Risk Factors for Enteric Pathogen Exposure among Children in Black Belt Region of Alabama, USA

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Learning Objectives

Upon completion of this activity, participants will be able to:

- Assess characteristics of the current study examining the prevalence of enteric pathogens among children and adolescents
- Distinguish the prevalence of enteric pathogens among children and adolescents in the current study
- Compare rates of positive tests for different enteric pathogens in the current study
- Evaluate variables associated with a higher rate of positive testing for at least 1 pathogen in the current study

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We collected stool from 488 children from 352 households living in the Black Belt region of Alabama, USA, where sanitation infrastructure is lacking. We used quantitative reverse transcription PCR to measure key pathogens in stool that may be associated with water and sanitation, as an indicator of exposure. We detected genes associated with ≥1 targets in 26% of specimens, most frequently *Clostridioides difficile* (6.6%), atypical enteropathogenic *Escherichia coli* (6.1%), and enteroaggregative *E. coli* (3.9%). We used generalized estimating equations to assess reported risk factors for detecting ≥1 pathogen in stool. We found no association between lack of sanitation and pathogen detection (adjusted risk ratio 0.95 [95% CI 0.55–1.7]) compared with specimens from children served by sewerage. However, we did observe an increased risk for pathogen detection among children living in homes with well water (adjusted risk ratio 1.7 [95% CI 1.1–2.5]) over those reporting water utility service.

Outside cities and towns served by conventional sewerage, many residents in the rural Black Belt region of Alabama, USA, have failing or inadequate sanitation infrastructure (*1*,*2*). This region was named after its rich black soils, which are typically high in clay content, limiting subsurface infiltration (*3*) and leading to surface discharge of domestic wastewater. Compounding those challenges is a high rate of poverty; 9 of the 10 poorest counties in Alabama are in the Black Belt region (*2*,*4*). Because common alternatives to septic systems are unaffordable (*5*,*6*), many residents use failing systems or lack systems altogether (*7*,*8*). Straight piping (i.e., direct discharge of untreated fecal wastes to the environment) of domestic wastewater is common (*7*).

When human fecal wastes are not safely managed, they may be transported to the environment through well-understood fecal–oral pathways (i.e., drinking water, soils, flies, food, fomites, and hands) (*9*,*10*). For households reliant on straight pipe discharge of wastewater, direct exposure to this waste may be more likely than for households served by a septic system (*8*). Those same households and their communities may also suffer from exposures further downstream. Inadequate treatment of fecal wastes can result in enteric pathogen transport through soil into groundwater and exposure through drinking water (e.g., well water) (*11*,*12*). Other exposures may include fecally contaminated soils (*13*), flies that feed on and reproduce in human feces (*14*,*15*), and contaminated food (*10*). Such exposures can result in infection with enteric pathogens, which is a necessary precondition for diarrheal disease and other sequelae, including environmental enteric dysfunction (*16*), growth deficits (*17*), cognitive impairment (*18*), and negative effects on the immune system (*19*).

Poor sanitation and persistent exposure to fecal wastes, particularly in the context of a state and nation with ample resources to address the issue (*20*), represents a public policy failure (*7*,*21*) affecting human health, dignity, and quality of life. Although the evidence base for public investment in sanitation on health grounds has a long history (*22*), the health burden attributable to poor rural sanitation in the United States remains poorly characterized, constraining the case for action. To determine the potential roles of rural sanitation improvements or other interventions in controlling disease transmission, a useful first step is estimating prevalence of enteric infections and identifying risk factors associated with them. Because of documented poor sanitation conditions in Alabama's Black Belt region (*5*,*7*,*8*) and the associated potential persistence of endemic enteric infection (*23*–*25*), we conducted a cross-sectional study to assess the prevalence of stool-based enteric pathogen detection in children using molecular methods, as an indicator of previous exposure. We further sought to identify potential household-level environmental risk factors for exposure to those pathogens to understand the potential role of infrastructure in protecting public health in this underserved region.

Methods

Study Site and Participants

This study was nested within a larger cross-sectional helminth surveillance study in rural Alabama (*26*). Participants were children 2–18 years of age living in 3 counties in the Black Belt (Lowndes, Wilcox, and Perry Counties). All children included in a household were requested to participate. The study used principles of community-based participatory research to work with stakeholders in co-creation of the project (*27*). Several meetings and focus groups were held with community partners and study collaborators to help guide study protocols, recruitment methods and materials, and participant enrollment. Participants were enrolled during January 2019–December 2021 (*26*).

