reported prevalence of toothache or cuts/scrapes (the negative control questions) among pupils attending intervention versus comparison schools (toothache OR: 0.64, 95%CI: 0.23, 1.84; cuts/scrapes RR: 1.06, 95% CI: 0.66, 1.72), indicating that any respondent bias that may have been present occurred equally between groups.

As-treated analysis

AT results are presented in **Table 3.3**. Intervention fidelity – meeting ≥75% of output indicators associated with water supply, toilets, handwashing facilities, promotion of group hygiene activities, group handwashing facilities, and filtered drinking water— was associated with roll call absence and prevalence of STH. Compared to students attending schools without intervention fidelity, students attending schools with intervention fidelity had a 23% lower risk of absence (RR: 0.76, 95% CI: 0.64, 0.91) and a 20% higher risk of STH prevalence (RR: 1.20, 95% CI: 1.01, 1.43). Diarrhea was significantly higher during the rainy season, but when stratified there was no significant difference by fidelity status (Dry season RR: 0.84, 95% CI: 0.48, 1.49; Rainy season RR: 1.65, 95% CI: 0.82, 3.33).

Intervention adherence – meeting outcome indicators associated with toilet use, handwashing with soap after toilet use, daily group handwashing, and daily group toilet cleaning — was not significantly associated with any impacts.

Structural nested model analysis

Results from the SNMs are presented in **Table 3.3**. Diarrhea was the only impact associated with fidelity or adherence. When stratified by season, diarrhea was lower in the dry season among students attending schools with intervention fidelity (RR: 0.45, 95% CI: 0.24, 0.85) and adherence

(RR: 0.42, 95% CI: 0.21, 0.87); there was no significant difference in diarrhea between groups during the rainy season.

Discussion

In the primary analysis, we found no evidence that the intervention had an effect on absence, school enrollment, dropout, grade progression, pupil-reported diarrhea, pupil-reported symptoms of respiratory infection, pupil-reported conjunctivitis, or prevalence of STH. These results contribute to the growing body of research showing limited or mixed impacts of WinS effectiveness trials on pupil health and education [13-16, 18]. Since 2010, access to WASH has been a fundamental human right recognized by the United Nations General Assembly [33]. As such, regardless of its potential education and health impacts, WinS access is an important objective, evidenced by its inclusion in the Sustainable Development Goals [10]. However, if improvements in education and health indicators are to be achieved, results from this and other rigorously evaluated WinS programs suggest that WinS interventions *alone*, and as currently delivered in many contexts, may be insufficient to achieve anticipated education and health impacts.

The theory of change for WinS programs posits that improved WASH access leads to reductions in pathogen exposure at the school level and the habitualization of hygiene behaviors that can be practiced both at school at and home, which in-turn leads to reduced illness and thus reduced school absence [8]. Numerous factors influence school absence, such as household wealth, distance to school, and number of siblings [34]. Lao PDR is a least-developed country, with over 65% of the population working in agriculture [35]. In Saravane Province, where over half of the population lives in poverty [35], the school calendar largely coincides with rice planting and harvesting seasons, and children are often kept home from school to assist in the fields and with

other household chores [22]. Indeed, in the current study, the leading pupil-reported cause of school absence was the need to stay home to support the family in economic activities (9.4% of pupils in intervention group and 8.7% of pupils in comparison group across all visits), not illness (5.1% of pupils in intervention group and 5.8% of pupils in comparison group across all visits), which may explain the lack of an impact of the intervention on absence. Thus, the role of school WASH in supporting an enabling environment may be critical, but ultimately not sufficient to reduce absence when other factors like household economic needs or food security is the main driver of truancy from school. Complementary approaches to WinS may be necessary to achieve improvements in absence and other educational impacts. For example, WinS may be successful in combination with school feeding programs [36] or conditional cash transfers [37], both of which have been associated with reduced absence and increased enrollment in other low- and middle-income contexts. Although our results did not reveal a significant interaction between the WFP school feeding program and absence or enrollment, our study was not designed or adequately powered to detect a difference.

Although there are potential mechanisms by which improved WASH may impact illness independently of measurable impacts on absence [13], we found no overall impact of the WinS intervention on pupil illness. These results contrast to previous WinS research that reported overall reductions in diarrhea [13], respiratory infection [13], and absence due to illness [11, 12], but are consistent with results from a WinS intervention in Lao PDR, Cambodia, and Indonesia that found no impact of the intervention on STH or underweight [18]. One explanation for the lack of an effect of the WinS intervention on pupil illness is low household WASH access; in this study context, the health benefits linked to improvements in school WASH conditions and behaviors provided by this intervention were likely not sufficient to overcome other potential transmission

pathways at home or elsewhere in the community. Environmental improvements in both the domestic and public domains may be required for successful control of infections targeted by environmental improvements, such as diarrhea [38]. As such, WinS alone may not achieve significant health gains without concurrent community and household WASH improvements.

Fidelity and adherence are fundamental antecedents to achieving intervention effects. It is possible that the lack of an effect of the intervention could be due, in part, to sub-optimal or unsustained fidelity and adherence. However, our secondary analyses yielded limited evidence of an effect of the intervention, even at high levels of intervention fidelity and adherence. Additionally, our sensitivity analysis showed no clear trend in impacts across the fidelity/adherence continuum. With two exceptions – the association between fidelity and lower absence (AT analysis) and the association between fidelity and adherence and lower diarrhea during the dry season (SNM analysis) – we did not find that fidelity and adherence led to improved education or health. These results support the above conclusion that factors other than WinS – such as low household WASH access or household economics – may supersede health and education benefits of a WinS intervention in low-income contexts.

However, the AT evidence should be should be interpreted cautiously due to the limited potential for causal inference resulting from breaking the randomization assignment in the AT analysis. The two fidelity and adherence analyses results were inconsistent and sometimes yielded estimates of effect in opposite directions (e.g. association between adherence and diarrhea, respiratory infection, and STH), which is likely due to unaccounted for confounding in the AT analysis. IV analyses are known to yield estimators with high variance, especially when compliance is low [30], which may also partially explain differences between the AT and SNM results. The choice

of which method to use depends on numerous factors, including study design, plausibility of meeting analysis assumptions, and available analytical resources; our conflicting estimates highlight the importance of testing the sensitivity of multiple fidelity analysis options [30].

Strengths and limitations

The design, methods and approach of the WASH HELPS Study were robust. Randomized controlled trials offer the greatest potential for causal inference. The longitudinal design allowed us to collect data across three full school years of in Group 1 schools and two full school years of in Group 2 schools, allowing us to capture inter-seasonal and inter-year variations in the outputs, outcomes, and impacts. All data were collected during unannounced school visits so that schools could not prepare for the visit and bias observations. Our primary measure of impact – roll-call absence - is an objective measure of school absence. This impact evaluation was conducted by external researchers, to foster an unbiased assessment of the project impact. Our field team was composed of experienced Laotian enumerators to ensure the tools were designed and delivered with cultural and contextual appropriateness. This robust study design lends strong internal validity, and results may be generalized to the larger, nationwide WinS project. This was an effectiveness trial evaluating an intervention as conducted in a real-world setting. The lessons from this project, taken with other recent WinS trials, reveal heterogeneity of findings that can inform programming across contexts. Lastly, in addition to comparing two methods to analyze the effect of intervention fidelity on WinS impacts, our fidelity analysis also examines adherence to intervention behaviors, which has not been previously included in WinS fidelity analyses.

There are a number of limitations to this evaluation. First, the secondary health impact measures (diarrhea, symptoms of respiratory infection, conjunctivitis) were based on self-report by pupils,

which may be subject to bias, and this evaluation was not blinded for either the beneficiaries or data collectors. More objective and robust measures of pupil health, such as molecular methods to detect enteric infection in stool samples, would improve our confidence in the reported impacts, these measures can be costly, time consuming, and require specialized equipment and laboratory staff. As a way to measure potential reporting bias, we included a negative control question about symptoms of illness unrelated to WASH access (cuts/scrapes and toothache) at the last survey visit. Differences in reported symptoms of these illnesses between intervention and comparison groups would indicate a potential reporting bias, but we found no evidence to suggest that any bias may have existed to a greater degree among either the intervention group or the comparison group. Additionally, schools in the comparison group did not have functional WASH facilities, so it is unlikely that the null results could be explained a change in behaviors among the comparison group. Second, the intervention was delivered across two different school years, so Group 1 schools had one more year of surveillance than Group 2 schools. Following a single cohort of schools over the same time period would have provided a more accurate measure of WinS hardware and software performance, sustainability, and impact. Third, implementation was delayed in many Group 1 schools. The intervention was fully implemented in Group 1 schools at visit 4, with the exception of Samoui district, in which the intervention was fully implemented at visit 9 [19]. Our analysis excludes visits prior to full intervention implementation, thus power may have been limited by dropping observations under incomplete intervention delivery. Last, we were unable to account for the quality of intervention design or dose of the intervention received, which are important components of fidelity and adherence [39, 40].

Conclusions

Our findings and those of other rigorous WinS trials suggest that WinS programs – as currently designed and delivered – do not have a population-level benefit on education and health. In this context, the WinS improvements alone were not sufficient to address the other powerful causes of absenteeism, enrollment, and dropout that are not related to— but possibly more influential than— school WASH. We believe this likely holds in many similar settings. Similarly, WinS improvements, though potentially critical for the enabling environment [7], may not be sufficient to overcome disease transmission in areas where community and household WASH coverage is poor. WinS, independent of its stated purpose of improving education and health, is an important objective for dignity, inclusivity, and development. However, if intended impacts are to be achieved, improving intervention fidelity and adherence and including other complementary approaches for WASH may be required. To better understand how to improve intervention fidelity and adherence, evaluations of WinS interventions need to better understand and adapt to contextual drivers of key impacts and outcomes, further develop and test theories of change, and conduct rigorous process evaluations to understand where along the causal pathways interventions are falling short.

Declarations

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Author contributions

Conception, MCF, ANC, and TC; design, MCF, ANC, TC, and HHC; data acquisition, ANC and JVG; analysis and interpretation, ANC, JVG, HHC; initial draft of article, ANC; revisions and final approval of article, ANC, JVG, HHC, TC, MCF.

Competing interests

All authors have completed the ICMJE uniform disclosure form at http://www.icmje.org/coi_disclosure.pdf (available upon request from the corresponding author) and declare no conflicts of interest.

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Figures

Figure 3.1. Association between intervention fidelity and adherence continuum and intervention impacts.

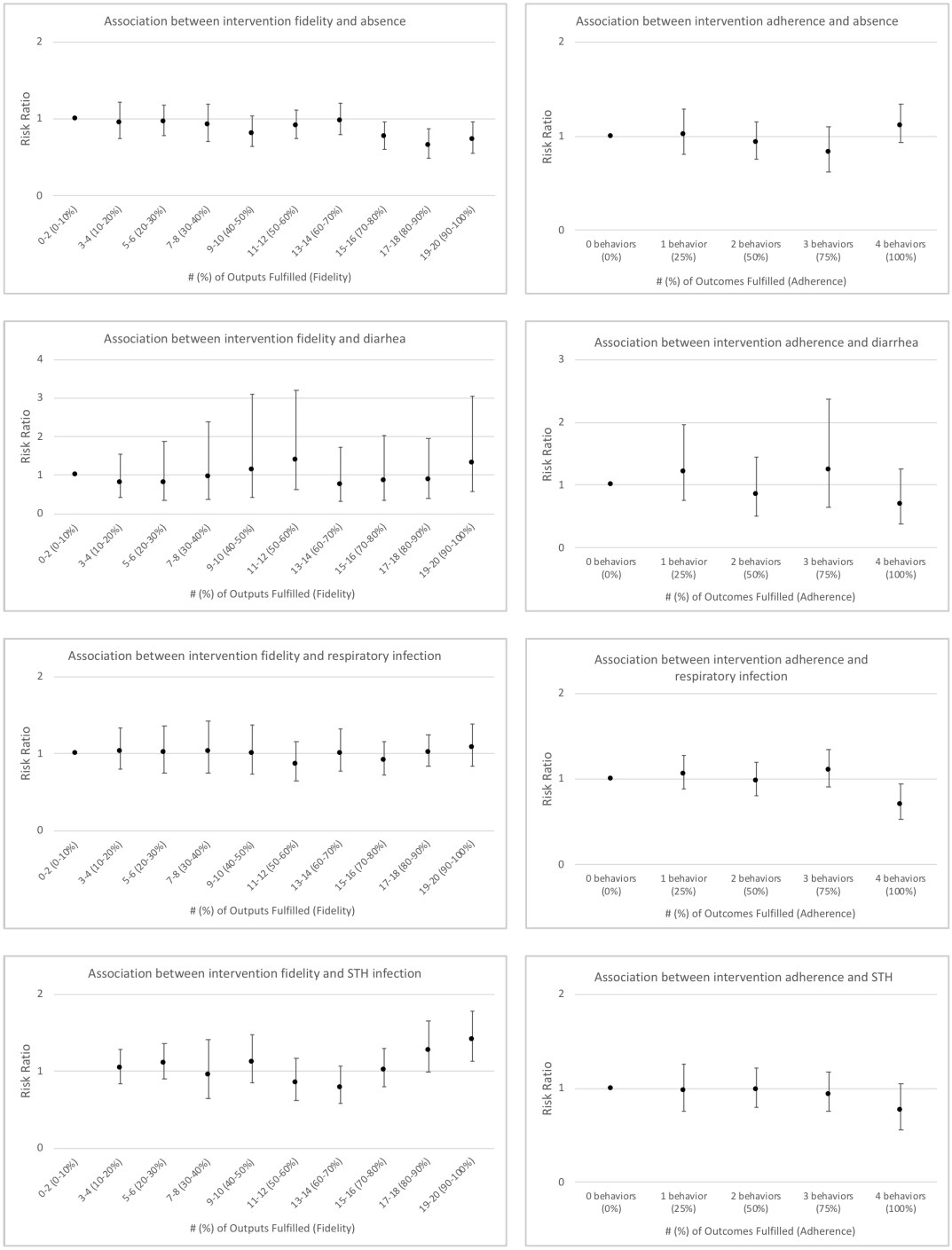
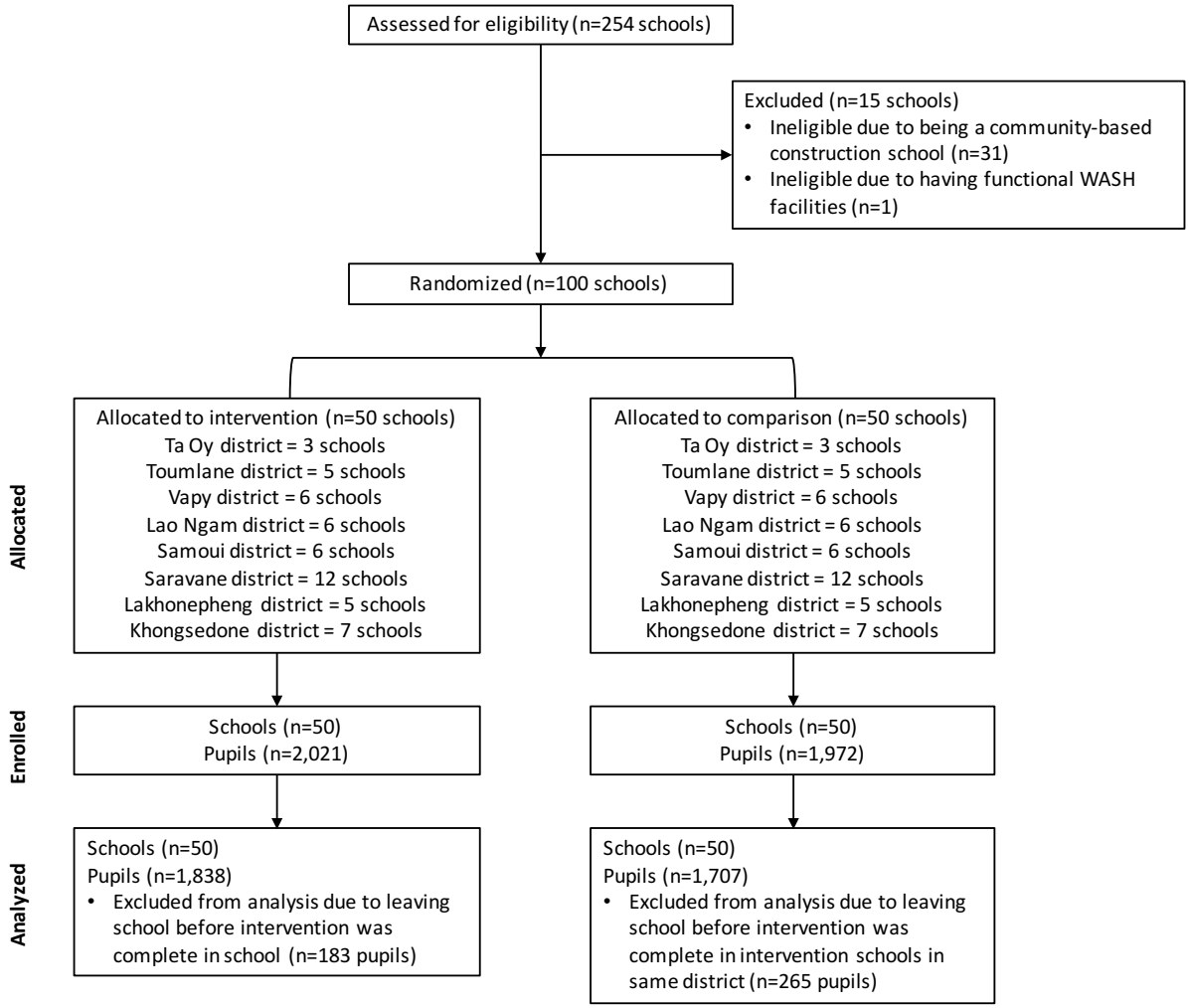


Figure 3.2. Flow diagram of school and pupil selection.



Tables

Table 3.1. Intervention outputs and behavioral outcomes and their measurement indicators

Output	**Indicator and Criteria**

Water supply	• Improved* water point on school compound
	  o Water point functional in the previous year (director reported)
	• Water tank to supply toilet and handwashing stations
	  o Water observed in tank

Toilets	• At least one improved* toilet compartment
	  o Toilet is sex separated (by designation)
	  o Toilet is unlocked
	  o Toilet is clean
	  o Toilet has water available inside compartment for flushing

Handwashing facilities	• At least one individual handwashing station available to pupils
	  o Water available at individual handwashing station
	  o Soap available at individual handwashing station

Promotion of daily group hygiene activities	• Daily group handwashing schedule posted in at least one classroom or near toilet • Daily group compound cleaning schedule posted in at least one classroom or near toilet • Daily group toilet cleaning schedule posted in at least one classroom or near toilet

Group handwashing	• Group handwashing facility available to pupils
	  o Water available at group handwashing facility
	  o Soap available at group handwashing facility

Water filters	• At least one drinking water filter available in a classroom for pupil use o Water in filter
Outcome	**Indicator**
Toilet use	• Percentage of students using toilet for defecation during school hours (pupil-reported)
Handwashing (individual)	• Percentage of students washing hands with soap and water upon exiting toilet (observation)
Daily group handwashing	• School conducted daily group handwashing the day of visit (observation)
Daily group toilet cleaning	• Percentage of students participating in daily group toilet cleaning within the previous five school days (pupil-reported)
Daily group compound cleaning	• Percentage of students participating in daily group compound cleaning within the previous five school days (pupil-reported)

* Defined according to Joint Monitoring Programme (JMP) standards.

Table 3.2. Association between WinS intervention and health and educational impacts, Saravane Province, Lao People's Democratic Republic, 2014-2017 (n=100 schools)

Impact	Comparison*	Intervention*	Adjusted Risk Ratio	95% Confidence Interval
Roll-call absence†	6,024 (32.2%)	7,147 (29.9%)	1.01	(0.84, 1.20)
Enrollment‡	68.2 (49.7)	71.6 (50.0)	1.07	(0.84, 1.35)
Dropout‡	0.8 (2.6)	0.4 (1.0)	0.56	(0.25, 1.24)
Grade progression‡	64.4 (48.6)	67.3 (48.6)	1.07	(0.91, 1.25)
Diarrhea†,§	1,032 (21.1%)	947 (14.7%)	0.80	(0.51, 1.26)
Symptoms of respiratory infection†,‖	1,414 (28.9%)	2,064 (32.1%)	1.08	(0.95, 1.23)
Conjunctivitis†,§	41 (0.8%)	48 (0.8%)	0.89	(0.53, 1.52)
Prevalence of any STH†,¶	1,833 (39.8%)	1,935 (41.6%)	1.00	(0.85, 1.17)

* Data are n (%) for impacts at the pupil level (roll-call absence, diarrhea, symptoms of respiratory infection, conjunctivitis, and prevalence of STH) and mean (SD) for impacts at the school-level (enrollment, dropout, grade progression) across study period.

† Risk ratios were calculated using a Poisson model with robust error variances and random intercepts at the school and pupil level. All models adjusted for district, visit number, pupil sex, pupil grade, school enrollment size, and season (rainy or dry). Absence model additionally controlled for and rice crop calendar (planting, growing, harvesting).

‡ Risk ratios were calculated using a Poisson model with random intercepts at the school level. All models adjusted for district and visit number.

§ Pupil-reported in previous week.

‖ Pupil-reported cough, runny nose, stuffy nose, or sore throat in previous week.

¶ Samples testing positive for *Ascaris lumbricoides*, *Trichuris trichuria*, and hookworm (*Ancyclostoma duodenale* and *Necatur americanus*).

Table 3.3. Association between WinS intervention fidelity and adherence and absence, diarrhea, respiratory infection, and soil-transmitted helminth infection (STH), Saravane Province, Lao PDR, 2014-2017 (n=100 schools)

	As-Treated Analysis		Structural Nested Model Analysis	
	Adjusted Risk Ratio*	**95% Confidence Interval**	**Adjusted Risk Ratio†**	**95% Confidence Interval**
Roll-call absence				
Fidelity‡	0.76	(0.64, 0.91)	0.97	(0.33, 2.81)
Adherence‡	0.91	(0.79, 1.05)	0.96	(0.19, 4.97)
Diarrhea§				
Fidelity, dry season‡	0.84	(0.48, 1.49)	0.45	(0.24, 0.85)
Adherence, dry season‡	1.00	(0.70, 1.44)	0.42	(0.21, 0.87)
Fidelity, rainy season‡	1.65	(0.82, 3.33)	1.03	(0.42, 2.51)
Adherence, rainy season‡	1.41	(0.61, 3.26)	0.50	(0.19, 1.30)
Symptoms of respiratory infection‖				
Fidelity‡	1.00	(0.89, 1.14)	1.41	(0.93, 2.13)
Adherence‡	0.97	(0.84, 1.11)	2.30	(0.54, 8.87)
Prevalence of any STH¶				
Fidelity‡	1.20	(1.01, 1.43)	1.10	(0.57, 2.13)
Adherence‡	0.93	(0.77, 1.12)	1.18	(0.37, 3.73)

* Risk ratios calculated using a Poisson model with robust error variances and random intercepts at the school and pupil level. All models adjusted for district, visit number, pupil sex, pupil grade, school enrollment size, season (rainy or dry). Absence models additionally controlled for rice crop calendar (planting, growing, harvesting).

† Risk ratios calculated using a Structural Nested Model with random intercepts at the school level. All models adjusted for district, visit number, pupil sex, pupil grade, school enrollment size.

‡ Fulfilling ≥75% of intervention outputs was considered fidelity. Fulfilling ≥75% of intervention outcomes was considered adherence.

§ Pupil-reported in previous week.

‖ Pupil-reported cough, runny nose, stuffy nose, or sore throat in previous week.

¶ Samples testing positive for *Ascaris lumbricoides*, *Trichuris trichuria*, and hookworm (*Ancyclostoma duodenale* and *Necatur americanus*).

Discussion

Although the health risks associated with inadequate access to water, sanitation, and hygiene (WASH) are well established [1-3], recent rigorously designed, large-scale, household-based WASH impact evaluations have not revealed that the improvements to WASH had an impact on diarrhea or STH [4-8]. Reducing the burden of enteric disease is complex due to a diverse range of pathogens, multiple transmission routes, and human-environment interactions. The aim of this dissertation was to better understand the potential for environmental improvements to mitigate enteric disease in low-resource settings. To that end, both laboratory- and field-based methods were employed to examine several environmentally mediated pathways associated with enteric infection.

Chapter 1 examined the leading pathogenic causes of enteric infections, as well as household- and village-level risk factors for those infections among study subjects of different ages living in the same households. Several noteworthy results emerged that may help explain the lack of significant impacts in recent impact evaluations [4-8]. First, our findings revealed heterogeneities in associations between household WASH access and enteric infection. Results highlighted the considerable influence of community-level factors—both WASH coverage and additional, contextual factors—that had a greater association with enteropathogen infection than did household-level WASH access. Second, enteropathogen infection was nearly universal (98.3%) among the study population, regardless of age. Diarrhea is typically considered a sequalae of importance for young children due to their elevated risk of mortality [9]. We found that age was not a significant predictor of odds of infection for half of the pathogens analyzed. Pathogens may circulate easily within a family or community, therefore the role of other household and village

members in disease transmission should not be overlooked. Third, animal ownership, which was used as a proxy for exposure to animal feces [10, 11], was associated with higher odds of protozoal infection, and trended towards higher odds of bacterial and STH infection, corroborating previous research implicating exposure to animal feces as a substantial risk factor for enteric disease [10-12]. This risk may substantially offset the positive benefits of animal ownership on livelihoods of poor families by yielding chronic enteropathogen exposure and infection and growth shortfalls.

Chapter 2 investigated how within-host biological interactions between STH and microparasites influence odds of infection. Given shared risk factors, including poor WASH access, economic marginalization, and tropical or subtropical climate zones, the geographic and demographic distribution of STH and diarrhea largely overlap and co-infection between STH and microparasites is common [13]. Our data revealed that STH infections were associated with higher odds of concurrent protozoal infections, lower odds of concurrent viral infection, and trended towards higher odds of concurrent bacterial infections, after controlling for WASH access and other shared risk factors. Together with findings from Chapter 1, these results suggest that interventions to control STH, such as increasing community sanitation coverage to eliminate the environmental reservoir for STH, combined with preventative chemotherapy (PC) with anti-helminthic drugs [14, 15], may have a spillover impact on reducing bacterial and protozoal infections. Though promising, the associations observed in Chapter 2 came from a cross-sectional study and additional research is needed to elucidate causation as well as the exact mechanisms by which STH immunomodulate microparasites.

Chapters 1 and 2 highlighted the numerous drivers of enteropathogen infection — including household- and community-level WASH access, exposure to animal feces, and

immunomodulation — and their heterogenous associations across enteropathogen taxa and species. It is unsurprising that the UNICEF Lao PDR WinS intervention had no overall effect on school absence, diarrhea, respiratory infection, or STH, as reported in Chapter 3. Key external factors along the WinS theory of change were not addressed: household- and community-level WASH access in the study communities was low, and the leading cause of school absence (needing to stay home to support the family) was not driven by illness. A recent systematic review of WinS evidence in low-resource countries emphasized how the heterogenous impacts of WinS on absence and health are very context-specific [16]. Our results corroborate this because even among schools with the *highest* levels of intervention fidelity and adherence, the impact of WinS on absence and health was minimal. This suggests that WinS improvements, though potentially critical for the enabling environment for learning and health for school children [17], may not be sufficient to overcome disease transmission in areas where community and household WASH coverage is poor.

Strengths and limitations

Strengths and limitations of the studies included in this dissertation have been described in each chapter. Key considerations are described in more detail below, along with reflections on areas for improvement.

Study design and random selection

The design of the WASH HELPS study was robust. Randomized controlled trials offer the greatest potential for causal inference; schools were randomly selected from a pool of all eligible schools and randomly allocated to either the intervention or comparison arm. The longitudinal design allowed us to collect data across three full school years, allowing us to capture inter-seasonal and inter-year variations in the outputs, outcomes, and impacts. All data were collected during

unannounced school visits so that schools could not prepare for the visit and bias observations. Collectively, this robust design supports strong external validity.

However, the eligibility criteria (primary public school, lacking functional WASH facilities, located in Saravane Province) may have limited the external validity of study results. Lao PDR is a least-developed country and Saravane Province, where this study took place, is the poorest province in Lao PDR [18]. Schools that were eligible for inclusion in the study were likely located in villages that were worse off than other villages in the province or country. Indeed, improved water and sanitation access among households in our study area (47% and 23%, respectively) was much lower than the estimates for the rest of the country (80% and 70%, respectively) [19]. The UNICEF WinS project was active in several provinces. However, the study was restricted to Saravane Province because it was the only province where intervention activities had not yet occurred prior to study design of the study, which allowed for development of an experimental design. We would have greater external validity of results if schools were selected from the larger implementation area.

The design of the cross-sectional sub-study has additional limitations. Villages were randomly selected from the pool of school-hosting villages from the parent trial, so our study population is not a random sample from the whole district or province. Additionally, households were eligible for inclusion only if they had a school-aged child attending a school participating in the WASH HELPS study, a child<5 years living in the household, *and* the household triad (child<5 years, school-aged child, and adult) all returned their stool sample on the same day. Characteristics of these households may be different in behaviors and exposures from those in the wider community.

Additionally, because the sub-study was cross-sectional we have only one measurement point for the PCR data; as such, we cannot assess causality. The results and conclusions in Chapter 2 would be strengthened if we had evidence that STH infection occurred before microparasite infection. We believe it is likely that STH infections preceded microparasite infections because STH are endemic in this population, data were collected prior to annual primary school-based PC, there is no routine community-based PC in this population, and re-infection often occurs rapidly after PC [20]. However, it is possible that results reflect an inverse association. Collecting a series of stool samples over time would be expensive, but would provide additional insight into helminth-microparasite co-infection patterns and give us more confidence in our conclusions. A longitudinal design could also provide valuable evidence of intra-household pathogen transmission dynamics.

Measurements

In the parent study, our primary measure of impact (roll-call absence) was an objective measure of school absence. However, with the exception of STH infection, our secondary health impact measures (diarrhea, symptoms of respiratory infection, conjunctivitis) were based on self-report by pupils, which may be subject to bias. We included negative control questions about symptoms of illness unrelated to WASH access (cuts/scrapes and toothache) as a way to measure potential reporting bias and found no evidence to suggest that any bias may have existed to a greater degree among either the intervention or the comparison group. However, these questions were only included at the last survey visit; in the future, these questions should be included from the outset of data collection.

Additionally, based on our observations during fieldwork, animal feces were pervasive within school compounds. We did not include any observations or questions related to animals or animal feces exposures, which was a missed opportunity to explore a pathogen transmission pathway that has not previously been included in WinS studies. We also did not include any environmental sampling or other measures of pathogen exposure (*e.g.* water quality testing or hand-rinses), which have been used in previous WinS studies [21, 22]. Although these data would have given us additional evidence to evaluate the ability of the WinS intervention to reduce pathogen exposure, a key causal pathway in the WinS theory of change, there were not sufficient resources (*i.e.* laboratory access or personnel) to feasibly carry out these measurements in the field.

In the sub-study, our measures of household improved/unimproved WASH access, as defined by the WHO/UNICEF Joint Monitoring Programme [23], may not be valid proxies of the conditions that result in enteric pathogens in the environment, and they may not sufficiently account for other possible exposure routes such as flies, food contamination, or contamination of stored drinking water. Also, we were unable to measure direct exposure to animal feces so we relied on animal ownership as a proxy, as has been done in the majority of previous studies on animal feces exposure [10, 11]. Collecting exposure data for flies, food, stored drinking water, or animal feces would have required much more extensive field resources and would have been logistically- and cost-prohibitive for this particular study. However, conducting structured observations for presence/quantification of flies, risk factors for food/water contamination, and presence/quantification of animal feces within household compound could have served as better proxies.

Perhaps the biggest limitation from the sub-study was that we could not link our measure of reported diarrhea to the qPCR pathogen data. Survey enumerators collected data on symptoms of reported diarrhea among all participants during the household survey. We also measured reported diarrhea on the day of data collection, which was recorded by the Ministry of Health/Ministry of Education and Sports collaborators that assisted with stool collection. In the household survey, reported diarrhea in the previous week was 8.8% across the household triad, but reported diarrhea on the day of stool collection was 0.5%. We believe there was a systematic bias for the diarrhea question when the samples were collected, likely having do to with Ministry officials collecting the data. However, because the household survey data were collected between one to seven days prior to stool collection, we could not reliably use the household survey reported diarrhea data. Due to this limitation, we cannot distinguish between symptomatic and asymptomatic enteric infections, pathogen shedding due to recent exposure, or pathogen carriage due to gut colonization. Although the detection of pathogens in stool indicates a person's exposure to the pathogen as well as an exposure risk to others, reliable symptomology data would have been valuable.

Fidelity and adherence to the WinS Intervention

Although we were able to collect data on fidelity to the hardware component of the WinS intervention per the stated design (*e.g.* toilets, handwashing facilities, water supply), as well as adherence to stated target behaviors (*e.g.* handwashing with soap), there are several gaps in our measurement of fidelity and adherence. Neither the protocol for capacity building nor data on the number of visits/intensity for the behavior change component were provided to us by the implementing organization (despite repeated requests) so we cannot assess intensity or dose related to the intervention components, nor are we in the position to evaluate the quality of implementation delivery. The lack of a discrete intervention protocol stymied our ability to carry out a rigorous

process evaluation, which would have provided a more accurate measure of intervention fidelity and adherence, and helped us to better understand why the intervention failed to achieve intended impacts.

Quality control of laboratory samples

We suspected contamination by one or more target pathogens of 66 samples in the field and 78 samples in the laboratory. These samples were excluded from taxa- and pathogen-specific analyses, which could have limited study power. The presence of contamination in the field highlights one of the many challenges of collecting biological data in low-resource settings. Practicing aseptic technique and ensuring sterility of samples and collection materials was challenging, particularly when the field laboratory was usually a simple table set up under an open-air roof, often without walls to block wind and dust. It was a challenge to ensure I was prepared with enough supplies for fieldwork (*i.e.* having enough gloves, ethanol, and other consumables) while also being realistic about having to transport all my supplies to each field site. Nonetheless, in the future I would consider additional resources to protect my field laboratory space from the elements and improve the sterility of my makeshift bench space. The presence of contamination in the lab was unfortunate and should have been preventable, but highlights the challenge and delicacy of DNA/RNA extraction with complete aseptic technique, even in highly controlled laboratory settings. With more time and resources, I would have done more extensive training and practice with DNA/RNA extraction prior to handling study samples. Conducting TAC analysis in tandem with DNA/RNA extraction could have also identified contamination risks earlier.

Policy and program recommendations

Since 2010, access to WASH has been a fundamental human right recognized by the United Nations General Assembly [24]. As such, regardless of its potential health impacts, access to WASH is an important development objective. The inclusions of WinS indicators in the Sustainable Development Goals (SDGs) in 2015 [25] may focus attention on improving WASH access for school-aged children, something that has been somewhat lacking in previous decades. However, if improvements in health indicators *are* to be achieved, the way in which WASH interventions are designed and delivered should be reconsidered.

First, previous evidence has established that comprehensive WASH programs do not provide additive benefits over single interventions for health outcomes [2, 26-28] or environmental fecal contamination [29]. Therefore, results from this dissertation suggest that focusing resources to achieve high uptake of a single, community-wide intervention targeted at the dominant transmission pathways of specific pathogens may be more effective than a comprehensive intervention delivered to a smaller population at a household level. Second, even if the SDGs for universal access to safe water, coverage of adequate sanitation and end to open defecation, and handwashing with soap are met [25], exposure to *human* feces may be eliminated, but enteric disease transmission will persist because exposure to *animal* feces will remain [11]. Therefore, the substantial, but often neglected, risk of animal feces should be taken into consideration in the design of WASH interventions. Third, global control strategies for neglected tropical diseases (NTDs) such as STH, schistosomiasis, and trachoma recognize the importance of improved WASH in disease control and elimination [30]. Though efforts to coordinate WASH improvements with PC have gained traction recently [31], most NTD control programs and grant funding focus principally on large-scale PC administration [32, 33]. Results from this dissertation support what

has been previously established in the WASH/NTD evidence base, but rarely put into practice: PC may be a necessary component to clear current infections, but alone it is not sufficient to prevent re-infection. WASH improvements, and in particular sanitation coverage, are essential for the sustained control and elimination of STH and other NTDs [14, 34]. To achieve this, better integration and collaboration between WASH and NTD sectors is required [15]. Last, WinS programs need to further develop and test theories of change, and conduct rigorous process evaluations to understand where along the causal pathways interventions are falling short. Additionally, WinS programs should better understand and adapt to contextual drivers of key impacts and outcomes (such as absence and diarrhea). Complementary approaches for WinS that address the external factors along the WinS theory of change– such as school feeding, conditional cash transfers to alleviate financial and labor demands, and/or increasing community WASH coverage– may be necessary to achieve intended impacts.

Recommendations for future research

This dissertation offers insights into the drivers of enteric disease in Saravane Province, Lao PDR — a poor, rural area of the country with very low WASH coverage — and includes the identification of the dominant pathogenic causes and environmental and biological drivers of enteric infection. This research can inform the development and targeting of future WASH interventions in similar low-resource contexts in order to improve their effectiveness and, ultimately, reduce the burden of enteric disease.

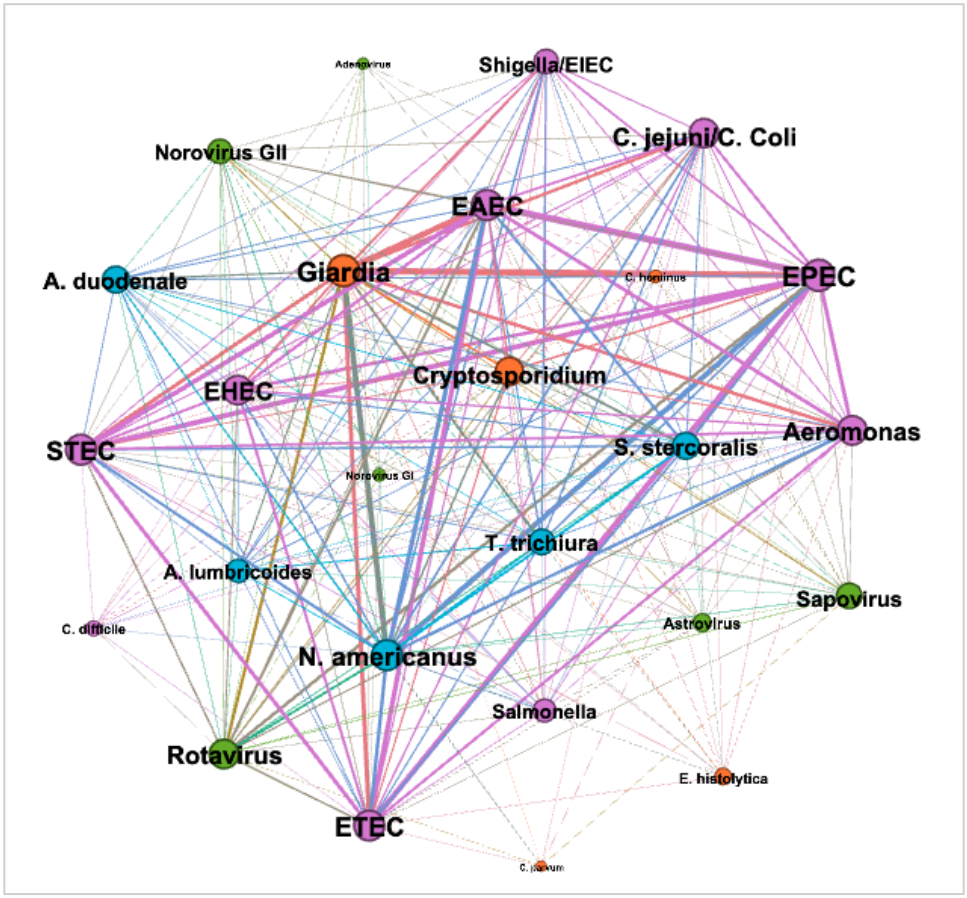
We observed heterogenous associations between WASH access and enteropathogens infection. Recent evidence has demonstrated a substantial risk of enteric infection from the public domain by quantifying a diversity of enteropathogens in soil, surface water, and drains [35, 36]. Future

research could expand the approach taken here to better elucidate where along the F-diagram specific pathogen contamination is most likely to occur [37]. In particular, integrating environmental sampling in both public and private domains, along with microbial source tracking for animal feces exposure may better inform current gaps in the evidence base.

Additionally, this study provided evidence that community-level WASH coverage was associated with lower odds of infection for many enteropathogens. There is some evidence that sanitation can provide community-level, or herd protection on health outcomes such as diarrhea, trachoma, nutritional status, and infant mortality [38-46], but is lacking for STH. Future research could explore community WASH thresholds for STH as well expand the evidence base for the association between community WASH coverage and the primary etiologies of diarrheal disease.