We provided participants with an at-home stool collection kit. For 3 separate bowel movements, participants filled and returned 1 50-mL collection tube containing 15 mL of zinc polyvinyl alcohol (Zn-PVA) (*28*) with ≈15 g of stool and another tube containing 15 mL of 10% formalin with an additional 15 g of stool (Parapak; Meridian Bioscience). Participants received $150 on a prepaid debit card for their participation. In

addition, participants completed a brief paper questionnaire regarding their demographic characteristics, household sanitation infrastructure, and potential exposures. Before March 2020, the questionnaire was completed at the time of enrollment; after March 2020, the questionnaire was completed by the participant at home and mailed to the study team.

Molecular Analysis

During January 2019–November 2020, specimens were shipped at ambient conditions to Georgia Institute of Technology (Atlanta, GA, USA); during December 2020–December 2021, specimens were shipped at ambient conditions to the University of North Carolina at Chapel Hill (Chapel Hill, NC, USA). Upon receipt, we visually screened specimens for indicators of nonhuman origin (i.e., animal hair, dirt, color, and atypical morphology), homogenized them with sterile inoculating loops (VWR; Radnor) and stored them at 4°C for further analysis. By using the QIAamp 96 Virus QIAcube HT Kit (QIAGEN), which included a pretreatment step with Precellys SK38 bead beating tubes (Bertin Technologies) (*29*–*31*), we extracted total nucleic acids from ≈150 mg of the stool Zn-PVA mixture. We typically extracted specimens within 1–4 weeks of receipt (median 15 days, interquartile range 8–28 days). We analyzed extracts from specimens suspected to potentially be from nonhuman sources by using dPCR (QIAcuity 4; QIAGEN) for human mitochondrial DNA, using a previously validated method that has high sensitivity and specificity for human feces (*32*). Among children who submitted >1 stool specimen, we randomly selected a single replicate for extraction. We randomly selected ≈5% of stools for duplicate extraction and another 3% for extraction from multiple replicates. We included ≥1 extraction-negative control (*33*) during each day of extractions. We spiked specimens with 10^7 copies of bacteriophage MS2 and 10^6 gene copies of synthetic DNA (IDT) as extraction-positive controls. We stored extracts at –80°C until analysis.

We measured 30 enteric pathogens in specimens by using a custom TaqMan Array Card (TAC) on a Quantstudio 7 Flex (ThermoFisher) at the University of North Carolina at Chapel Hill, according to the methods described by Liu et al. (*34*). Targets were *Acanthamoeba* spp., adenovirus 40/41, astrovirus, *Balantidium coli*, *Blastocystis* spp., *Cystoisospora belli*, *Cyclospora cayetanensi*, *Campylobacter jejuni* or *C. coli*, *Clostridioides difficile*, *Cryptosporidium* spp., *Enterocytozoon bieneusi*, *Escherichia coli* O157:H7, *Encephalitozoon intestinalis*, *Entamoeba hystolytica*, *Entamoeba* spp., enteroaggregative *E. coli*, enteropathogenic *E. coli*, enterotoxigenic *E. coli*, *Giardia* spp., *Helicobacter pylori*, hepatitis A virus, *Shigella* spp. or enteroinvasive *E. coli*, norovirus, *Plesiomonas shigelloides*, rotavirus, *Salmonella* spp., sapovirus, SARS-CoV-2, Shiga toxin–producing *E. coli*, and *Yersinia enterocolitica*. We prepared the TAC by combining 40 µL of template with 60 µL of AgPath-ID One-Step RT-PCR Reagents (Applied Biosystems). We evaluated TAC performance by using an 8-fold dilution series (10^9–10^2 gene copies/reaction) of an engineered combined positive control developed by using methods from Kodani and Winchell 2012 (*35*). We used 2 plasmids (GeneArt), including 1 specifically for DNA targets. We linearized the other with a BshT1 restriction enzyme (ThermoFisher) and transcribed it (MEGAscript T7 Transcription Kit and MEGAclear Transcription Clean-Up Kit, both from ThermoFisher) to generate RNA control material, which we quantified by using a Qubit RNA HS Assay Kit on Qubit 4 Fluorometer (ThermoFisher). The linearity and efficiency for 28 of the 30 targets were within normative standards (linearity 0.97–1.0, efficiency 87%–102%) (Appendix Tables 1–3, Figure 1, https://wwwnc.cdc.gov/EID/article/29/12/23-0780-App1.pdf). The assays for hepatitis A virus and adenovirus 40/41 did not perform well, and we excluded them from our analysis.

Each day of TAC analysis, we included ≥1 positive and negative control (either an extraction-negative control or a PCR-negative control). We determined quantification cycle values through manual thresholding and included comparison of each specimen's fluorescent signal against the daily negative and positive controls (Appendix Figure 1). We categorized any target that amplified past a quantification cycle of 35 as negative to reduce the potential for false positives (*34*). To examine the effect of our preservation medium on the probability of detecting our targets of interest, we measured recovery of *Giardia duodenalis* and *Shigella sonnei* from stool by using different preservative conditions over a period of 8 weeks (Appendix).