We described patterns of helminth-microparasite co-infections in which we observed that STH infections were associated with higher odds of bacterial and protozoal infections and lower odds of viral infections. Helminth-microparasite co-infections are just one of many possible mixed infections that may be of interest to the WASH sector. **Figure C.1** depicts a network map of co-infections from this study, and shows that that co-infections were frequent both across and within taxa. Evidence of the pathogenic consequences of these mixed infections is limited [47], but as multiplex tools such as Luminex and TAC are becoming more widely utilized, the potential for a better understanding of the prevalence, patterns, and implications of enteric pathogen co-infection is enhanced. Additional research on enteric pathogen co-infection and their interactions is warranted.

Figure C.1. Network map of pathogen co-infection among children <5, school-aged children, and adults in Saravane Province, Lao PDR, 2017



Legend: purple node=bacteria, green node=virus, orange node=protozoa, blue node=STH

Finally, ours is the third large-scale, rigorously conducted, comprehensive WinS trial to show limited or mixed impacts of a WinS intervention on pupil education and health. To build on this work, additional studies could examine the role of external factors in the WinS theory of change. Evaluating how community WASH access and/or concurrent programming (*e.g.* school feeding or conditional cash transfers) complement WinS interventions would provide important evidence for the development sector as they work to help countries achieve the SDGs.

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Appendices of articles published during PhD

Included in the Appendices are three manuscripts, written and published during my tenure as a PhD Student and Candidate. These papers further explore the role of WASH in Schools as a modality to improve child health, and are included here to show the breadth of work conducted during my PhD).

Appendix 1. Design, Intervention Fidelity, and Behavioral Outcomes of a School-Based Water, Sanitation, and Hygiene Cluster-Randomized Trial in Laos[4]

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Article

Design, Intervention Fidelity, and Behavioral Outcomes of a School-Based Water, Sanitation, and Hygiene Cluster-Randomized Trial in Laos

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Abstract: Evidence of the impact of water, sanitation, and hygiene (WASH) in schools (WinS) interventions on pupil absence and health is mixed. Few WinS evaluations rigorously report on output and outcome measures that allow for comparisons of effectiveness between interventions to be made, or for an understanding of why programs succeed. The Water, Sanitation, and Hygiene for Health and Education in Laotian Primary Schools (WASH HELPS) study was a randomized controlled trial designed to measure the impact of the United Nations Children's Fund (UNICEF) Laos WinS project on child health and education. We also measured the sustainability of intervention outputs and outcomes, and analyzed the effectiveness of group hygiene activities on behavior change and habit formation. Here, we present the design and intermediate results from this study. We found the WinS project improved the WASH environment in intervention schools; 87.8% of schools received the intervention per design. School-level adherence to outputs was lower; on average, schools met 61.4% of adherence-related criteria. The WinS project produced positive changes in pupils' school WASH behaviors, specifically increasing toilet use and daily group handwashing. Daily group hygiene activities are effective strategies to improve school WASH behaviors, but a complementary strategy needs to be concurrently promoted for effective and sustained individual handwashing practice at critical times.

Keywords: water; sanitation; hygiene; WASH; primary schools; handwashing; toilet use; behavior change; intervention fidelity

1. Introduction

Access to water, sanitation, and hygiene (WASH) facilities and behavior change education in schools are critical for a strong learning environment, and contribute to inclusion, dignity, and equity [1]. WASH in schools (WinS) programs also support feeding programs and preventive chemotherapy to reduce reinfection with soil-transmitted helminths and trachoma [2]. As such, WinS programs are increasingly incorporated in political and development agendas as a modality to improve children's health and boost educational attendance and achievement [3–5]. However, evaluations assessing the health and educational impacts of WinS have found mixed results. In Kenya, a hygiene and sanitation intervention reduced absences for girls by 58%, but not for boys [6], and had an impact on some soil-transmitted helminths [7], but not on diarrhea [8]. The arm that included water found reductions in diarrhea among both school children and their younger siblings [8,9] as well as increased enrollment and gender parity [10]. A matched-control trial of a comprehensive WinS intervention in Mali found no impact on reduced absence, but did show a reduction in self-reported diarrhea and respiratory infection [11]. In China, a comprehensive hygiene campaign where soap and peer monitoring was

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Abstract

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There are many potential reasons for these mixed results, including environmental conditions, disease transmission dynamics, and background coverage rates [13]. WinS improvements may simply be insufficient to overcome other drivers of absence and illness, though intervention effectiveness inherently also plays a crucial role, as has been noted in several recent large-scale WASH studies [14]. Many impact evaluations report on an intervention as it would have been

delivered at scale (i.e., an effectiveness study), yet few report on rigorous output and outcome measures that allow for comparison of effectiveness between interventions [15], or to understand why programs succeeded and in what context. In the Kenya and Mali WinS studies discussed above, intervention schools with higher intervention fidelity had better outcomes [16, 17].

Poor sanitation and hygiene in Lao People's Democratic Republic (Lao PDR) account for three million disease episodes and 6000 premature deaths each year [18]. In 2015, 80% percent of Laotians had access to an improved water source, while 73% of the total population had access to improved sanitation, with estimates lower in rural areas (73% and 60%, respectively) [19]. Water and sanitation access in primary schools is even worse, with functioning water and sanitation facilities available in between 29.4% and 38.9% of centers [20, 21]. In the 2005 National Education Sector Development Plan, the Government of Lao PDR (GoL) set a target for improved water and sanitation access in 50% of schools by 2015. In 2013, the United Nations Children's Fund (UNICEF) and the GoL began the Laos Basic Education, Water, Sanitation and Hygiene Programme, a four-year WinS improvement project in Lao PDR. The objective of the program was to increase school attendance through the delivery of WASH facilities to 492 schools in 13 provinces across the country, promote health and hygiene behaviors in 100 primary schools in Saravane Province, and provide improved and sustainable water access to more than 80 school-hosting villages (villages whose school received WinS programming).

The Water, Sanitation, and Hygiene for Health and Education in Laotian Primary Schools (WASH HELPS) study employed a cluster-randomized control trial with longitudinal data collection to quantify the impact of UNICEF's WinS project on pupil learning and health in Lao PDR. Here we assessed the project's intervention fidelity—defined as how the intervention was delivered per the

stated objectives, as well as downstream school-level adherence to the intervention by teachers and students [22, 23]. This paper also serves to describe the study design for our forthcoming paper assessing impact of the intervention.

Materials and Methods

Intervention

The intervention included both infrastructure (hardware) and behavior change (software) components. The hardware consisted of: (1) provision of a school water supply (borehole, protected dug well with pump, or gravity-fed system) and a water tank with connections to supply the toilet block and handwashing facilities; (2) school sanitation facilities, consisting of three toilet compartments designated for boys, girls, and disabled students; and (3) handwashing facilities, consisting of two sinks with taps connected to the water supply. The software component, called Hygiene Action led by Pupils in Schools (HAPiS), was implemented after the installation of the hardware components and consisted of: (1) clean drinking water, where each classroom received a ceramic water filter that was maintained and filled with water by teachers; (2) group handwashing with soap at critical times, in which schools were provided with three group handwashing tables and children were instructed to wash their hands with soap twice per day, guided by teachers in charge of hygiene activities; (3) toilet cleanliness, where pre-organized teams of students (boys and girls) performed light routine cleaning and maintenance of toilets; and (4) school compound maintenance, where teams of boys and girls cleaned the school compound, and garbage bins were used for light collection of waste. The approximate materials and labor cost of hardware installation (water supply, sanitation facilities, and handwashing facilities) per school, as estimated by UNICEF, was US $11,500 for schools that received a borehole or protected well with pump

and US $16,000 for schools that received a gravity fed system; the approximate cost of software implementation was US $1,500. These were paid for by UNICEF and do not include UNICEF staff costs.

Study Design

Though the parent UNICEF project was active in several provinces, this impact evaluation focused on Saravane, a province in the southern part of the country. Saravane was the only province where intervention activities had not yet occurred prior to the design of the study, which allowed for development of an experimental design. We employed a cluster randomized controlled trial (RCT) among 100 randomly selected schools (50 intervention, 50 comparison).

Due to the size and scope of the intervention, it was delivered in two phases. Group 1 schools received the intervention during the 2014–2015 school year, and included schools in the Ta Oy, Toumlane, Vapy, Lao Ngam, and Samoui Districts. Group 2 schools received the intervention during the 2015–2016 school year, and included schools in the Saravane, Lakhonepheng, and Khongsedone Districts. We collected data throughout the school year to account for temporal and seasonal variability (specifically, absenteeism, diarrhea, and respiratory illness). Data were collected over two (Group 2 schools) to three (Group 1 schools) years to track uptake and sustainability of facilities and behavior change. None of the school hosting villages participating in the impact evaluation received community-level WASH interventions or programming from UNICEF as part of the larger WinS project.

School Selection

Schools were randomly selected from a list of 222 eligible schools provided by UNICEF Lao PDR. Schools were eligible for inclusion if they met the following criteria: (1) they were located in Saravane Province; (2) were public primary schools; (3) not community-based construction schools; and (4) were lacking functional WASH facilities. Using a random number generator in Excel (Microsoft Corporation, Redmond, WA, USA), 100 schools were selected from this list for inclusion in the evaluation. The number of schools selected in each district was proportional to the number of eligible schools in each district. Following selection, schools were randomly assigned by the research manager to either the intervention group (50 schools) or the comparison group (50 schools) using a random number generator in Excel, and using stratified random sampling to ensure equal representation of control and intervention schools in each district. Given the need to plan for the intervention, we randomized the schools prior to baseline. Enumerators were blinded to this allocation at baseline.

Participant Selection

Within each school, a sample of 40 students from grades 3–5 were randomly selected from class registers by study enumerators using systematic stratified sampling to select equally among boy and girl pupils and among classes; however this was not always possible due to unequal enrollment in some schools. We interviewed students in grades 3–5 based on the ability of children at this grade level to reliably answer survey questions. This cohort of pupils was followed throughout the evaluation period. If a pupil in the cohort left the school during the evaluation period due to abandonment or transfer, that pupil was replaced the following academic year by another randomly selected pupil, maintaining equal pupil sex and class ratios as much as possible. Pupils in the fifth

grade who advanced to secondary school at the end of each academic year were replaced by pupils in the third grade at the start of the following academic year. Some schools had fewer than 40 pupils in grades 3–5, in which case all students in grades 3–5 were included.

Power Calculation

Given a paucity of data on school absence in Lao PDR, we were not able to determine an estimate of the daily absence (primary outcome) within Lao PDR. As such, we utilized data from our evaluation of a school-based WASH program in Mali to estimate the necessary sample size [11]. We calculated the sample size of 40 pupils/school using Monte Carlo simulations of roll-call data, assuming 250 pupils per school, a daily absence rate of 5.6%, a within-school intra-class correlation (ICC, a measure of variability within versus between schools/pupils) of 0.09 and within pupil ICC of 0.36, and seven rounds of data collection.

Following collection of baseline data, we conducted a power analysis to calculate the minimum effect we were able to detect in absences (roll-call) and diarrhea (self-reported) among the study population using data from our true study population as opposed to the previous work in Mali. With 80% power, we will be able to detect a 1.9 percentage point (or 15%) change in absence and a 2.3 percentage point (or 21% change) in diarrhea. The power analysis was based on the estimated sample size for the entire study, projected from the sample size from Group 1 (4633 pupils for the roll-call/1323 pupils for the interview, 54 schools); baseline levels of absenteeism and diarrhea (12.4% and 10.8%, respectively); ICC for absenteeism (within-school ICC: 0.25, within-pupil ICC: 0.41) and diarrhea (within-school ICC: 0.17, within-pupil ICC: 0.36); and the projected number of rounds of data collection (including baseline) for each school (eight rounds for Group 1 schools and four rounds for Group 2 schools).

Ethics

The study was approved by Emory University's Institutional Review Board (IRB0076404) and the Lao Ministry of Health's National Institute of Public Health National Ethics Committee (No. 043 NIOPH/NECHR). Both Institutional Review Boards approved consent *in loco parentis* (in the place of the parent) signed by the school director. Pupils who were selected for the evaluation provided informed verbal assent. The evaluation is registered at clinicaltrials.gov (NCT02342860). The intervention was delivered to control schools in April 2017, after research activities ended.

Data Collection

Data were collected by a team of experienced enumerators who underwent rigorous training on research ethics, minimization of bias, and study tools and protocols. All data were collected using the Open Data Kit application [24] on Android-enabled mobile devices, except for the roll-call absence data, which were recorded on paper-based ledgers.

The evaluation was designed such that construction in intervention schools would occur after baseline data collection, which took place at the beginning of the school year in September/October 2014 (Group 1 schools) and September/October 2015 (Group 2 schools). Construction was expected to take approximately 8–10 weeks, with completion deadlines at the end of December (2014 for Group 1 schools and 2015 for Group 2 schools). Longitudinal surveillance of outputs, outcomes, and impacts began in February 2015 (Group 1) and 2016 (Group 2), following school exams and the January school holidays. However, given delays in construction in some schools and districts, construction was not complete in all schools by the second data collection visit, as depicted in the timeline in Figure A1.1.

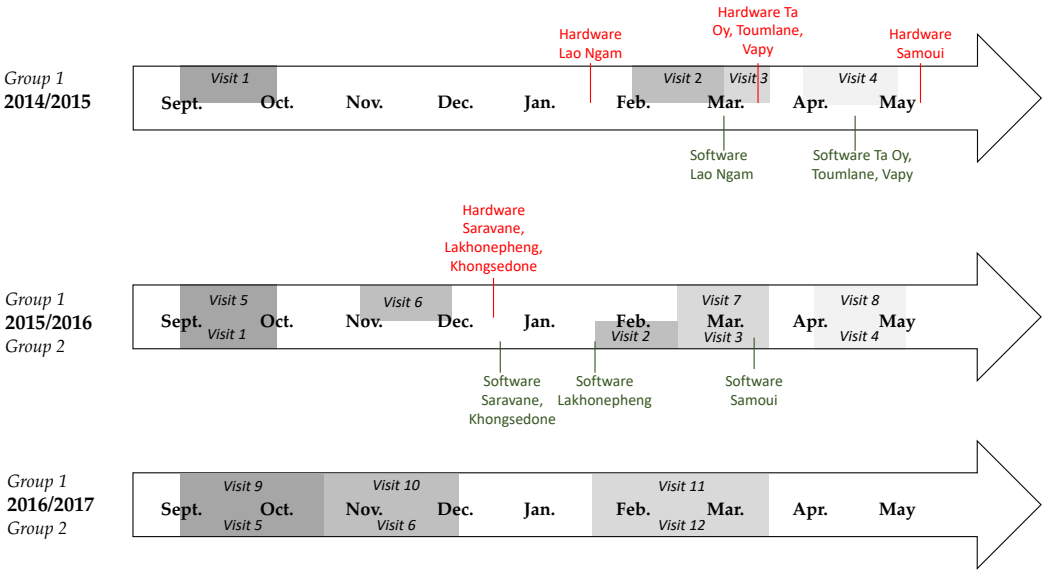


Figure A1.1 Project delivery and data collection visit timeline.

Enumerators visited study schools every 6–8 weeks during the school year (September–May) through March 2017, for a total of 11 (Group 1) or 7 (Group 2) visits per school. On average, data were collected for 8 visits (2 years) following hardware completion in Group 1 schools, and 5 visits (1.25 years) following hardware completion in Group 2 schools. All visits were unannounced. At each visit, enumerators interviewed the school directors; interviewed up to 40 pupils in grades 3–5; observed conditions and functionality of WinS hardware; observed individual and group handwashing practices; and conducted a roll call of all students enrolled in the school.

All outputs, outcomes, and impacts, as well as their indicators and evaluation criteria were jointly developed between Emory University and UNICEF (the implementing partner) prior to the start of the study. Many, but not all, of these indicators align with the World Health Organization's water, sanitation, and hygiene standards for schools in low-cost settings [25]. For example, the toilets were sex-separated and accessible to disabled students, but given the standard design and delivery of the toilet block and the small enrollment size of schools, we did not consider the pupil-

to-latrine ratio. We measured accessibility and reliability of water points, but given that an on-site, improved water source was provided by the intervention, we did not measure water quantity. Additionally, we did not monitor water quality, which was conducted by the local water authority. We did not measure vector control or food storage/preparation, as these were beyond the scope of the intervention.

Baseline Measures

Baseline levels of enrollment, gender parity, school WASH access (presence of a toilet, water point in school compound, presence of handwashing facilities), school wealth, pupil demographics (age, household wealth, household presence of a toilet, use of an improved water source, and presence of a handwashing facility equipped with soap and water), and primary and secondary impacts were evaluated to ensure there were no significant differences across intervention and comparison groups and that the randomization process was successful.

Gender parity was calculated by dividing the number of boys enrolled by the number of girls enrolled in each school. School wealth was determined by the amount of money received through the School Block Grant, which is the operational budget given schools each year and is dependent on the number of pupils enrolled. Household wealth was determined through a series of questions about household construction materials (roof, floor, and walls), ownership of a mobile phone, and presence of electricity. These variables were chosen based on those used in the Demographic and Health Surveys for measures of wealth in Laos (Ministry of Health and Lao Statistics Bureau 2012). We used principal component analysis methods to derive one single wealth metric from all of the wealth assets combined [26].

Presence and Functionality of WinS Outputs

We collected data to measure the presence and functionality of the WinS project hardware and software outputs (Table A1.1). The WinS indicators and criteria defined for each output for the purpose of this evaluation go beyond the presence of infrastructure, as often defined in WASH and in evaluations, and encompass functionality and condition of the infrastructure over time, as well as adequate use (water tanks and filters must be filled; individual and group handwashing stations must be accompanied with water and soap; toilets must be kept unlocked, clean and with water available for flushing). This data was also used to assess intervention fidelity, which was defined as how well the intervention was delivered and adhered to as intended [22, 23].

Table A1.1. Water, sanitation, and hygiene (WASH) in schools (WinS) project outputs and indicators**.**

Output	Indicator and Criteria
Hardware	
Water Supply	Improved[1] water point on school compound [2] Water point functional in the previous year (director reported) [3] Water tank to supply toilet and handwashing stations [2] Water observed in tank [3]
Toilets	At least one improved[1] toilet compartment [2] Toilet is sex separated (by designation) [3] Toilet is unlocked [3] Toilet is clean [3] Toilet has water available inside compartment for flushing [3]
Handwashing facilities	At least one individual handwashing station available to pupils [2] Water available at individual handwashing station [3] Soap available at individual handwashing station [3]
Software	
Promotion of daily group hygiene activities	Daily group handwashing schedule posted in at least one classroom or near toilet [3] Daily group compound cleaning schedule posted in at least one classroom or near toilet [3] Daily group toilet cleaning schedule posted in at least one classroom or near toilet [3]

Group handwashing	Group handwashing facility available to pupils [2] Water available at group handwashing facility [3] Soap available at group handwashing facility [3]
Water filters	At least one drinking water filter available in a classroom for pupil use [1] Water in filter [2]

[1] Defined according to Joint Monitoring Programme (JMP) standards. [2] Classified as quality of project delivery. [3] Classified as school-level adherence.

To measure intervention fidelity, an index score was created where one point was given for each of the 20 output criteria fulfilled. As such, for each visit, the maximum score for intervention fidelity was 20, whereas the minimum score was 0.

Pupil Behavioral Outcomes

We monitored five outcomes related to pupil WASH behavior change and habit formation among students: toilet use, individual handwashing, daily group handwashing, daily group toilet cleaning, daily group compound cleaning. These outcomes and their indicators are described in Table A1.2.

Table A1.2. WinS behavioral outcomes and indicators.

Outcome	**Indicator**
Toilet use	Percentage of students using toilet for defecation during school hours (pupil-reported)
Handwashing (individual)	Percentage of students washing hands with soap and water upon exiting toilet (observation)
Daily group handwashing	School conducted daily group handwashing the day of visit (observation)
Daily group toilet cleaning	Percentage of students participating in daily group toilet cleaning within the previous five school days (pupil-reported)
Daily group compound cleaning	Percentage of students participating in daily group compound cleaning within the previous five school days (pupil-reported)

Health and Educational Impacts

The primary impact of interest was school absence, measured through roll-call collected by study enumerators (rather than relying on school records). Secondary impacts included pupil-reported

absence, pupil-reported diarrheal incidence, pupil-reported symptoms of respiratory infection, pupil-reported absence due to illness, and soil-transmitted helminth infection. Both intention to treat and as-treated impact results from this trial will be reported in a forthcoming paper.

Statistical Analysis

Data were analyzed using STATA Statistical Software: Release 13 (StataCorp, College Station, TX, USA). To test for equality among intervention and comparison groups at baseline, school-level indicators were evaluated using linear (enrollment, gender parity, wealth) and logistic (school WASH access) regression. Pupil-level indicators were evaluated using linear (age, household wealth) and logistic (roll-call absence, household WASH access, reported absence, reported diarrhea, reported symptoms of respiratory infection, soil transmitted helminth infection) regression with random intercepts at the school level to account for clustering.

To measure if achievement of output and outcome indicators significantly changed among intervention schools across the evaluation period, we used logistic (binary outcomes) and linear (continuous outcomes) regression models, with random intercepts at the pupil and school levels to adjust for repeated (longitudinal) measurements, and linear splines at 7 months, 13 months, and 19 months. Programmatic adjustments were made for Group 2 schools based on lessons learned from Group 1, which led to different levels of achievement at output and outcome levels. As such, we stratify output and outcome results by implementation group. All associations were evaluated for significance at $p < 0.05$.

Results

Baseline

There were neither substantial nor statistically significant differences in key school- or pupil-level indicators between intervention and comparison groups at baseline, indicating that the groups were balanced after randomization allocation (Tables S1 and S2).

Presence and Functionality of WinS Outputs

Achievement of the six project outputs across the evaluation period by intervention status and implementation group is depicted in Figure A1.2. Intervention schools were more likely to meet each of the indicators and evaluation criteria for the six project outputs (as described in Table A1.1) than were comparison schools. Generally, Group 2 intervention schools met project outputs more often than Group 1 intervention schools.

Intervention schools' achievement of the six project outputs and their evaluation criteria throughout the evaluation period are described in Table A1.3. The odds of achieving project outputs and their evaluation criteria either increased or did not significantly change throughout the first six months of hardware/software implementation, with the exception of the hygiene promotion output and related criteria, the odds of which reduced throughout the first six months of software implementation in Group 1 schools. Among Group 1 schools, the odds of achieving project outputs and their criteria either continued to increase beyond six months of project implementation, or did not significantly change, indicating improved or sustained achievement, respectively. The group handwashing facility was the only criteria where odds of achievement decreased, which occurred 13–18 months after project implementation. Among Group 2 schools, achievement of most outputs and their evaluation criteria did not significantly change beyond six

months. However odds of achieving some outputs/criteria increased (7–12 months: water supply output, water in tank; 13–18 months: water point did not malfunction) while others decreased (7-12 months: water point did not malfunction, water tank present, sex-separated toilets, drinking water output and associated criteria).

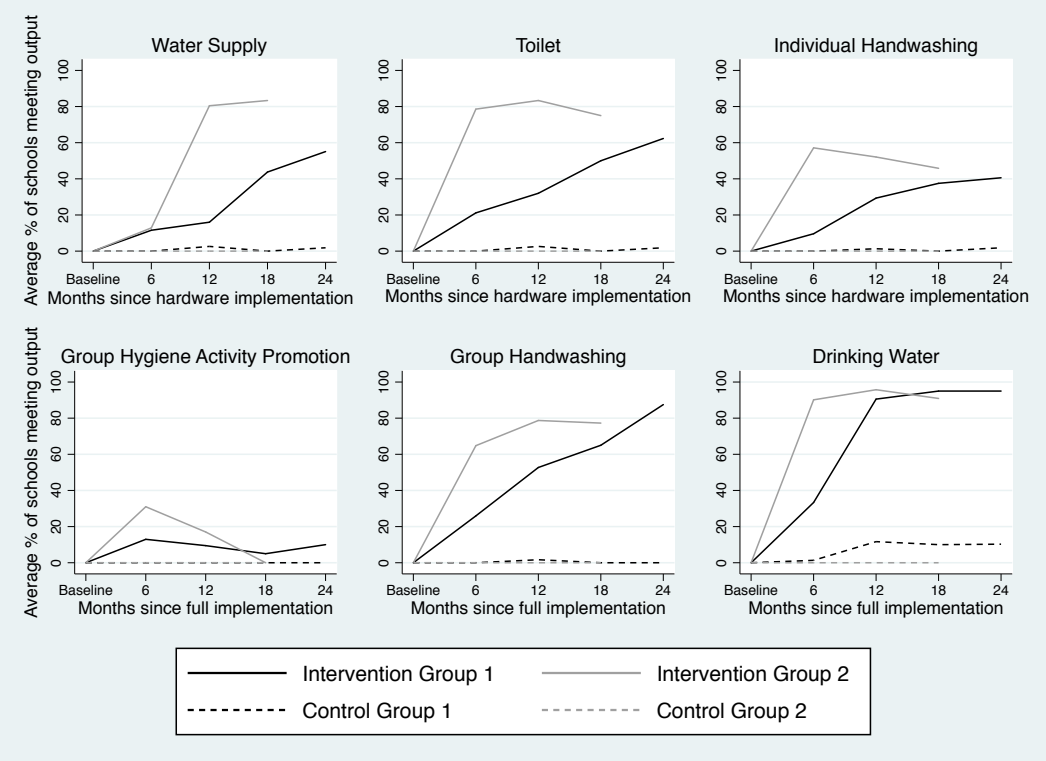


Figure A1.2. Achievement of project outputs by time since project implementation, stratified by intervention and implementation groups.

Of the hardware-related outputs, intervention schools were most likely to meet the toilet output (56.1% of visits after hardware implementation), followed by the handwashing output (38.6%), and the water supply output (36.4%). Of the software-related outputs, intervention schools were most likely to achieve the drinking water output (82%), followed by the group handwashing output

(61%). Intervention schools were least likely to meet the promotion of group hygiene activities output (15%).

Table A1.3. Per month change in odds of intervention schools achieving project output or evaluation criteria by time since project implementation.

	Group 1 Intervention Schools (n = 26)				Group 2 Intervention Schools (n = 24)		
Output or Evaluation Criteria	**1–6 Months**	**7–12 Months**	**13–18 Months**	**19+ Months**	**1–6 Months**	**7–12 Months**	**13–18 Months**
Water supply output [1]	1.1 (0.8, 1.4)	0.9 (0.7, 1.2)	***1.6* (*1.2*, *2.1*)**	1.0 (0.8, 1.4)	1.3 (0.8, 2.0)	***2.6* (*1.4*, *4.8*)**	0.3 (0.0, 1.9)
Water point located on school grounds	***1.8* (*1.2*, *2.8*)**	1.2 (0.6, 2.2)	2.3 (0.9, 6.4)	0.5 (0.1, 1.7)	--	--	--
Did not malfunction in previous year	***1.4* (*1.1*, *1.6*)**	1.2 (0.9, 1.6)	0.8 (0.6, 1.1)	1.1 (0.7, 1.5)	***3.1* (*1.9*, *5.0*)**	***0.2* (*0.1*, *0.5*)**	***7.1* (*1.1*, *47*)**
Water tank	***4.4* (*1.3*, *15*)**	0.4 (0.1, 1.4)	1.7 (0.6, 4.7)	0.8 (0.2, 3.1)	***8.8* (*1.7*, *46*)**	***0.2* (*0.0*, *0.9*)**	--
Water in tank	1.3 (1.0, 1.6)	0.8 (0.6, 1.1)	***2.6* (*1.7*, *3.9*)**	0.9 (0.6, 1.5)	0.8 (0.5, 1.3)	***7.8* (*3.5*, *18*)**	--
Toilet output [1]	1.0 (0.8, 1.3)	***1.4* (*1.1*, *1.9*)**	1.2 (0.9, 1.6)	1.1 (0.7, 1.5)	***1.6* (*1.2*, *2.0*)**	0.7 (0.5, 1.1)	1.1 (0.3, 4.7)
At least one improved toilet compartment	***4.4* (*1.9*, *10*)**	--	--	--	--	--	--
Sex separated	***1.4* (*1.1*, *1.8*)**	***1.5* (*1.0*, *2.1*)**	1.4 (0.8, 2.2)	0.9 (0.5, 1.9)	***7.9* (*1.7*, *37*)**	***0.1* (*0.0*, *0.9*)**	--
Unlocked	***1.3* (*1.0*, *1.6*)**	1.2 (0.9, 1.6)	***1.8* (*1.1*, *3.1*)**	0.8 (0.4, 1.6)	***1.8* (*1.4*, *2.5*)**	0.7 (0.4, 1.2)	1.7 (0.2, 13)
Clean	1.2 (1.0, 1.4)	***1.4* (*1.0*, *1.8*)**	1.3 (0.9, 1.8)	0.9 (0.6, 1.5)	***1.7* (*1.3*, *2.3*)**	0.7 (0.4, 1.2)	2.0 (0.3, 14)
Water available inside for flushing	***1.2* (*1.0*, *1.5*)**	1.0 (0.8, 1.3)	***1.3* (*1.0*, *1.7*)**	1.1 (0.8, 1.7)	***1.6* (*1.2*, *2.0*)**	0.7 (0.5, 1.1)	1.1 (0.3, 4.7)
Handwashing output [1]	***1.4* (*1.1*, *1.8*)**	1.2 (0.9, 1.7)	1.0 (0.8, 1.4)	1.0 (0.7, 1.5)	***1.3* (*1.0*, *1.7*)**	0.7 (0.5, 1.1)	1.7 (0.5, 5.8)
At least one individual handwashing station	***4.8* (*1.5*, *16*)**	0.5 (0.2, 1.5)	1.3 (0.5, 3.6)	0.9 (0.2, 3.9)	***3.4* (*1.6*, *7.5*)**	0.4 (0.1, 1.2)	--
Water	***1.4* (*1.1*, *1.8*)**	1.0 (0.8, 1.4)	***1.4* (*1.0*, *1.9*)**	1.2 (0.7, 1.8)	***1.7* (*1.3*, *2.3*)**	0.7 (0.4, 1.0)	1.2 (0.3, 5.3)
Soap	1.3 (1.0, 1.7)	1.2 (0.9, 1.7)	1.0 (0.8, 1.3)	1.0 (0.7, 1.5)	***1.3* (*1.0*, *1.7*)**	0.7 (0.5, 1.1)	1.7 (0.5, 5.8)
Hygiene promotion output [2]	***0.5* (*0.3*, *0.8*)**	***2.2* (*1.2*, *4.0*)**	0.7 (0.5, 1.0)	1.0 (0.6, 1.7)	1.0 (0.7, 1.3)	0.8 (0.4, 1.6)	--
Group compound cleaning schedule	***0.9* (*0.8*, *1.0*)**	1.0 (0.8, 1.3)	1.1 (0.9, 1.3)	1.1 (0.8, 1.6)	1.0 (0.8, 1.2)	0.9 (0.6, 1.3)	3.4 (0.3, 39)
Group toilet cleaning schedule	***0.7* (*0.6*, *0.9*)**	***1.4* (*1.1*, *2.0*)**	0.9 (0.7, 1.2)	1.2 (0.8, 1.6)	1.0 (0.8, 1.3)	0.9 (0.6, 1.4)	0.1 (0.0, 2.9)
Group handwashing schedule	***0.7* (*0.6*, *1.0*)**	1.3 (0.9, 1.9)	0.9 (0.6, 1.2)	1.0 (0.6, 1.6)	0.9 (0.7, 1.1)	1.0 (0.6, 1.8)	--
Drinking water output [2]	***5.4* (*3.0*, *9.9*)**	1.2 (0.7, 2.0)	0.8 (0.5, 1.4)	1.1 (0.5, 2.4)	***3.1* (*1.9*, *5.1*)**	***0.3* (*0.1*, *0.6*)**	--
At least one drinking water filter	***8.2* (*4.8*, *14*)**	1.0 (0.5, 2.0)	--	--	***4.5* (*2.0*, *10*)**	***0.2* (*0.1*, *0.6*)**	--
Water in filter	***5.4* (*3.0*, *9.9*)**	1.2 (0.7, 2.0)	0.8 (0.5, 1.4)	1.1 (0.5, 2.4)	***3.1* (*1.9*, *5.1*)**	***0.3* (*0.1*, *0.6*)**	--
Group handwashing output [2]	1.0 (0.8, 1.2)	***1.6* (*1.2*, *2.1*)**	0.9 (0.7, 1.2)	1.3 (0.8, 2.0)	***1.2* (*1.0*, *1.5*)**	1.2 (0.8, 1.8)	0.6 (0.0, 9.9)
Group handwashing facility	1.6 (1.2, 2.1)	***1.9* (*1.1*, *3.0*)**	***0.8* (*0.5*, *1.5*)**	--	***1.9* (*1.3*, *2.6*)**	0.8 (0.4, 1.8)	--
Water	***1.3* (*1.1*, *1.6*)**	1.2 (0.9, 1.5)	1.4 (1.0, 2.1)	0.9 (0.5, 1.9)	***1.4* (*1.1*, *1.8*)**	1.0 (0.6, 1.5)	0.6 (0.0, 13)
Soap	1.0 (0.8, 1.2)	***1.7* (*1.3*, *2.2*)**	0.9 (0.7, 1.2)	1.3 (0.8, 2.0)	1.2 (1.0, 1.5)	1.1 (0.8, 1.7)	0.6 (0.0, 11)
Number of outputs met (range 0–20) [3]	***1.2* (*1.0*, *1.4*)**	0.1 (−0.1, 0.4)	0.2 (0.0, 0.5)	0.1 (−0.3, 0.5)	***3.5* (*3.1*, *4.0*)**	***−2.9* (*−3.9*, *−1.9*)**	***8.7* (*2.3*, *15*)**
School-level adherence outputs met (range 0–14) [3]	***0.7* (*0.6*, *0.9*)**	0.2 (0.0, 0.4)	0.2 (0.0, 0.4)	0.1 (−0.2, 0.4)	***1.4* (*1.0*, *1.8*)**	***−1.0* (*−1.6*, *−0.4*)**	2.3 (−1.5, 6.1)

Bold italicization indicates significant change in odds within time strata ($p < 0.05$). -- indicates data were too sparse to calculate odds. [1] Analyzed by time since hardware implementation. [2] Analyzed by time since full implementation. [3] β coefficients represent the per month change in the number of outputs met within time strata.

Our measure of intervention fidelity was based on achievement of the 20 criteria used to evaluate the 6 project outputs. On average, Group 1 intervention schools achieved 12.2 output criteria (95% Confidence Interval (CI) = 11.5, 12.9) after project implementation; the number of output criteria met increased by 1.2 criteria per month through the first six months following full implementation

(β = 1.2, 95% CI = 1.0, 1.4), and was sustained thereafter. On average, Group 2 schools achieved 15.4 output criteria (95% CI = 14.9, 16.0); the number of output criteria met increased by 3.5 criteria for the first six months following full implementation (β = 3.5, 95% CI = 3.1, 4.0), decreased by 2.9 criteria per month between 7–12 months following full implementation (β = −2.9, 95% CI = −3.9, −1.9), and increased again 13–18 months after implementation (β = 8.7, 95% CI = 2.3, 15). Intervention schools fulfilled all six WASH outputs and their associated indicators and criteria at 2.4% of visits after project implementation; fulfillment of all 20 criteria was higher among Group 2 schools (3.6%) than Group 1 schools (1.6%, $p < 0.01$).

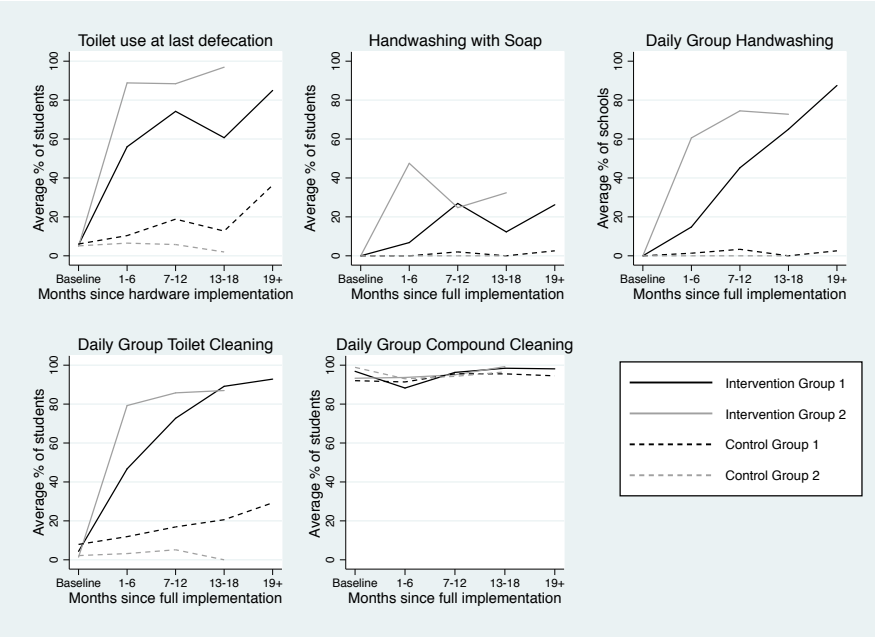
Quality of WinS project delivery was high; of all intervention schools (n = 50), 42 (87.8%) received the intervention infrastructure per design. Two (4%) did not receive a water point, three (6%) did not receive water tanks, three (6%) did not receive individual handwashing facilities, and three (6%) did not receive group handwashing facilities. School-level adherence to the outputs provided by the project (e.g., water and soap availability at handwashing facilities) was sub-optimal; of the 14 criteria related to school-level adherence, intervention schools met an average of 8.6 (Standard deviation (SD) = 3.5) criteria (61.4%) during visits following full project implementation. School-level adherence was higher among Group 2 intervention schools than Group 1 intervention schools (β: 2.3, 95% CI: 1.0, 3.7).

Pupil Behavioral Outcomes

Achievement of each of the five project outcomes by intervention status and implementation group across the evaluation period is depicted in Figure A1.3. After project implementation, group compound cleaning was the most commonly achieved behavioral outcome (94.8%), followed by

toilet use (75.5%), group toilet cleaning (68.3%), group handwashing (48.7%), and individual handwashing with soap after toilet use (23.9%).

Figure A1.3. Achievement of project outcomes by time since project implementation, stratified by intervention and implementation groups.



Trends in achievement of project outcomes among intervention schools are presented in Table A1.4 and described in detail below.

Toilet Use

At baseline, only 5.9% of pupils attending intervention schools reported using a toilet at last defecation during the school day. In both implementation groups, pupil-reported toilet use at last defecation during the school day increased in the first six months following hardware implementation. In Group 1, toilet use at last defecation increased 8.5% per month between baseline and 6 months after hardware implementation ($\beta = 8.5$, 95% CI = 6.8, 10) and did not

significantly change thereafter, indicating sustained behavior. In Group 2, toilet use at last defecation fluctuated across the evaluation period; it increased 20% per month from baseline to 6 months after hardware implementation (β = 20, 95% CI = 16, 24), decreased 18% per month (β = −18, 95% CI = −23, −12) from 7–12 months after hardware implementation, and increased again 34% per month (β = 34, 95% CI = 17, 50) from 13–18 months after hardware implementation.

In intervention schools, the percentage of pupils reporting toilet use at last defecation during the school day was higher among schools that met the toilet output criteria (β = 20.1, 95% CI = 14.0, 26.2). Having at least one unlocked toilet, at least one toilet with water available for flushing, and at least one clean toilet were all associated with increased prevalence of pupil-reported use of a toilet at last defecation during the school day. Having at least one gender-separated toilet compartment was not associated with reported use of a toilet at last defecation during the school day (Table S3).

Table A1.4. Per month change in achievement project outcomes among intervention schools by time since project implementation.

	Group 1 Schools (*n* = 26)				Group 2 Schools (*n* = 24)		
	1–6 Months	**7–12 Months**	**13–18 Months**	**19+ Months**	**1–6 Months**	**7–12 Months**	**13–18 Months**
Percentage of students reporting toilet use at last defecation during school day [1]	***8.5* (*6.8*, *10*)**	0.1 (−2.2, 2.5)	0.3 (−1.8, 2.4)	1.8 (−1.1, 4.6)	***20* (*16*, *24*)**	***−18* (*−23*, *−12*)**	***34* (*17*, *50*)**
Percentage of students observed handwashing with soap [2]	1.1 (−0.1, 2.3)	0.0 (−2.5, 2.5)	−1.5 (−3.7, 0.7)	***−3.7* (*−7.2*, *−0.2*)**	***4.5* (*1.9*, *7.1*)**	***−9.8* (*−15*, *−4.7*)**	***34* (*1.1*, *67*)**
School observed conducting group handwashing [2]	1.2 (0.9, 1.5)	***1.8* (*1.3*, *2.4*)**	1.0 (0.7, 1.2)	1.4 (0.9, 2.1)	***1.3* (*1.0*, *1.7*)**	1.1 (0.7, 1.6)	***0.6* (*0.0*, *7.3*)**
Percentage of students reporting participating in group toilet cleaning in previous week [2]	***8.5* (*6.7*, *10*)**	1.8 (−1.1, 4.8)	0.7 (−2.1, 3.5)	1.4 (−2.9, 5.7)	***16* (*12*, *20*)**	***−12* (*−19*, *−5.6*)**	39 (−0.1, 79)
Percentage of students reporting participating in group compound cleaning in previous week [2]	***11* (*9.3*, *14*)**	−2.4 (−5.9, 1.1)	1.0 (−2.4, 4.3)	0.0 (−5.2, 5.2)	0.5 (−1.2, 2.1)	0.2 (−2.7, 3.0)	4.6 (−13, 22)

All β coefficients represent the per month change in percent of students engaging in behavior within time strata, except for group handwashing, which is a per month change in odds of school conducting group handwashing. Bold italicization indicates significant change in outcome within time interval ($p < 0.05$). [1] Analyzed by time since hardware implementation. [2] Analyzed by time since full implementation.