Data Analysis

To perform Poisson regression, we used generalized estimating equations with robust SEs that accounted for clustering among children living in the same household. This method estimated unadjusted and adjusted risk ratios with 95% CIs. We created a directed acyclic graph on the basis of the variables included in the questionnaire where independent variables were biologically plausible predictors of the dependent variable, which was the detection of nucleic acids from ≥1 enteric pathogen in stool (Appendix Figure

2). Independent variables that met this criterion were the household's sanitation infrastructure, whether the household paid a water bill (i.e., a proxy measure indicating a connection to a water utility), reported raw sewage in the home, and the child's screen time, sex, history of international travel, and age. We generated adjusted estimates from a single model that included all independent variables. We used multiple imputation by chained equations (MICE package in R [*36*]) with 10 multiple imputations and the predictive mean matching method to account for missing data in the generalized estimating equations model.

Ethics Considerations

We obtained written informed consent from each participant's legal guardian and assent from children ≥7 years of age. The study protocol was approved by the Institutional Review Boards of the University of Alabama at Birmingham (approval no. 300002219), Georgia Institute of Technology (approval no. H19021), and the University of North Carolina at Chapel Hill (approval no. 20-3212).

Results

Questionnaire

We enrolled 488 children from Wilcox (237 participants from 181 households), Lowndes (101 participants from 50 households), and Perry Counties (86 participants from 55 households) (Table 1). Most children identified as Black or African American (91% [444/488]); few identified as White (2% [9/488]), preferred not to answer (1% [6/488]), identified as Black and White (<1% [2/488]), or were unsure (<1% [2/488]). Almost half of households (47% [164/352 households) enrolled multiple children (63% [306/488] participants). The median age of enrolled children was 11 years (range 2–18 years, interquartile range 8–14 years). A septic tank system was the most reported sanitation infrastructure (42% [207/488] of participants, 39% 137/352 of households), followed by a sewer connection (23% [111/488] of participants, 20% [72/352] of households,), whereas 11% (56/488) of respondents (11% [39/352] of households) reported straight piping wastewater onto their property. Few participants reported not paying a water bill (14% [67/488] of participants, 14% [48/352] of households), an indicator of household-based well water usage. As a proxy for time spent indoors, participants most often reported >4 hours of screen time per day (42% [203/488]), followed by 2–4 hours (37% [182/488]) and <2 hours (15% [72/488]).

Reverse Transcription Quantitative PCR

We detected target-specific nucleic acids from ≥1 pathogen in 26% (127/488) of children's stool specimens

Table 1. Demographic characteristics of 488 children and water infrastructure summary based on self-administered surveys conducted in Lowndes, Wilcox, and Perry Counties, Alabama, USA, January 2019–December 2021*

Variable and response	Value
Race	
Black or African American	444 (91)
White	9 (1.8)
Prefer not to answer	6 (1.2)
Black and White	2 (0.4)
Unsure	2 (0.4)
No response	25 (5.1)
Ethnicity	
Not Hispanic or Latino	407 (83)
Prefer not to answer	16 (3.3)
Hispanic or Latino	7 (1.4)
Unknown	6 (1.2)
No response	52 (11)
County	
Wilcox	237 (56)
Lowndes	101 (21)
Perry	86 (17)
No response	66 (14)
Household receives water bill	
Yes	385 (79)
No	67 (14)
Don't know	6 (1.2)
No response	430 (6.1)
Household sanitation	
Septic tank	207 (42)
Sewer connection	111 (23)
Don't know	80 (16)
Straight pipe	56 (11)
Cesspit	2 (0.4)
Other	1 (0.1)
No response	31 (6.3)
Raw sewage in yard or home in past year	
No	400 (82)
Yes	38 (7.8)
No response	50 (10)
History of international travel in past year	
No	448 (92)
Yes	13 (2.7)
No response	27 (5.5)
Sex	
M	236 (48)
F	229 (47)
No response	23 (4.7)
Daily screen time, h	
<2	72 (15)
2–4	182 (37)
>4	203 (42)
No response	31 (6.4)
Age, y	
Mean (SD)	11 (4.1)
Median (interquartile range)	11 (8–14)
Range	2–18
No response	37 (17.6)
Ever treated for an intestinal parasite	
No	418 (86)
Don't know	45 (9.2)
Yes	12 (2.5)
No response	13 (2.7)

*Values are no. (%) except as indicated.