Handwashing with Soap after Toilet Use

Handwashing with soap (HWWS) after toilet use fluctuated across the evaluation period. No schools had handwashing facilities as baseline, thus HWWS was not possible. In Group 1 intervention schools, the percentage of students HWWS did not significantly change until 18+ months after software implementation, when it decreased 3.7% per month ($\beta = -3.7$, 95% CI = −7.2, −0.2). Among Group 2 intervention schools, the percentage of students HWWS increased 4.5% per month in the first six months following software implementation ($\beta = 4.5$, 95% CI = 1.9, 7.1), then decreased 7–12 months after software implementation ($\beta = -9.8$, 95% CI = −15, −4.7), and increased again 13–18 months after software implementation ($\beta = 34$, 95% CI = 1.1, 67, Table A1.4). The percentage of students observed to HWWS after toilet use was higher among schools that practiced group handwashing on the day of the visit ($\beta = 31.7$, 95% CI = 24.0, 39.5).

Group Handwashing

Among Group 1 intervention schools, the odds of intervention schools conducting group handwashing did not increase until 7–12 months after software implementation (OR = 1.8, 95% CI = 1.3, 2.4), and was sustained thereafter. Among Group 2 schools, the odds of intervention schools conducting group handwashing (GHW) increased in the first 6 months after software implementation (OR = 1.3, 95% CI = 1.0, 1.7), was sustained 7–12 months after software implementation, and slightly decreased 13–18 months after software implementation (OR = 0.6, 95% CI = 0.0, 7.3). Intervention schools were more likely to conduct GHW on the day of the visit if they had a posted schedule for GHW (Odds Ratio (OR) = 4.1, 95% CI = 2.0, 8.1).

Group Toilet Cleaning

In Group 1 intervention schools, the percentage of students reporting participating in group toilet cleaning (GTC) in the previous week increased in the first six months following software implementation, and was sustained thereafter (β = 8.5, 95% CI = 6.7, 10). In Group 2, the percentage increased in the first six months after software implementation (β = 16, 95% CI = 12, 20), declined 7–12 months after software implementation (β = −12%, 95% CI = −19, −5.6), and was sustained thereafter. Odds of pupils in intervention schools reporting participating in GTC in the previous week were higher in schools where a GTC schedule was posted (OR = 3.2, 95% CI = 2.7, 3.8). Further, there was a positive association between toilet cleanliness and GTC; toilets were more likely to be observed to be clean in intervention schools among schools where a greater percentage of students reported participating in GTC in the previous week (β = 0.4, 95% CI = 0.1, 0.6).

Group Compound Cleaning

Student-reported participation in group compound cleaning (GCC) was high at baseline (96.9%). In Group 1 intervention schools, the percentage of students reporting participating in GCC in the previous week increased in the first six months after software implementation (β = 11, 95% CI = 9.3, 14), and was sustained thereafter. There was no significant change in the percentage of students in Group 2 schools reporting participating in GCC across the evaluation period. Odds of pupils in intervention schools reporting participating in GCC in the previous week were higher in schools where a GCC schedule was posted (OR = 2.4, 95% CI = 1.8, 3.3).

Discussion

This impact evaluation provided evidence that the UNICEF Lao PDR WinS project improved the WASH environment in intervention schools by increasing access to toilets, handwashing facilities, and safe drinking water and these improvements were sustained over two years after implementation of the project. We found that the project produced positive changes in pupils' WASH behaviors. Specifically, the project led to increases in pupils reporting using the toilet for defecation during the school day (as opposed to open defecation), increased prevalence of pupils' handwashing with soap following toilet use, and habitualization of daily group handwashing. Quantifying intervention fidelity is a critical component of assessing the impact of large-scale public health interventions. A priori determined output and outcome indictors agreed between government, implementation, and evaluation partners facilitated a better understanding of context specific intervention impact and provides important information to policy makers and donors.

Intervention Fidelity: Presence and Functionality of WinS Outputs

We found that quality of WinS project delivery was high, with 87.8% of schools receiving the intervention per stated design. School-level adherence to the outputs provided by the project was lower, but generally improved across the evaluation period. Similar results of high project delivery but low school-level adherence have been reported for school WASH projects in Mali and Kenya [16, 17, 27] and may be a key reason for inconsistent impact findings. WinS projects must focus on higher adherence; possibly through more appropriate technology, improving behavior change, or more accountability within the schools.

The greatest barrier to meeting the water supply and toilet outputs was water availability. Although functionality of the water point was relatively high (82% of post-hardware implementation visits), and consistent with other low-income school settings [28-30], schools were sometimes unable to fill the water tanks. Since the water tank supplied the handwashing facilities and the toilet compartments, water was often not available for handwashing or toilet flushing/cleaning. One reason for this was that the initial intervention design delivered to Group 1 schools consisted of a rainwater tank to supply the toilets with water. However, rainwater could not provide a consistent supply of water to fill the tank, causing pupils to have to manually fill the water tank. Thus, UNICEF revised the design, incorporating the lessons learned from the first year of intervention delivery, and detached the water tank from rain water harvesting system and connected tanks with motorized hand pumps or gravity-fed water supply systems. These results highlight the importance of routine monitoring to ensure that intervention technologies are contextually specific and appropriate. Following this adjustment, the presence of water in the water tank, in toilet compartments, and supplying the handwashing facilities improved, but was still not universal, probably because operating the pumps still required some action on part of the schools, which were not consistently performed.

Provision of soap was another adherence-related challenge; soap was observed at individual handwashing facilities during only 39.7% of post-hardware implementation visits, and the provision of soap at handwashing facilities showed little improvement as time since implementation passed. Each intervention school received one bar of soap per pupil, which was estimated to be a sufficient supply for an entire school year. Schools were expected to provide their own soap beyond this initial supply. Anecdotally, school directors reported difficulty in keeping soap by the individual handwashing facilities because of theft and of consumption by

animals. Purchasing soap to supply the handwashing facilities once the initial supply ran out could have also been a financial challenge for schools or an indicator of poor buy-in from teachers and parents. Having a sufficient and consistent supply of soap is a requisite to ensure that HWWS is a habitualized practice among students. Future WinS programming could explore strategies for protecting soap from theft or animal consumption. WinS implementers should also consider additional ways to help schools maintain a consistent supply of soap that is sustainable and is not a financial burden, such as including soap making in project activities.

Lastly, few schools had schedules for daily group hygiene activities (handwashing, toilet cleaning, compound cleaning), an output that relied solely on school adherence. However, for all of the group hygiene activities, odds of the respective activity being observed (group handwashing) or reported by pupils (group toilet and compound cleaning) were significantly higher in intervention schools that had a schedule posted for the respective activity. These results suggest that posting daily group activity schedules may serve as a visual cue for school directors and students, leading to increased adherence to these activities. Given the minimal cost and time needed to make and post schedules for the daily group activities output, as well as the direct linkage to positive WASH behaviors, meeting this output could be a focus in future programming.

Pupil Behavioral Outcomes

The WinS project was effective in achieving behavior change on the part of the pupils. Reported toilet use for defecation during the school day increased among both intervention groups. Toilet use at last defecation during the school day increased as the number of unlocked toilets increased, a trend that has also been reported in Kenya [31]. Beyond toilets being unlocked (which is necessary for pupil use), cleanliness and water availability were the largest predictors of whether

pupils reported using the toilet at last defecation during the school day. The few existing studies examining the links between toilet cleanliness and toilet use corroborate these results. In two different WinS studies in Kenya, dirty toilets were also found to be deterrents for toilet use, particularly among girl pupils [31, 32]. These results suggest that promoting toilet cleanliness is an important component of WinS interventions. Interventions utilizing pour flush toilets (such as this one) should also prioritize water availability, which is necessary for flushing and maintaining clean toilet environments.

Handwashing with soap (HWWS) is a notoriously difficult behavior to improve and sustain. Three school-based studies—two in Kenya and one in Mali—have reported HWWS rates of 38%, 32–38%, and 58%, respectively [33-35]. In Laos, improvements in HWWS after toilet use were observed among students in intervention schools 1-6 and 13-18 months following software implementation (Group 2), but these improvements were not sustained across the evaluation period. A similar overall trend was reported in Mali, where peak handwashing was observed 7–12 months following intervention implementation, and declined thereafter [35]. Thus, although HWWS showed a positive change among pupils in intervention schools, these results point to the need to reinforce HWWS behaviors periodically throughout the school year and from one year to the next one, beyond the timeframe of any externally-supported project. Activities such as regular teacher training, administrative incentives, and appropriate follow-up, monitoring, and supervision, can be employed so that the HWWS education and promotion persists despite frequent turnover of pupils and teachers. Additionally, our results indicate what is well known in the sector: due to lack of soap, handwashing projects are unlikely to be sustained beyond the direct implementation period. While handwashing with soap is considered a cost-effective way to prevent

illness, an assessment of long-term cost-effectiveness of HWWS interventions at schools may not indicate that current approaches are effective.

Daily group handwashing (GHW) was integrated into the UNICEF and German Corporation for International Development (GIZ) Three-Star Approach to WinS in 2013, however, few projects have evaluated behavioral outcomes associated with this approach. We found evidence of improved and sustained GHW behavior change across the evaluation period. Additionally, pupils attending schools where GHW was conducted on the day of the visit were more likely to practice individual HWWS after toilet use. These results point to the success of the WinS project in promoting HWWS through GHW, and suggest that GHW is an effective approach for promoting HWWS at critical times. However, more robust evaluations on the effectiveness, cost-effectiveness, and sustainability of these programmatic approaches are warranted to verify and complement the external validity of results from this evaluation.

Strengths and Limitations

The presence and functionality of the water point relied on report by the school director. We intended to include both reported and observed functionality of the water point, but due to an oversight the observation component was not included. We did observe whether the handwashing (group and individual) taps and the taps within the toilet compartments were functioning, as well as whether water was present in the water tank. Since these taps are connected to the school water supply, we were able to use handwashing and toilet functionality data to triangulate and confirm the reported water point functionality data. A second limitation was the staggered delivery of the intervention across two different school years. This could be seen as a strength of the intervention approach, as lessons learned from evaluation of the first implementation group (Group 1) were

used to improve delivery to the second implementation group (Group 2). However, this did create minor limitations to the analysis; Group 2 schools often performed better than Group 1 schools in meeting output and outcome indicators. Additionally, differences in delivery also limit our ability to report on the sustainability of the intervention, as Group 1 had a full extra year of surveillance but implementation was also delayed in some districts. In order to have an accurate measure of WinS hardware and software performance and sustainability, we ideally would need to follow a single cohort of schools over the same time period. Lastly, given the quantitative design of the study, we were unable to take into account some dimensions of project delivery and adherence, specifically the dose of hygiene education received and participant responsiveness to the project [22, 23]. Additionally, we were unable to explore possible socio-cultural explanations for why certain behaviors improved (e.g., toilet use), while others, such as handwashing, did not. Previous research has shown that emotional drivers and social norms can be motivators for handwashing behaviors, whereas heath or fear of disease generally are not [36, 37]. WinS programming should consider these drivers prior to program design in order to ensure the Theory of Change is contextually and culturally targeted.

Despite these limitations, the design, methods, and approach of the WASH HELPS Study were robust. This is the first evaluation of a comprehensive school WASH project in Laos and one of the largest and most comprehensive evaluations to date of a school WASH project in low-income settings. Our study design—a randomized-controlled trial—is the gold standard of epidemiological evidence, and we followed schools over 2 to 3 years in order to account for inter-seasonal and inter-year variations.

Conclusions

Our results describe the success of the UNICEF Laos WinS project in improving the WASH environment in schools that were lacking WASH facilities and the effectiveness of the intervention in positively changing WASH behaviors. Similar to previous WinS impact evaluations in Mali and Kenya, we report high quality of project delivery such as provision of a functional water supply, toilets, and handwashing facilities. Conversely, there was sub-optimal school-level adherence to project outputs such as soap provision, water availability, and promoting group hygiene activities. Despite these shortcomings, most behavioral outcomes (toilet use and daily group hygiene activities) improved and/or were sustained across the evaluation period. Strategies to sustain handwashing behaviors beyond the initial 6 to 12 months of project implementation and to sustain a consistent supply of soap warrant further exploration and should be a priority for policy makers and WinS project implementers.

Supplementary Materials: The following are available online at www.mdpi.com/link, Table S1: Key school-level indicators by intervention status at baseline, Table S2: Key pupil-level indicators by intervention status at baseline, Table S3: Associations between school toilet output criteria and percentage of pupils reported toilet use for last defecation during the school day.

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Author Contributions: A.N.C. and M.C.F. conceived and designed the study; A.N.C. analyzed the data; A.N.C and M.C.F. wrote the paper.

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Appendix 2. The impact of school water, sanitation, and hygiene improvements on infectious disease using serum antibody detection[5]

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

The impact of school water, sanitation, and hygiene improvements on infectious disease using serum antibody detection

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Abstract

Background

Evidence from recent studies assessing the impact of school water, sanitation and hygiene (WASH) interventions on child health has been mixed. Self-reports of disease are subject to bias, and few WASH impact evaluations employ objective health measures to assess reductions in disease and exposure to pathogens. We utilized antibody responses from dried blood spots (DBS) to measure the impact of a school WASH intervention on infectious disease among pupils in Mali.

Methodology/Principal findings

We randomly selected 21 beneficiary primary schools and their 21 matched comparison schools participating in a matched-control trial of a comprehensive school-based WASH intervention in Mali. DBS were collected from 20 randomly selected pupils in each school (n = 807). We analyzed eluted IgG from the DBS using a Luminex multiplex bead assay to 28 antigens from 17 different pathogens. Factor analysis identified three distinct latent variables representing vector-transmitted disease (driven primarily by dengue), food/water-transmitted enteric disease (driven primarily by *Escherichia coli* and *Vibrio cholerae*), and person-to-person transmitted enteric disease (driven primarily by norovirus). Data were analyzed using a linear latent variable model. Antibody evidence of food/water-transmitted enteric disease (change in latent variable mean (β) = -0.24; 95% CI: -0.53, -0.13) and person-to-person transmitted enteric disease (β = -0.17; 95% CI: -0.42, -0.04) was lower among pupils attending beneficiary schools. There was no difference in antibody evidence of vector-transmitted disease (β = 0.11; 95% CI: -0.05, 0.33).

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Abstract

Background: Evidence from recent studies assessing the impact of school water, sanitation and hygiene (WASH) interventions on child health has been mixed. Self-reports of disease are subject to bias, and few WASH impact evaluations employ objective health measures to assess reductions in disease and exposure to pathogens. We utilized antibody responses from dried blood spots (DBS) to measure the impact of a school WASH intervention on infectious disease among pupils in Mali.

Methodology/Principal Findings: We randomly selected 21 beneficiary primary schools and their 21 matched comparison schools participating in a matched-control trial of a comprehensive school-based WASH intervention in Mali. DBS were collected from 20 randomly selected pupils in each school (n=807). We analyzed eluted IgG from the DBS using a Luminex multiplex bead assay to 28 antigens from 17 different pathogens. Factor analysis identified three distinct latent variables representing vector-transmitted disease (driven primarily by dengue), food/water-transmitted enteric disease (driven primarily by *E. coli* and *V. cholerae*), and person-to-person transmitted enteric disease (driven primarily by norovirus). Data were analyzed using a linear latent variable model. Antibody evidence of food/water-transmitted enteric disease (change in latent variable mean (β)=-0.24; 95% CI: -0.53, -0.13) and person-to-person transmitted enteric disease (β=-0.17; 95% CI: -0.42, -0.04) was lower among pupils attending beneficiary schools. There was no difference in antibody evidence of vector-transmitted disease (β=0.11; 95% CI: -0.05, 0.33).

Conclusions/Significance: Evidence of enteric disease was lower among pupils attending schools benefitting from school WASH improvements than students attending comparison schools. These findings support results from the parent study, which also found reduced incidence of self-reported diarrhea among pupils of beneficiary schools. DBS collection was feasible in this resource-poor field setting and provided objective evidence of disease at a low cost per antigen analyzed, making it an effective measurement tool for the WASH field.

Author summary

Water, sanitation, and hygiene (WASH) in schools is promoted as an intervention to improve child health in low-resource settings. However, evidence of the impact of school WASH interventions on child health is mixed. One reason could be that most studies rely on self-reported disease symptoms, which are prone to bias. In order to objectively measure evidence of disease, we collected dried blood spots (DBS) from pupils attending schools participating in an impact evaluation of a comprehensive school WASH intervention in Mali, and analyzed the DBS for antibody responses to 28 antigens from 17 different pathogens. We found that evidence of enteric disease was lower among pupils attending beneficiary schools compared to pupils attending comparison schools. These results are consistent with those from the parent study, which also found reduced self-reported diarrhea among pupils attending beneficiary schools. Our results support WASH in schools as an effective intervention to improve child health. Further, DBS are a feasible measurement tool for the WASH field to provide objective evidence of disease.

Background

Diarrhea is among the leading causes of morbidity and mortality among children in developing communities [1], and children are often disproportionately affected by other infectious diseases,

including soil-transmitted helminths (STHs) and trachoma [2]. The role of improvements in household safe water, sanitation access, and hygiene (WASH) behaviors on the reduction of infectious diseases among children and adolescents is well documented [1, 3-5]. Despite the biological plausibility supporting the role of improvements in school WASH conditions on pupil health, results from school WASH evaluations have been mixed [6-12]. There is some evidence of associations between WASH in schools programs and reductions in diarrhea, acute respiratory infection, soil-transmitted helminth re-infection, and school absence, but results are inconsistent and effects are sometimes evident only among sub-populations [6, 8, 9, 13-17].

One limitation to health impact evaluations of WASH interventions in low-resource settings is the existing methods and tools used to measure diarrheal and other infectious disease incidence. A common approach for measuring diarrheal disease is self-report, an approach prone to recall and social desirability biases. Respondents' variable and often subjective interpretations of the definition of "diarrhea" may also lead to imprecise measurements of incidence [18, 19]. Further, the definition of "diarrhea" and the recall period are not uniform across studies [18, 20, 21], making inter-study comparison of disease incidence and intervention effectiveness difficult. Stool collection is another, more accurate approach to assessing enteric infections, however conventional methods lack sensitivity for many pathogens [22]. The cost and logistical implications for this approach are considerable. Stool must be collected, often by return visit, and samples must be transported to laboratory equipment and trained laboratory personnel for the identification of pathogenic agents [22, 23], making it a challenging and expensive way to assess infectious disease prevalence in low-resource field settings.

Antibody detection assays are used to detect immune responses to past infections from a variety of different organisms by detecting signal intensity due to the presence of antibodies [24-28]. Luminex multiplex bead assay (MBA) technology detects antibodies in a range of biological specimens including eluted antibodies from dried blood spots (DBS) obtained through a single finger-prick. As such, this technology has significant potential for providing reliable measures of infections in low-resource settings, as DBS samples are stable at ambient temperatures and can be collected, transported, and stored easily, negating the need for expensive equipment and skilled laboratory technicians in the field [23, 29]. Because the multiplexing capacity allows for the simultaneous analysis of up to 100 different antigens from one sample, and because samples can be analyzed off-site in a reference laboratory, Luminex MBAs have been shown to be an effective method for data collection in low-resource settings and at a low cost per antigen analyzed. Previous studies have used Luminex MBAs to detect serum antibody responses to tuberculosis, lymphatic filariasis, chikungunya, dengue, malaria, and enteric protozoa (giardia, *E. histolytica*, and cryptosporidium) [25-27, 30-32].

We collected DBS to evaluate the impact of a school WASH program in Mali on infectious disease by analyzing immunoglobulin (Ig) G responses to 28 antigens from 17 different pathogens using the Luminex MBA platform. Although this serological platform has been widely used to evaluate drug treatment programs [25, 31] and as a disease diagnostic and surveillance tool [26, 27, 33], it has had limited employment within WASH program impact evaluations [34]. In addition to providing evidence for the impact of school WASH interventions on pupil health, data from this study also provide evidence for the feasibility of using the Luminex platform as an objective measurement of enteric pathogen exposure. Further, this study highlights the benefit of utilizing

the Luminex MBA platform's multiplexing capacity to simultaneously assess serological episodes for a range of infectious diseases.

Methods

Setting

This study was nested within a longitudinal impact evaluation of the Dubai Cares Water, Sanitation, and Hygiene in Schools Initiative in Mali (DCIM WASH) project, a comprehensive school-based WASH intervention in 900 schools in Bamako Capital District and the Koulikoro, Mopti and Sikasso regions of Mali. Using stratified random sampling based on region, a subset of 21 of 100 beneficiary primary schools from the impact evaluation were selected for inclusion, as well as their 21 matched comparison schools, for a total of 42 schools participating in the study (**S1 Figure**). Matched comparison schools were located within the same educational district and matched to beneficiary schools based on baseline enrollment size and school WASH characteristics. Detailed methods of the parent study and the as-treated analysis are described elsewhere [14, 17]. In each school, 20 pupils were randomly selected from a list of all pupils enrolled in classes 1-6 using stratified random sampling based on pupil sex and grade. Pupils were interviewed about their household WASH access, school absence, and recent illness. Capillary whole blood in the form of a dried blood spot (DBS) was collected from each pupil. Data from a total of 807 participants aged 4-17 years were collected between January and May 2014.

Dried blood spot collection

Students' ring or middle fingers were cleaned and sanitized with an alcohol wipe, allowed to air dry, and then punctured with a new single-use lancet. The first drop of blood was wiped away [35, 36]. Fingertip whole capillary blood specimens were then collected onto a filter paper wheel with

six circular extensions (TropBio Pty Ltd, Townsville, Queensland, Australia), each designed to absorb 10 µl of whole blood. One filter paper wheel was collected per child. The filter paper wheels were air dried for up to 4 hours and placed in a sealed plastic bag with a desiccant. Between 1-3 months following collection, samples were shipped to a laboratory at the Centers for Disease Control and Prevention in Atlanta, Georgia for storage at -20 °C and analysis [35, 36].

Antigen coupling and antibody analysis

Purified antigens were coupled in various buffers as indicated in **S1 Table**, and some antigens were linked with glutathione-S-transferase (GST). For each antigen and GST, carboxyl groups on the surface of specifically classified-spectral magnetic polystyrene microspheres (MagPlex Beads; Luminex Corporation, Austin, TX) were converted to reactive esters using the 1-ethyl-3-(3-imethylaminopropyl) carbodiimide method (Calbiochem, Woburn, MA). The esters readily react with available primary amine structures on the antigens to form a covalent amide bond between antigen and bead. Coupling efficiency was determined using sera and reagent known to be highly reactive to the antigens and GST.

One circular extension from each child's DBS wheel was placed in 0.5 mL of elution buffer consisting of PBS with 0.5% bovine serum albumin, 0.3% Tween 20, 0.1% sodium azide, 0.5% polyvinyl alcohol, 0.8% polyvinylpyrrolidone, and 0.1% casein, and allowed to elute overnight at 4 °C. Afterward, the elution was further diluted 1:4 with the same elution buffer, containing sufficient amounts of crude *Escherichia coli* extract for a final concentration of 3 µg/mL. The *E. coli* extract is used to absorb *E. coli* antibodies that could react with any extraneous *E. coli* proteins coupled to the beads. After overnight storage at 4 °C, the eluate was exposed to antigen-coupled beads for 1.5 hours at room temperature. Bound antigen-specific IgG was detected on the coupled

beads as previously described [26]. Between steps, the magnetic beads were washed three times with 0.05% Tween 20 PBS, using a Bio-Plex Pro II Wash Station (Bio-Rad, Hercules, CA). Data were acquired using a Bio-Plex 100 reader with Bio-Plex Manager 6.1 software (Bio-Rad). For each antigen, the median fluorescence intensity (MFI) with a range of 1 – 32,766 channels was determined, and the average from duplicate wells was obtained. From a primary antibody blank, background (bg) was subtracted (MFI-bg) and used as data.

Measures and statistical analysis

Univariate analysis. To measure pupils' household WASH access, we created an index score using pupil responses to three survey questions on their household access to 1) an improved drinking water source, classified according to the Joint Monitoring Programme definition [37]; 2) any on-site sanitation facility; and 3) soap for handwashing. Affirmative responses were assigned one point and all responses were summed, creating an index score ranging from 0 (no household WASH access) to 3 (maximum household WASH access).

Differences in pupil demographics (age, sex, grade) and household WASH access by intervention status were evaluated using logistic (sex) and linear regression models (age, grade, household WASH access), with random intercepts at the school level. Associations with a p <0.05 were considered statistically significant.

Factor analysis and latent variable development. Factor analysis is a statistical tool commonly used in behavioral and health sciences to assess complex inter-relationships among large numbers of variables, including non-independent or correlated variables. The theory behind factor analysis is that multiple observed variables with similar patterns of response are all associated with an

underlying latent variable. Thus, the goal of factor analysis is to yield a small number of new variables (factor constructs) that adequately express the communality of – and can substitute for – a larger number of variables [38, 39]. Given that we had data on antibody responses from 28 different antigens for infectious disease, some of which may be correlated, we employed factor analysis to identify latent variables representing groupings of antibody responses.

The 28 variables representing antibody responses to infectious diseases were normalized by taking the natural log and standardized [40]. We used an iterative approach, specified *a priori*, for selecting antibody response variables to include in the factor analysis. First, we restricted the analysis to include only antibody responses that were prevalent in the population; all unique antibody response variables for which a cutoff value for infection was available and for which <10% of samples exceeded the cutoff value were excluded (chikungunya, *B. malayia*, *W. bancrofti*, *T. solium*, and yellow fever). The following unique antibody response variables were included in the initial factor analysis: *E. histolytica*, *G. intestinalis* VSP3, *G. intestinalis* VSP5, *P. falciparum* MSP-1_{19}, *P. falciparum* MSP-1_{42}, *P. falciparum* AMA-1, *P. vivax* MSP-1_{19}, entero-toxigenic *E. coli* (ETEC), *V. cholerae*, Dengue 2, Dengue 3, norovirus GI.1 (Norwalk strain), norovirus GII.4 (Sydney strain), norovirus GIV.1 (St. Cloud strain), *Cryptosporidium* 17-kDa, *Cryptosporidium* 27-kDa, *S. mansoni*, *C. jejuni* p18, *C. jejuni* p39, *S. typhimurium*, *S. enteritidis*, *C. trachomatis* Pgp3, and *C. trachomatis* CT694. Second, we evaluated antigen response variables for uniqueness, a measure of the variance that is not shared with other variables in the model; the higher the uniqueness, the lower the relevance of the variable in the factor model [38, 39]. Since the goal of factor analysis is to identify groupings of variables with similar responses, variables with uniqueness ≥0.6 (thus implying the majority of the variable's response is not shared with other variables in the model) were dropped from the factor analysis until a reduced factor model

consisting only of variables with uniqueness <0.6 was achieved. An oblique rotation was applied given the assumption that factors were correlated, and factors with Eignenvalues >1 were retained [38, 39, 41]. The rotated factor loadings from the reduced factor model became latent variables representing disease responses; from here forward, we refer to these latent variables as disease response variables.

Latent variable models. We elected to use a latent variable modeling approach over a more conventional modeling strategy, such as linear regression, because latent variable models allow multiple, correlated outcomes (disease response variables) to be analyzed simultaneously in one model rather than running individual models for each outcome [42, 43]. The association between disease outcomes and intervention status were analyzed using the generalized linear latent and mixed model (gllamm) package [44] in Stata version 13 (StataCorp, College Station, TX).

The latent variable modeling framework consisted of 1) a measurement model of the child-specific latent variables identified through factor analysis, clustered at the school level, and 2) a structural model of the regression of the intervention on the latent variables, controlling for pupil grade, sex, and household WASH access. Pupil grade was included as a proxy for pupil age due to a large number of missing pupil age data (n=338). Associations with a p <0.05 were considered statistically significant.

Linear regression models. To cross-validate the linear latent model results, we independently evaluated differences in antibody responses between pupils attending intervention versus comparison schools for each antigen response included in the initial factor model using mixed effects linear regression models with random intercepts at the school level. To facilitate comparison between the linear regression model results and the latent model results, the

normalized antibody response variables were used as the outcomes and the same control variables— pupil grade, sex, and household WASH access— were included. Because we *a posteriori* used linear regression to cross-validate the linear latent model results, we included a Bonferroni correction to adjust for multiple comparisons. Associations were considered statistically significant if they had a $p<0.002$, the alpha necessary to reach 95% significance with 23 hypotheses.

Ethics

This study was approved by the Ethics Committee of the National Institute of Public Health Research in Mali (*Comité d'Ethique de l'Institut National de Recherché en Santé Publique*, 02/2014/CE-INRSP) and the Institutional Review Board of Emory University (IRB00060756). The trial was registered at ClinicalTrials.gov (NTC01787058). We obtained informed written consent (signature or fingerprint) from the parents of all participants and oral assent from all participants prior to any interview data or blood spot collection. Laboratory staff from the United States Centers for Disease Control and Prevention had no contact with children nor access to personal identifiers.

Results

Bead coupling efficiency

All coupled beads showed high MFI-bg from sera or reagents known to be highly reactive to the antigens, indicating sufficient antigen coupling and the excellent condition of the DBS.

Student characteristics

DBS were collected from 807 primary school students attending 42 schools (21 beneficiary, 21 comparison). Survey data from 7 pupils, all attending the same beneficiary school, were not collected and these pupils were subsequently dropped from analysis. The final sample population was 800 students. There were no significant differences in age, sex, grade, or household WASH access between beneficiary and comparison groups (**Table A2.1**). Demographic characteristics of the students were similar to those in the full parent study [14, 17].

Table A2.1. Demographic characteristics of study population

	Beneficiary (n=393) Mean (SD) or n(%)	Comparison (n=407) Mean (SD) or n(%)	p[1]
Age[2]	10.9 (0.14)	11.1 (0.17)	0.56
Female	183 (46.6%)	188 (46.2%)	0.93
Grade	3.8 (0.08)	3.9 (0.08)	0.65
Household WASH access scale index score	2.1 (0.03)	2.3 (0.03)	0.28

[1]Differences across strata were evaluated using logistic (sex) and linear (age, grade, household WASH access) regression models, with random intercepts at the school-level. P<0.05 was considered statistically significant.
[2]Age missing for 142 pupils in beneficiary group and 196 pupils in comparison group

Factor analysis

The final factor model included antibody response variables for ETEC, cholera, Dengue 2 VLP, Dengue 3 VLP, norovirus Norwalk strain, and norovirus St. Cloud strain. This factor model resulted in the development of 3 distinct factors, or disease response variables (**Table A2.2**).

Table A2.2. Rotated factor loadings and unique variances

Antigen	Factor 1	Factor 2	Factor 3	Uniqueness
ETEC	-0.0112	0.872	-0.0124	0.2425
V. cholerae	0.0046	0.8701	0.0146	0.2402
Dengue 2	0.876	-0.0223	0.0324	0.2376
Dengue 3	0.871	0.0161	-0.0341	0.2345
Norovirus Norwalk strain	-0.0481	-0.0493	0.7426	0.4449
Norovirus St. Cloud strain	0.0477	0.0524	0.7422	0.4418

A factor loading can be interpreted as a Pearson correlation coefficient between the original variable and the factor. Factor 1 was strongly correlated with Dengue 2 (0.876) and Dengue 3 (0.871). Based on these variables loading highly with Factor 1, and given that Dengue is transmitted by mosquitoes [45], we classified Factor 1 as a latent variable representing vector-transmitted disease. Factor loadings for ETEC and *V. cholerae* were strongly correlated with Factor 2 (ETEC=0.872, cholera=0.871). Given that ETEC and *V. cholerae* are transmitted when food or water are contaminated with feces [45, 46], we classified Factor 2 as a latent variable representing food/water-transmitted enteric disease. Lastly, norovirus Norwalk and St. Cloud strains were strongly correlated and loaded most highly with Factor 3 (Norwalk=0.7426, St. Cloud=0.7422). Norovirus infection occurs by ingesting stool or vomit from an infected person. Although foodborne and waterborne transmission is possible, norovirus is considered primarily a person-to-person transmitted disease [47-49]; as such, we classified Factor 3 as a person-to-person transmitted enteric disease latent variable.

Linear latent model results

Results from the linear latent model indicate that there was a 0.24 reduction in the latent variable mean of food/water-transmitted enteric disease, and a 0.17 reduction in the latent variable mean of person-to-person transmitted enteric disease among pupils attending beneficiary schools versus

pupils attending comparison schools (**Table A2.3**). We found no difference in the evidence of vector-transmitted disease between pupils attending beneficiary versus comparison schools (β=0.11, p=0.141).

Table A2.3. Linear latent model results of the association between the school WASH intervention and disease response variables

	β	**95% CI**	***p***
Vector transmitted disease	0.11	(-0.05, 0.33)	0.141
Food/water transmitted enteric disease	-0.24	(-0.53, -0.13)	<0.001
Person to person transmitted enteric disease	-0.17	(-0.42, -0.04)	0.019

β represents change in latent variable mean
Model controls for pupil age, grade, and household access to WASH and includes a random intercept for school
p<0.05 is considered statistically significant

Linear regression model results

Results from the linear regression models are similar to those from the linear latent model. Among the antibody response variables included in the linear latent model, Dengue 2 and Dengue 3 (antigens making up the vector transmitted disease latent variable) were higher among the intervention group; results for Dengue 3 were not significant once we applied the Bonferroni correction (β=0.29, p=0.02). Antigen responses for *E. coli* and *V. cholerae* (food/water transmitted enteric disease) and the two Norovirus strains (person to person transmitted enteric disease) were lower among the intervention group, but not statistically significant. Among antibody response variables not included in the linear latent model, only *Chlamydia trachomatis* (CT-694) was higher among the intervention group (β=0.39, p=0.001) (**Table A2.4**).

Table A2.4. Linear regression model results of the association between the school WASH intervention and antibody responses

	β	95% CI	*p*
Campylobacter jejuni (P18 Antigen)	0.02	-0.20, 0.24	0.88
Campylobacter jejuni (P39 Antigen)	-0.12	-0.31, 0.06	0.18
Cryptosporidium parvum (17 KdA Antigen)	0.29	0.09, 0.49	0.01
Cryptosporidium parvum (27 KdA Antigen)	0.09	-0.11, 0.28	0.41
Dengue 2	0.09	-0.21, 0.39	0.55
Dengue 3	0.29	0.04, 0.54	0.02
Entamoeba histolytica	-0.06	-0.31, 0.20	0.65
Escherichia coli	-0.18	-0.40, 0.05	0.12
Giardia intestinalis (VSP 3)	-0.02	-0.20, 0.16	0.84
Giardia intestinalis (VSP 5)	-0.19	-0.37, -0.01	0.04
Norovirus (Norwalk strain)	-0.01	-0.24, 0.22	0.92
Norovirus (St. Cloud strain)	-0.02	-0.25, 0.22	0.88
Norovirus (Sydney strain)	0.12	-0.28, 0.04	0.14
Plasmodium falciparum (MSP_{19})	0.07	-0.13, 0.27	0.51
Plasmodium falciparum (MSP_{42})	0.16	-0.13, 0.46	0.29
Plasmodium falciparum (AMA_1)	0.16	-0.20, 0.52	0.38
Plasmodium vivax (MSP_{19})	0.14	-0.06, 0.34	0.16
Salmonella enteritidis	0.10	-0.05, 0.25	0.20
Salmonella typhimurium	0.06	-0.11, 0.23	0.50
Schistosoma mansoni	0.22	-0.02, 0.45	0.07
Chlamydia trachomatis (CT-694)	0.39	0.20, 0.58	<0.001
Chlamydia trachomatis (Pgp3)	0.15	-0.03, 0.33	0.10
Vibrio cholerae	-0.07	-0.27, 0.13	0.49

Models control for pupil age, grade, and household access to WASH, and include a random intercept for school
Shaded rows represent antigens included in the final linear latent model
Due to the Bonferroni correction for multiple comparisons, $p<0.002$ is considered statistically significant

Discussion

This study utilized dried blood spots and the Luminex MBA as a tool to evaluate the impact of a school WASH intervention in Mali on infectious disease among pupils. Antibody evidence of both food/water-transmitted enteric disease and person-to-person transmitted enteric disease was lower among pupils attending beneficiary schools, while the intervention had no impact on antibody

evidence of vector-transmitted disease. This study was innovative in its use of antibody responses from DBS to measure the impact of a WASH program. Additionally, utilizing factor analysis on antibody responses to identify latent groupings of disease is a novel approach to the analysis of antibody data.

Consumption of microbiologically safe drinking water, handwashing with water and soap, and use of sanitation facilities that safely contain feces are all strategies for stopping enteric disease transmission along the fecal-oral route. Indeed, we found evidence that food/water-transmitted enteric disease and person-to-person transmitted enteric disease was lower among pupils attending schools benefitting from a comprehensive WASH intervention compared to those attending comparison schools, supporting the idea that school WASH can interrupt disease transmission among pupils. These results also corroborate results from the longitudinal parent study, which found a 29% reduction in the odds of reported symptoms of diarrhea among pupils attending beneficiary schools compared to pupils in the comparison schools [14], and a 35% reduction in the odds of diarrhea among pupils attending beneficiary schools that met all WASH targets compared to pupils attending beneficiary schools that met none of the WASH targets [17]. Additionally, these results contribute to the growing body of evidence supporting the association between WASH in schools and reduced pupil diarrheal incidence [9, 13, 14, 17] and other poor health outcomes [8, 13-17].

We found no impact of the intervention on evidence of vector-transmitted disease, which is more commonly linked to environmental conditions than to WASH access, and is generally controlled through the use of insecticides and elimination of breeding sites [50-52]. Given that a school WASH intervention is unlikely to alter the transmission pathways of vector-borne disease [51],

our finding that the intervention did not have a significant impact on evidence of vector-transmitted disease is not surprising. There is little biologic plausibility of an impact of a school WASH program on vector-transmitted disease and indeed we found none; as such, the absence of an impact of the intervention on vector-transmitted disease supports the validity of our findings on enteric infections, and can be considered a negative control.

This study employed novel methodology in the use of antibody data to assess the impact of WASH interventions. The Luminex MBA serologic platform has had limited use as a WASH program impact evaluation tool. We found that the collection of capillary blood in the form of DBS was feasible in a low-resource field context and acceptable by participants and their guardians and therefore serves as a viable alternative to current methods of biological assessment of WASH-related disease such as stool collection or venipuncture that are labor-, time-, and cost-intensive. Additionally, our results suggest that objectively measuring WASH-related disease might be useful for identifying biomarkers that could serve as proxies for access to WASH. Further, given the multiplexing capacity of the Luminex technology, we were able to capitalize on the DBS antibody data collected for the purpose of the WASH program impact evaluation by including antibody measures for diseases beyond the scope of the program – such as lymphatic filariasis, measles, tetanus, and rubella – at a minimal additional cost; with a total of 36 antigens included in the assay, the cost was ~USD $0.54 per antigen/sample, excluding the costs of labor and antigens. Ongoing sub-analyses from this data are providing valuable information on the effectiveness of mass drug administration [25] and vaccination programs, and could identify areas where these programs have been successful or should be scaled up; additional analyses examine patterns of malaria [27] and neglected tropical disease [25] transmission.

This study also employed novel analysis methods for antibody data. Factor analysis is commonly used in behavioral, health, and life sciences [38, 39]. However, our use of factor analysis on antibody responses to classify latent variables of disease responses is a novel approach. It is important to emphasize that while we labeled each factor as vector-transmitted disease, food/water-transmitted enteric disease, and person-to-person transmitted enteric disease, we did not select how the original antibody response variable loaded into each factor/disease response variable. The finding that the original antibody response variables loaded into three distinct pathways of disease transmission validates our use of factor analysis, which assumes that variables share a common factor due to their similar patterns of response. The validity of this method is also highlighted by the antibody response variables that dropped out of the factor model. For example, pathogens such as trachoma and *S. mansoni* have largely unique transmission vectors and intermediate hosts relative to the other pathogens retained in the model (flies and snails, respectively) [45], contributing to a high unique variance ("uniqueness") and subsequent elimination from the model. Like dengue, malaria is also a vector-borne disease. However, the high uniqueness of the malarial pathogens (*P. falciparum* MSP-1_{19}, *P. falciparum* MSP-1_{42}, *P. falciparum* AMA-1, *P. vivax* MSP-1_{19}) in the factor model could be explained by the extremely high prevalence of malaria in this population; nearly all children had antibody responses exceeding the cutoff values for *P. falciparum* (78.4%, 90.7%, 91.6%, respectively), with little variance in antibody response (**S2 Figure, S2 Table**). While there was greater variance in antibody response for *P. vivax,* it may have dropped out of the factor model due to different dengue and *P. vivax* mosquito vectors (*Aedes aegypti* and *Anopholes*, respectively) and vector behaviors (day biters and night biters, respectively) [45]. Lastly, while *E. histolytica, Giardia*, *Campylobacter*, *Salmonella* and *Cryptosporidium* share a similar transmission pathway to that of ETEC and *V.*

cholerae (food/water) [45], these pathogens also dropped out of the factor model. Antibody reactivity for *Campylobacter, E. histolytica, Giardia*, and *Salmonella* are particularly predominant in the first few years of life, and wane thereafter [26, 53, 54], a trend that is also evident in our sample (**S2 Figure**). These variables likely dropped out of the factor model given low antibody responses among our school-aged participants. *Cryptosporidium* antibody response is not associated with age [26], but it is less pathway specific than other antigens in the model; for example, in addition to the fecal/oral route, *Cryptosporidium* can also be transmitted via inhalation [55, 56], which could explain the high uniqueness of *Cryptosporidium* responses.

Results from the linear models of association between intervention status and antibody responses further strengthen the linear latent modeling approach. In these models the trends for all antibody response outcomes were in the same direction as their respective latent variable. The Dengue 3 outcome was only statistically significant at $p<0.05$ and prior to the use of the Bonferroni correction. This suggests that analyzing the antigens simultaneously— as is done in the linear latent model —may give us more power to detect an effect as opposed to running each outcome individually. Further, because linear latent models allow multiple outcomes to be analyzed simultaneously, they also eliminate the need for a multiple comparisons correction [42, 43]. Of the antibody response variables included in the linear latent model, Dengue 3 ($p=0.02$) was only significant prior to the Bonferroni correction. It is possible that this association was due to a Type I error, especially given that there is little biological plausibility that a school-based WASH intervention would lead to increased incidence of dengue. Indeed, under the Bonferroni correction, the p-value needed to be <0.002 to achieve statistical significance. All but one of the antibody response variables that were eliminated from the factor model were statistically insignificant in the linear model results. Antibody response for *Chlamydia trachomatis* (CT-694) was significantly

higher among pupils attending intervention schools. There is some evidence that WASH in schools interventions have the potential to *increase* exposure to fecal pathogens when the intervention is incompletely delivered or adherence is low. An evaluation in Kenya found that in a trial where sanitation was provided at schools, but handwashing was poor, children had higher fecal hand contamination than children at schools without new sanitation facilities. Researchers hypothesized that pupils' increased use of toilets led to higher fecal contamination, but that a lack of handwashing behaviors put children at risk [15]. Thus, it is possible that *Chlamydia trachomatis* (CT-694) was indeed higher among beneficiary schools, considering that fidelity and adherence to the intervention was varied [17]. However, it is more likely that this was a spurious association given that there was no significant difference in *Chlamydia trachomatis* (Pgp3).

We found that factor analysis was useful in identifying common patterns of disease response in our study population. Future studies examining multiple, and possibly correlated disease outcomes should consider the factor analysis approach as a complement to more conventional modeling techniques. By focusing on the underlying phenomena driving the measured results, factor analysis allows researchers to generalize their findings to a larger measurement domain and improve practical applicability.

Limitations

There are a number of limitations to this study, mostly associated with the use of antibodies as a measure of infection. First, the assay may detect antibodies for infections that were asymptomatic, which may lead to an over-estimation of morbidity. Second, we measured antibody levels for enteric disease among children over 5 years old, who may have already been repeatedly exposed to a variety of pathogens and developed effective immune responses other than IgG. An example

of this may have been shown in a study showing IgG responses to these same *Giardia* antigens that decreased in children > 4.5 years of age [26]. Other immune arms, such as cell-mediated immune responses may allow a more rapid clearing of the antigen and shorter IgG responses. However, these two limitations would likely be similar across beneficiary and comparison groups, thus biasing the estimate towards the null. Third, antibody kinetics vary by pathogen, and the current cross-sectional analysis may have captured antibody responses from infections that occurred prior to the intervention. This could have caused us to underestimate the protective benefit of the WASH program.

There are also limitations associated with the use of factor analysis and linear latent models. Our three measures of disease response are factor variables, and the beta coefficient represents a change in the latent variable mean. As such, the model measures a larger construct than the original antibody response variables, and we are not able to calculate the odds or risk ratios for reductions in specific diseases associated with the intervention. Also, many antibody response variables dropped out of the factor model due to low prevalence or a high unique variance. While there is limited biological plausibility of a WASH intervention impacting transmission of some of these pathogens (e.g. chikungunya, lymphatic filariasis, yellow fever), other pathogens, such as schistosomiasis and trachoma, have been directly linked to WASH access [57, 58]. It is possible that these pathogens could have been impacted by the WASH intervention, but were not included in the final analysis. Another limitation is that not all antigens have a known cut-off value for infection, so whether the original antibody response variable exceeded the cutoff for disease was not taken into consideration when constructing the factors. Lastly, linear latent models assume that the latent variables arise from a normal distribution, which is difficult to verify.

Conclusion

Our results describe evidence of infectious disease among pupils attending schools benefitting from a comprehensive school WASH program in Mali compared to pupils attending matched comparison schools. We found that evidence of enteric disease (both food/water-transmitted and person-to-person transmitted) was lower among pupils attending beneficiary schools, results which are supported by the parent study, which found reductions in self-reported diarrhea among pupils attending beneficiary schools compared to pupils attending comparison schools. Collecting accurate data on biologic evidence of infectious disease in low-resource field settings can be logistically challenging, expensive, and laborious. We collected DBS and analyzed pupil antibody response for 28 antigens from 17 pathogens using a Luminex MBA, a method that has had limited employment in evaluation of WASH interventions. Our study demonstrates the feasibility and applicability of this method in the WASH field as an objective measure of disease.

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Supporting Information

S1 Table. Purified antigen, antigen format, amount used in coupling, GST linked, and coupling buffer.

S2 Table. Prevalence of enteric and neglected tropical diseases among primary school children in Mali (n=800).

S1 Figure. Map of study school locations. Light gray regions were included in study sample; dark gray regions were not included. Dark gray circles indicate location of beneficiary schools; white circles indicate location of comparison schools.

S2 Figure. Mean pupil antibody response by grade level. Red line indicates cut-off value for infection (if it exists).

Appendix 3. The impact of water consumption on hydration and cognition among schoolchildren: Methods and results from a crossover trial in rural Mali[6]

PLOS ONE

RESEARCH ARTICLE

The impact of water consumption on hydration and cognition among schoolchildren: Methods and results from a crossover trial in rural Mali

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Abstract

Adequate provision of safe water, basic sanitation, and hygiene (WASH) facilities and behavior change can reduce pupil absence and infectious disease. Increased drinking water quantity may also improve educational outcomes through the effect of hydration on attention, concentration, and short-term memory. A pilot study was conducted to adapt field measures of short-term cognitive performance and hydration, to evaluate levels of hydration, and to investigate the impact of providing supplementary drinking water on the cognitive performance of pupils attending water-scarce schools in rural Mali. Using a cross-over trial design, data were collected under normal school conditions (control condition) on one visit day; on the other, participants were given a bottle of water that was refilled throughout the day (water condition). Morning and afternoon hydration was assessed using specific gravity and urine color. Cognitive performance was evaluated using six paper-based tests. Three percent of pupils were dehydrated on the morning of each visit. The prevalence of dehydration increased in the afternoon, but was lower under the water condition. Although there was a trend indicating drinking water may improve cognitive test performance, as has been shown in studies in other settings, results were not statistically significant and were masked by a "practice effect."

Introduction

Health and educational benefits associated with improved water, sanitation, and hygiene (WASH) in schools include reduced diarrhea, absence, acute respiratory infection, and soil-transmitted helminth infection [1–5]. The availability of water during the school day is essential for supporting personal hygiene, sanitation, and maintaining a clean school environment. Increased access to water for drinking at school may also directly affect pupils' academic performance through the cognitive benefits associated with decreased dehydration [6–8].

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A recent UNICEF report found that only 53% of schools in least developed and other low-income countries had access to adequate water facilities, highlighting a gap in access to year-round, reliable, and safe water supply in sufficient quantities to support students' needs [9]. Two studies assessing dehydration prevalence among school-age children living in hot, arid regions found that approximately two-thirds of children were in a state of moderate to severe dehydration [10, 11].

The impact of dehydration on cognitive performance is well studied among adults in experimental settings. Dehydration induced through exercise or heat stress has been associated with decreased short-term memory [6, 8], long-term memory [8, 12], arithmetic efficiency [6], visuospatial function [6], and attention [7]. Few studies have investigated the relationship between dehydration and cognition in children. Evidence from three intervention studies in the United Kingdom corroborate findings among adults, suggesting that drinking water was associated with better scores of attention [13, 14], short-term memory [14-16], and visual search [13]. However, these

studies did not collect biometric measures of hydration status. Two additional studies conducted among children in Israel and Italy that assessed hydration status through urine osmolality found that dehydration was associated with decreased short-term memory [10, 16].

Linking drinking water availability directly to cognitive skills among children in water-scarce areas would have important public health and policy implications. A deeper understanding of the relationship between hydration and cognition could provide significant and novel evidence for the importance of improving water access in schools. Here, we aim to address the gaps in existing literature by assessing the relationship between water consumption, hydration, and cognition in a setting where children do not commonly have water access during the school day.

We assessed the prevalence of dehydration among children attending schools in Mali, West Africa, and examined the effect of drinking supplementary water during the school day on hydration status and on cognitive test scores. Our hypothesis was that the majority of students would be dehydrated and that the provision of supplementary water would be associated with improved hydration and improved cognition. Methods included the piloting and refining of cognition measurements that had not been previously used in sub-Saharan African field settings. In addition, to our knowledge we collected one of the first sets of data indicating biometric levels of dehydration and reporting on the cognitive effects of dehydration in sub-Saharan Africa or elsewhere in the global South, where access to water is the poorest.

Materials and Methods

Setting

We conducted a pilot study to investigate the impact of providing supplementary drinking water on the cognitive performance of pupils in water-scarce schools in rural Mali. The purpose of this study was to 1) pilot measures of short-term cognitive performance, 2) pilot field measures of hydration, 3) pilot data collection procedures for potential inclusion in a larger trial, 4) evaluate levels of dehydration among primary school students in water-scarce settings, and 5) test the association between drinking water and hydration on various measures of cognitive performance.

Data collection took place between January 7-10 and March 4-7, 2013 at two rural primary schools within 20 km of Sikasso town, Mali. Data collection at the second school was delayed due to armed conflict within the country. The maximum high temperature for data collection was 29°C in January and 40°C in March.

School eligibility, school selection, and participant selection

Schools were eligible for inclusion if they had no water point access within 0.5 kilometers, were within 1.5 hours drive from Sikasso town, and had at least 60 students in grades three through six. Two schools meeting eligibility requirements were purposively selected based on logistical considerations.

A total of 120 pupils in grades five (ages 9-13) and six (ages 10-16) were recruited. At each school, 30 pupils from each grade were randomly selected from school rosters using random number lists. In the event a pupil was absent or did not wish to participate, we continued to select pupils randomly from the class rosters until a sample size of 30 was reached for each grade.

Study design

We employed a crossover trial design in which each pupil in the study served as his or her own control. A crossover design was selected over a randomized controlled trial design due to the logistical challenge of randomizing water distribution within classrooms. Given the novel study procedures, crowded school setting, and limited timeframe, we were not certain that we could ensure water was not shared between pupils in intervention and control groups.

Hydration and cognition measurements were collected on two different days at each school. On one of the visit days we collected data without changing any conditions at the school (the control condition). On the other visit day we provided all pupils, regardless of participation in the study, with a 1.5 litre bottle of water in the morning, encouraged them to drink throughout the day, and refilled their bottle upon request (the water condition). We did not track the amount of water each pupil consumed. To account for confounding due to becoming familiar with the test (henceforth referred to as "practice effect"), the order of intervention days was counterbalanced between

schools so that one school received water on the first day, while the other received water on the second day. Additionally, we included a separation of three days between visits.

To evaluate potential confounders or effect modifiers of hydration and cognition, participants were asked if they had anything to eat or drink that morning and reported drinking water availability at school. Staff members also made observations of drinking water availability at the school on the day of the visit. The majority of pupils went home at noon and returned for afternoon classes. We did not record lunch practices.

Measures of hydration

We collected three measures of hydration: urine specific gravity (U_{sg}), urine color (U_{col}), and self-reported thirst. Both U_{sg} and U_{col} are inexpensive measurements that can be easily conducted in the field with minimal training. They are strongly correlated with urine osmolality [17, 18], a common measure of hydration in non-laboratory settings [10, 11, 16]. U_{sg} measures urine density compared with water and was measured with ATAGO MASTER-URC/NM urine specific gravity analog refractometers (model 2793, ATAGO U.S.A. Inc., Bellevue, WA) [18]. The refractometers were calibrated using distilled water and were recalibrated at least every 15 readings, according to manufacturer instructions. U_{col} was measured against a validated scale of eight colors [17, 18]. Two trained enumerators independently evaluated each sample, and re-evaluated the sample together if their independent values differed; a third trained enumerator was consulted if no

consensus was reached. Self-reported thirst [13, 19] was collected on a five-point pictorial scale based on the Wong-Baker FACES pain rating scale [20]. For analysis, the least-thirsty image was assigned a value of 5 and values decreased to 1 as reported thirst increased.

Pupils provided urine samples between 8 and 9 am and again between 2-3 pm on each day of data collection. All urine analyses were conducted on the school grounds by trained study enumerators. Pupils self-reported thirst in the afternoon, after the completion of cognitive testing.

Measures of cognition

Cognition was measured using six tasks that assessed visual attention, visual memory, short-term memory, and visuomotor skills. These tests were taken from previous research on hydration and cognition that was conducted with children in Israel and the United Kingdom [10, 13, 14], piloted in Mali, and adapted to the Malian context.

Letter cancellation. This test assesses *visual attention.* Pupils were given a grid containing target letters randomly dispersed among non-target letters and were given one minute to cross out as many target letters as possible. Scores were calculated by subtracting the number of non-target letters identified from the number of target letters identified; the maximum test score was 38.

Direct image difference. This test assesses *visual attention*. Two nearly identical pictures were presented side-by-side. Pupils were given one minute to circle differences between the two images.

Scores were calculated by subtracting the number of incorrect differences identified from the number of correct differences identified; the maximum test score was 9.

Indirect image difference. This test assesses *visual memory*. Two nearly identical pictures were presented in sequence. Pupils were given ten seconds to study the first image. They were then briefly presented with a blank page, followed by a second image, and given one minute to circle the differences between the two images on the second image, without returning to the first. Scores were calculated by subtracting the number of incorrect differences identified from the number of correct differences identified; the maximum test score was 9.

Forward digit recall. This test assesses *short-term memory*. Twelve sequences of numbers two to seven digits in length were read aloud to pupils at a rate of one number per second. Pupils were asked to write down the sequence in order after the sequence was read aloud. Two scores were derived from this test: the total number of correctly recalled sequences (maximum score of 12) and the maximum digit span of the correctly recalled sequence (maximum score of 7).

Reverse digit recall. This test assesses *short-term memory*. Ten sequences of numbers two to five digits in length were read aloud to pupils at a rate of one number per second. Pupils were asked to write down the sequence in reverse order after the sequence was read aloud. Two scores were derived from this test: the total number of correctly recalled sequences (maximum score of 10) and the maximum digit span of the correctly recalled sequences (maximum score of 5).

Line tracing task. This test assesses *visuomotor skills*. Pupils were presented with two curved parallel lines. They were given fifteen seconds to draw a line between them as quickly as possible while attempting not to touch the printed lines. Scores were calculated by subtracting the number of times the pupil's line touched the side from the total length of the line in centimeters; the maximum test score was 29.

All cognitive tests were paper-based and administered by trained study staff in a group setting within the school classrooms. Testing sessions were standardized using written scripts. Staff introduced each test with a scripted explanation and an example, with no breaks between tests. Testing sessions lasted a total of 60-75 minutes and began at 3:00 pm in the afternoon of each visit. Each pupil in the study completed the testing session twice, once on the control condition day and once on the supplementary water condition day. Four parallel versions of each test were developed so that individual pupils did not receive the same test twice and pupils sitting next to each other did not receive the same test. All four test versions were distributed at each testing session. Tests were independently graded by two different staff members using fixed criteria. Grading criteria also provided guidelines to indicate whether or not pupils understood the tasks according to instruction. Tests with conflicting scores were examined by the study coordinator, who decided the final score for the task.

Data analysis

Data were entered into MS Excel and analyzed using STATA 13 SE. We tested both the impact of treatment condition (whether student was provided water or not during the day) and hydration status on change in test score. U_{sg} was used to test the impact of hydration on change in test score because it was the only of our three hydration measures based on biomarkers, and is the most accurate of those three measures of hydration status [21]. A higher U_{sg} indicates increased dehydration. Pupils were classified as dehydrated if they had a U_{sg} of 1.020 or higher, which is equal to the dehydration threshold of urine osmology>800 mOsmol kg-1 H_2O that has been used in previous studies of dehydration among children [10, 11, 16]. A total of eight scores for the six cognitive tests were calculated according to grading criteria. Scores were coded such that higher test scores on all cognitive tests represented better performance.

Univariable analysis. As proof of concept of the effect of water provision on hydration, we evaluated univariable differences in morning and afternoon hydration, U_{sg}, and U_{col} by treatment group using McNemar's test statistic (binary variables) and paired sample t-tests (continuous variables). To evaluate the correlation between U_{sg}, U_{col}, and self-reported thirst, as well as the correlation between each of the cognitive test scores, pairwise tests of correlations between cognitive test scores were conducted using the pwcorr command. Lastly, to measure the presence of a "practice effect," paired sample t-tests were used to assess differences in cognitive test scores between school visits.

Multivariable analysis. We examined the association between the provision of supplementary drinking water (treatment) and cognitive test scores as well as the association between pupil hydration (regardless of treatment) and cognitive test scores. These associations were assessed using separate mixed-effects linear regression models, where each cognitive test was the outcome, while treatment condition or hydration status, respectively, was the predictor covariate. Models included a random intercept at the pupil level to account for pupils acting as their own control. Unstandardized Beta coefficients are presented.

All models adjusted for multiple comparisons using the Bonferroni correction; as such, associations were considered significant if they had a p-value <0.006, the alpha necessary to reach 95% significance with eight hypotheses. Models were assessed for interaction and confounding with the following variables chosen *a priori*: pupil sex, pupil grade, reported drinking in the morning, reported eating in the morning, reported thirst, and morning hydration.

Interaction was assessed by running models of each cognitive test outcome with each predictor variable, potential interaction covariate, and an interaction term for the predictor and covariate (e.g. treatment*sex). Some variables initially indicated interaction at p<0.05. However, after adjusting for multiple comparisons using the Bonferroni correction, the only effect modifier to retain significance was pupil sex, which modified the relationship between afternoon dehydration and forward number recall- maximum digit span test score. Stratified results from this model are presented. All other associations were then tested for confounding; covariates significantly

associated with the predictor variable as well as the outcome variable in independently run fixed-effect models were considered to be confounding variables. At p=0.006, grade confounded the association between treatment and direct image difference & indirect image difference test scores, so was included as a control variable in these models. All models controlled for the visit day in order to account for a "practice effect" on cognitive tests.

We compared models from all pupils to models that excluded scores from pupils who did not complete cognitive tests according to instruction. There were no significant differences between model results, thus, we present the former results in order to maximize sample size. Only students with complete data for all measures of interest were included in analysis. We dropped 13 pupils due to absence on the second day of data collection, not being able to provide a urine sample, or inability to match pupils test scores and hydration measures due to improper identification procedures.

Ethics

This study was approved by Emory University's Institutional Review Board (IRB00062354), the Mali Ministry of Education, and the National Technical and Scientific Research Center (*Centre National de la Recherche Scientifique et Technique*) in Mali (001/2013-MESRS/CNRST). All three institutions approved consent *in loco parentis* (in the place of parents) due to the logistical challenges of finding and contacting parents in their homes, risk of lost wages to parents if they

were summoned to school, and low levels of literacy making letters unfeasible. Permission for study activities and approval of a waiver of parental consent was also obtained from the *Centres d'Animation Pédagogique* (Center for Pedagogical Activity) and *Académie d'Enseignement* (Academy of Education) in Sikasso, both local government representatives responsible for education in the area where the study was conducted. Prior to commencing study activities at each school, we obtained consent in *loco parentis* from the school director and the *Comité de Gestion Scolaire* (school management committee), the organization empowered to oversee management and activities at the school, on behalf of the community that school serves. Pupils who were selected for the study provided informed verbal assent in a private setting prior to the start of data collection activities.

Results

Study population

Data were collected from 120 pupils in two schools; of these, 107 (89.2%) pupils had complete data and were included in analysis. The sample was initially comparable in terms of sex, grade, and school. After removing pupils with incomplete data (n=13), the final sample included 46 (43.0%) girls, 61 boys (57.0%); 58 (54.2%) pupils from grade five, 49 (45.8%) pupils from grade six; 47 (43.9%) from School 1, and 60 (56.1%) from School 2. The mean (sd) age was 11.6 (1.0) years in School 1 and 12.1 (1.7) years in School 2.

Univariable estimates of association with hydration

Only 3% of pupils were classified as dehydrated in the morning according to U_{sg} (U_{sg}>1.019), regardless of visit day or study condition. The difference between water and control condition mean morning U_{sg} or U_{col} was not statistically significant, and we found no difference in the prevalence of dehydration prior to distribution of water.

Pupils became more dehydrated throughout the school day under both study conditions. There was no significant difference in U_{col}, self-reported thirst, or the prevalence of pupils classified as dehydrated in the afternoon under the water condition compared to the control condition. However, mean afternoon U_{sg} was significantly higher under the control condition compared to the water condition (Table A3.1).

U_{sg} and U_{col} were strongly correlated both in the morning (r=0.777, p<0.001) and afternoon (r=0.734, p<0.001). Self-reported thirst, which was only measured in the afternoon, was not significantly correlated with either afternoon U_{sg} (r=0.089, p=0.20) or afternoon U_{col} (r=-0.003, p=0.97).

Table A3.1. Univariable associations between hydration indicators and study condition (n=107)

	Water	**Control**	***p****
Mean (SD) morning urine specific gravity (U_{sg})	1.008 (0.01)	1.007 (0.01)	0.35
Mean (SD) morning U_{col} (scale 1-7)	2.34 (1.54)	2.29 (1.24)	0.77
Dehydrated† in morning	4 (3.7%)	2 (1.9%)	0.69
Mean (SD) afternoon U_{sg}	***1.010 (0.01)***	***1.014 (0.01)***	***<0.01***
Mean (SD) afternoon U_{col} (scale 1-7)	3.10 (1.82)	3.44 (1.43)	0.11
Dehydrated† in afternoon	12 (11.2%)	17 (15.9%)	0.38
Mean (SD) afternoon self-reported thirst (scale 1-5)	3.2 (1.5)	3.1 (1.6)	0.21

**p*-value based on McNemar's test statistic for binary variables and paired sample t-tests for continuous variables

†Pupils with a U_{sg} >1.019 classified as mildly dehydrated

Bold values indicate a significant association at α=0.05

Univariable estimates of association with cognition

Results from pairwise tests of correlations between cognitive test scores and results from the paired t-tests of the association between test score and visit day are shown in Table A3.2. Most tasks were significantly correlated with at least one other task included in the battery of cognitive tests. Students achieved significantly higher scores on the second visit compared to the first visit for six of the eight cognitive tests, regardless of treatment condition.

Multivariable estimates of association between cognitive test scores and treatment condition

In adjusted models, the provision of supplementary drinking water was significantly associated with two cognitive tests: reverse number recall (total) and line trace. Under the water condition, pupils performed better on the reverse number recall test. However, pupils had lower scores on the line trace test under the water condition (Table A3.3).

Multivariable estimates of association between cognitive tests scores and hydration status

We examined the impact of hydration on cognitive test performance, regardless of treatment condition. Neither hydration status, where a Usg greater than 1.019 indicated dehydration, nor Usg were significantly associated with any cognitive test score (Table A3.3). The test for interaction indicated that pupil sex significantly modified the association between forward number recall (maximum) and afternoon dehydration. When stratified by sex, males performed worse when dehydrated (β=-0.14; 95% CI -0.54, 0.27; p=0.501) and females performed better when dehydrated (β=1.10; 95% CI 0.31, 1.89; p=0.006); only the association between hydration and forward number recall among female pupils approached statistical significance.

Table A3.2. Correlation matrix of cognitive test scores' pairwise correlation coefficients and univariable associations between visit day and mean (standard deviation) of cognitive test scores

	Pairwise Correlation Coefficients								**Mean (SD) test scores**		
	LC[1]	**DID[1]**	**IID[2]**	**NFC[3]**	**NFM[3]**	**NRC[3]**	**NRM[3]**	**LT[4]**	**Visit 1**	**Visit 2**	***p****
Letter cancellation (LC)[1]	1								***18.9 (7.6)***	***26.3 (7.0)***	***<0.01***
Direct image difference (DID)[1]	0.1890	1							***1.4 (1.4)***	***2.3 (1.6)***	***<0.01***
Indirect image difference (IID)[2]	0.1668	***0.3137***	1						***1.6 (1.5)***	***2.3 (1.6)***	***<0.01***
Forward number recall- total (NFC)[3]	0.1465	0.1775	***0.2985***	1					***5.0 (1.4)***	***5.5 (1.5)***	***<0.01***
Forward number recall- maximum digit span (NFM)[3]	0.1360	***0.2228***	***0.2975***	***0.7235***	1				***4.1 (1.0)***	***4.3 (1.0)***	***0.04***
Reverse number recall- total (NRC)[3]	0.0870	0.0885	0.2052	***0.3310***	***0.2473***	1			3.4 (1.8)	3.6 (1.6)	0.64
Reverse number recall- maximum digit span (NRM)[3]	0.1013	0.0234	***0.2128***	***0.2208***	0.1705	***0.8440***	1		3.2 (1.3)	3.3 (1.1)	0.41
Line trace (LT)[4]	***0.2302***	0.1049	0.0947	0.0704	0.0961	-0.0693	-0.0858	1	***13.4 (7.8)***	***17.2 (6.0)***	***<0.01***

Bold values indicate a significant association at α=0.05

**p*-value based on paired t-tests

Target skills assed by test: [1]visual attention; [2]visual memory; [3]short-term memory; [4]visuomotor skills

Table A3.3. Mixed effects linear regression models of associations between treatment group, afternoon measures of hydration, and cognitive performance (n=107)

Cognitive Test	Treatment			Dehydrated*			Urine Specific Gravity (U_{SG})		
	Beta	95% CI	p	Beta	95% CI	p	Beta	95% CI	p
Letter cancellation	0.36	-0.81, 1.53	0.545	-1.95	(-4.23, 0.32)	0.092	-64.98	(-177.47, 47.51)	0.258
Visit (ref: Visit 1)	***7.43***	***6.26, 8.59***	***<0.001***	***7.40***	***(6.26, 8.54)***	***<0.001***	***7.28***	***(6.12, 8.44)***	***<0.001***
Image difference, direct	0.25	-0.10, 0.60	0.163	-0.47	(-1.05, 0.10)	0.103	-18.22	(-46.28, 9.84)	0.203
Visit (ref: Visit 1)	***0.90***	***0.55, 1.25***	***<0.001***	***0.87***	***(0.52, 1.22)***	***<0.001***	***0.84***	***(0.49, 1.19)***	***<0.001***
Pupil grade (ref: 5th grade)	***0.77***	***0.36, 1.19***	***<0.001***	--	--	--	--	--	--
Image difference, indirect	0.26	-0.09, 0.61	0.151	0.30	(-0.29, 0.89)	0.323	6.81	(-22.33, 35.95)	0.647
Visit (ref: Visit 1)	***0.74***	***0.39, 1.09***	***<0.001***	***0.71***	***(0.36, 1.06)***	***<0.001***	***0.72***	***(0.37, 1.08)***	***<0.001***
Pupil grade (ref: 5th grade)	***0.70***	***0.25, 1.15***	***0.002***	--	--	--	--	--	--
Number recall total, forward	-0.01	-0.32, 0.30	0.943	0.22	(-0.32, 0.77)	0.420	17.35	(-9.31, 44.02)	0.202
Visit (ref: Visit 1)	***0.52***	***0.21, 0.83***	***0.001***	***0.52***	***(0.21, 0.83)***	***0.001***	***0.55***	***(0.24, 0.86)***	***0.001***
Number recall maximum digit span, forward	0.01	-0.20, 0.22	0.936	0.17	(-0.20, 0.55)	0.359	4.61	(-13.70, 22.93)	0.621
Visit (ref: Visit 1)	***0.23***	***0.01, 0.44***	***0.038***	0.22	(0.01, 0.44)	0.040	0.23	(0.02, 0.45)	0.034
Number recall total, reverse	***0.61***	***0.19, 1.03***	***0.005***	0.19	(-0.48, 0.87)	0.577	-8.29	(-41.39, 24.80)	0.623
Visit (ref: Visit 1)	0.18	-0.25, 0.60	0.412	0.10	(-0.33, 0.53)	0.648	0.09	(0.35, 0.53)	0.687
Number recall maximum digit span, reverse	0.17	-0.15, 0.48	0.296	0.08	(-0.39, 0.54)	0.746	3.12	(-19.64, 25.89)	0.788
Visit (ref: Visit 1)	0.15	-0.16, 0.47	0.346	0.13	(-0.18, 0.44)	0.415	0.14	(0.18, 0.45)	0.399
Line trace	***-4.48***	***-5.87, -3.08***	***<0.001***	1.29	(-1.38, 3.96)	0.344	79.16	(-53.10, 211.41)	0.241
Visit (ref: Visit 1)	***-4.48***	***1.93, 4.73***	***<0.001***	***3.81***	***(2.20, 5.43)***	***<0.001***	***3.96***	***(2.34, 5.58)***	***<0.001***

*Pupils with a U_{SG} >1.019 classified as dehydrated

Bold values indicate a significant association at α=0.006, the level of 95% significance after correcting for multiple comparisons

Models include a random intercept at the pupil level to account for clustering

Discussion

We conducted a cross-over trial as part of a pilot study to examine the associations between water consumption, hydration, and cognition among pupils attending water-scarce schools. We successfully adapted measures of cognitive performance that could be completed by children in rural Malian schools and tested the feasibility of field hydration measures and data collection procedures within schools in Sub-Saharan Africa. Results demonstrated that supplementary water

provision within a school setting significantly decreased U_{sg}, even within a short time period. However, we found no effect of the impact of supplementary water provision on cognitive test scores.

This research refined a battery of cognitive tests for use with children in Mali which can be adapted to other developing settings. Research conducted in the U.K. concluded that their cognitive test of visual memory was too easy for the target population, indicated by many children achieving the maximum score on the test, and thus modifying study results [13]. Our results show that the percentage of children achieving the maximum score or the minimum score on any of the cognitive tests ranged from 0.5%-15.4% and 0.5-4.2%, respectively, indicating that the cognitive tests adapted for this trial were neither too difficult nor too hard. However, results from our pairwise tests of correlation indicate that the two tests measuring visual attention (letter cancellation and direct image difference) were not significantly correlated, suggesting that further adaptation may be needed on these tests to measure this target skill. Furthermore, while scores for each of the four tests measuring short-term memory were significantly associated with at least one other score in the suite of tests measuring that domain, they were very similar tests in that they all incorporated number recalls. Thus, correlation does not necessarily indicate that they were in fact measuring the cognitive skill they were intended to measure.

This is one of the first studies to employ existing field methodology to collect urine samples and measure dehydration among school children in low-resource school settings. Results from this

pilot study were further refined in a subsequent trial in Zambia [22]. Prior research on dehydration among schoolchildren has relied predominantly on self-reported thirst as their measure for dehydration. Although evidence- particularly among healthy individuals- is limited, research has concluded that one's thirst response is not an accurate measure of hydration [23, 24]. We found no research investigating this association among children. Our results demonstrated no significant difference between self-reported thirst among pupils under the water condition compared to the control condition, even though the measurements of U_{sg} indicated that pupils under the water condition had significantly higher levels of hydration than pupils under the control condition. Additionally, self-reported thirst and the biometric measurement of U_{sg} were not significantly correlated. These findings support previous literature concluding that self-reported thirst is not an accurate measure of hydration. Given our findings, future research should consider utilizing only measurements that provide biometric evidence of dehydration. Data also revealed that U_{col}, although strongly correlated with U_{sg}, did not capture a significant difference in afternoon hydration between water and control conditions. We believe this may have been due to the subjective nature of matching urine color to the color chart. The use of refractometers to measure U_{sg} required less training and took less time than measuring U_{col}, and thus is recommended for future studies investigating dehydration levels of subjects in low-resource settings.

Our finding that only 2.8% of pupils were dehydrated in the morning stands in stark contrast to previous research which reported that 84% of Italian school children [16], 68% of Israeli school

children [11], and 43% of Zambian schoolchildren [22] were dehydrated at the beginning of the school day. While this result was initially surprising, it may be partly explained by evolutionary mechanisms. In their research, Bar-David reported that among their sample of Israeli schoolchildren, Bedouin children, who originate from a population that has lived in the desert for many generations, had the lowest mean urine osmolality (the lowest prevalence of dehydration), possibly because their bodies adapted over time to have a lower threshold of thirst [10, 11]. Thus, Malian children, who reside in hot, arid, and water-scarce environments, may have also adapted a greater resistance to dehydration, leading to a lower prevalence of dehydration at the beginning of the school day. Extremely low levels of morning dehydration may also be partly explained by the fact that a vast majority of students (93%) reported drinking something in the morning before going to school. We do not believe that pupils intentionally consumed more water than usual in preparation for participation in the research. Neither school officials nor pupils were aware of the study topic, activities, or pupil selection prior to the first day of the study. Thus, participants would not have had the foreknowledge to alter their normal drinking behaviors. Although school officials and pupils were aware of the date of the second visit, given that no significant differences in the prevalence of dehydration or U_{sg} were observed between the first and second visits, it is unlikely that students changed their drinking practices for the second day.

Under both treatment conditions, dehydration increased throughout the day. Pupils had significantly lower U_{sg} in the afternoon under the supplementary water condition than under the

control condition, demonstrating the "proof of principle" that supplementary water provision improves hydration. However, there was no significant difference in the prevalence of afternoon dehydration among pupils in the water group compared to pupils in the control group. Nonetheless, when the significant impact of water consumption on increasing U_{sg} is considered in light of findings of the relationship between drinking water and cognition from other contexts [13-16], there is evidence that providing drinking water at school may create a positive impact on pupil learning.

We found some evidence that supplementary water provision was associated with higher scores on cognitive tests, but few results were significant. These results are consistient with those from our follow-up trial among primary school children in Zambia [22]. Treatment was significantly associated with higher scores on the letter cancellation task, a result supported by previous literature that also found a positive relationship between provision of drinking water and performance on visual attention tasks [14, 22]. While previous studies have reported no significant association between water provision and visuomotor skills [13, 14], we found that scores on the line trace test were significantly, but negatively associated with supplementary water provision. Although this result was unexpected, it may be largely explained by a practice effect, in which pupils performed significantly better the second time they took the test, regardless of treatment condition. Although pupils took a different version of the test on each day, a practice effect was evident, as test scores significantly improved when pupils performed each task the second time.

One possible reason for this difference could be that pupils in Mali are not accustomed to the types of activities performed during the tests, which were adapted from tests used in Western settings. Although the distribution of test scores and the correlation of tests measuring the same domain do indicate that the tests were suitably adapted to the context, the novelty of the tests may have caused a much lower baseline score at the first testing session. Pupils may need to practice completing the tasks several times in order to fully understand the tests before their scores are measured.

Lastly, evidence on the degree and duration of dehydration necessary to impact cognitive performance is limited. It is possible that the lack of significant improvements in cognitive performance following treatment is because one school day of supplementary water provision is not sufficient to reverse the impacts of chronic dehydration and impart cognitive benefits on schoolchildren; perhaps more long term water consumption is necessary for these benefits to be measurably improved [22]. Further, although the U_{sg} data provide evidence that pupils drank under the treatment condition, we did not measure the volume of water consumed by subjects. Measuring the volume of water consumed by subjects and including a dose-response measure in the analysis could contribute to the discourse on how much water consumption is needed to improve hydration, and how much hydration is needed to improve cognition.

Limitations

There are several limitations to the current research. First and most crucial was the impact of the practice effect, in which pupils performed significantly better on cognitive testing during the

second visit, regardless of treatment condition. Approaches to limit or account for the practice effect on cognitive testing in primary school populations residing in settings where this type of testing is uncommon requires additional attention; future research should focus on alternative trial designs to minimize this impact. Additionally, the fixed test order could have led to a learning effect across tests, where certain tests- conceivably later on in the series- revealed a more significant association due students becoming more comfortable with testing in general, rather than due to the skill tested. Students in both the intervention and control would have had the same learning effect, which would bias our results to the null, but there is no way to control for this within the individual models. However, we observed no trend where students performed differently on tests administered in the end of the suite on either testing day. Further, we reviewed the estimates of effect and do not find any effect modification. Second, because this was a pilot study, the sample was limited to 120 pupils in two schools. As such, the study may not have been sufficiently powered to detect significant but less strong impacts of supplementary water provision or hydration status on cognitive performance. Low levels of dehydration across study groups may have also further limited our ability to detect an impact. Third, we conducted an intention-to-treat analysis and did not measure or control for the volume of water consumed by the participants in the treatment group. We did not measure whether pupils in the control group consumed water brought from home, and we could not ethically restrict them from drinking water. We also did not record lunch practices among students, and cannot guarantee that children did not consume water when they went home for lunch. As such, we cannot unequivocally state that the intervention and

control groups were separated by water consumption, or lack thereof. However, afternoon U_{sg} was collected regardless of treatment condition, and results validate the degree of water consumption under treatment. Additionally, lunch practices among individual students would likely be similar across days, thus the influence of lunch practices would be consistent across test conditions since pupils act as their own controls. Fourth, due to external events, data collection at the second school was delayed for two months and occurred during a warmer period. The higher temperatures during the second data collection period may have impacted study results. Evidence suggests that exposure to heat may independently impact cognitive functions, however this research has not been conducted among children [25-27]. Although significantly more pupils in the second school were dehydrated in the afternoon compared to pupils in the first school, due to the crossover design, it is not possible to quantify the effect that temperature may have had on study outcomes. Last, the methodology, including the duration of tests, were adapted from cognitive tests previously used among primary school children [13, 14, 28], but the total testing time was longer than in previous studies due to the novelty of the tests in the population and our emphasis on explanation and examples. However, because there was no significant trend in scores across the testing suite, there is no evidence that performance worsened due to fatigue among students.

We suggest a two-step approach for collecting further evidence on hydration and cognition among pupils in water-scarce schools. First, we recommend implementing a second trial with cognitive testing methodology that addresses the challenges of the practice effect in order to increase the

evidence base on the link between hydration and cognition among schoolchildren in water scarce areas. Once the link between improved hydration and cognition among schoolchildren has been established under experimental conditions, we recommend carrying out cross-sectional hydration testing in a larger sample of schools. Considering the apparent invalidity of self-reported thirst and the subjective nature of urine color evaluation, we recommend the use of urine specific gravity or another objective biometric measure for hydration testing. Given the evidence previously established, hydration in this case would serve as an easily quantified and measured proxy for pupil attention, memory, and concentration. Findings from this investigation could provide evidence of the benefit of drinking water access, and specifically on the construction of water points on school grounds, for pupils' educational attainment.

Conclusions

This study represents novel research across multiple scientific disciplines and development sectors, and is an important step in developing clear and direct linkages between provision of WASH in schools and learning. Results demonstrated the proof of principle that increased water access improves hydration. Although we found no evidence for our hypothesis that improvements in hydration status leads to improvements in cognitive performance among pupils in water-scarce schools, results may have been masked by a strong practice effect, and the power to detect significant differences was limited. We demonstrated the feasibility of collecting biometric measurements of hydration status and testing cognitive abilities in resource-poor settings. Findings

from this research and subsequent studies of hydration and cognition have broad significance for advocacy for international development and health sectors for increased attention to insufficient access to water supply for school children.

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Supporting Information

S1 File. Data.