(Table 2), most frequently *C. difficile* (6.6% [32/488]), atypical enteropathogenic *E. coli* (6.1% [30/488]), and enteroaggregative *E. coli* (3.9% [19/488]). We detected each viral, protozoan, fungal, and algae targets in <1.0% of specimens except for *Blastocystis* (3.7% [18/488]) and norovirus genotype group I or II (1.4% [7/488]). We observed perfect agreement in target detection among 26 specimens analyzed in duplicate (same child, same bowel movement) and 80% (12/15) agreement in pathogen detection among replicates (same child, different bowel movement). We did not observe contamination among extraction-negative controls (n = 19) and PCR-negative controls (n = 2), and our PCR-positive controls (n = 30) exhibited the expected amplification for all targets except hepatitis A virus and adenovirus 40/41.

Risk Factor Analysis

We found no association between pathogen detection in samples from participants who reported using a straight pipe or septic tank compared with those served by a sewer connection (Table 3). The only statistically significant association we observed, according to the conventional definition of significance (*37*), was that participants from households that did not pay a water bill (a proxy for well water consumption) had a greater risk (adjusted risk ratio [aRR] 1.7 [95% CI 1.1–2.5]) of detection of ≥1 pathogen than did participants from households that reported paying a water bill. Although not meeting conventional definitions of statistical significance (*37*), the point estimates for 2–4 hours of screen time (aRR 0.79 [95% CI 0.51–1.2]) and >4 hours of screen time (aRR 0.73 [95% CI 0.47–1.1]) suggest that time spent indoors could be protective against enteric pathogen detection, although this observation should be interpreted with caution. We found minor differences in the regression results using only complete cases (n = 341) compared with the model that used MICE (Appendix Table 4); not paying a water bill was associated with increased risk for detecting ≥1 pathogen targets (aRR 1.8 [95% CI 1.3–2.6]), and >4 hours of reported daily screen time had a greater protective effect at the margin of significance (aRR 0.64 [95% CI 0.41–1.0]).

Table 2. Prevalence of enteric pathogens in stool specimens of children in a study conducted in Lowndes, Wilcox, and Perry Counties, Alabama, USA, January 2019–December 2021*

Type and pathogen	Prevalence, no. (%)
Any	
≥1 pathogen gene detected	127 (26)
Bacteria	
Clostridioides difficile	32 (6.6)
EPEC (atypical)	30 (6.1)
EAEC	19 (3.9)
Helicobacter pylori	11 (2.3)
EPEC (typical)	7 (1.4)
Yersinia enterocolitica	5 (1.0)
E. coli O157:H7	4 (0.8)
Plesiomonas shigelloides	2 (0.4)
ETEC	2 (0.4)
Shigella or EIEC	1 (0.2)
Salmonella	1 (0.2)
STEC	1 (0.2)
Campylobacter jejuni or coli	0
Fungus/algae	
Blastocystis	18 (3.7)
Enterocytozoon bieneusi	0
Encephalitozoon intestinalis	0
Protozoa	
Balantidium coli	3 (0.6)
Acanthamoeba	2 (0.4)
Giardia spp.	2 (0.4)
Entamoeba hystolytica	1 (0.2)
Cystoisospora belli	0
Cyclospora cayetanensi	0
Cryptosporidium	0
Entamoeba	0
Virus	
Norovirus GI or GII	7 (1.4)
SARS-CoV-2	3 (0.6)
Rotavirus	2 (0.4)
Sapovirus	2 (0.4)
Astrovirus	1 (0.2)

*EAEC, enteroaggregative *Escherichia coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; GI/GII, genotype group I and II; STEC, Shiga toxin–producing *E. coli*.

Human Stool Specimen Confirmation

One stool specimen was flagged by technicians as potentially nonhuman because of atypical morphology. In addition, we prospectively selected 51 additional specimens for screening to determine origin. All specimens were positive for human mitochondrial DNA at concentrations indicating human origin (*32*). The median concentration was $10^{3.3}$ gene copies human mitochondrial DNA per nanogram of double-stranded DNA (range $10^{1.2}$–$10^{4.7}$ gene copies/nanogram double-stranded DNA).

Zn-PVA Validation

The concentration of *Giardia* DNA we recovered from Zn-PVA decreased by 0.034 $\log_{10}$/day at ambient conditions and by 0.0037 $\log_{10}$/day in Zn-PVA at 4°C. The concentration of *Shigella* DNA we recovered from Zn-PVA decreased at ambient conditions by 0.030 $\log_{10}$/day and by 0.0085 $\log_{10}$/day in Zn-PVA at 4°C (Appendix Table 5, Figure 3).

Discussion

We detected various enteric pathogens in stool specimens from children living in the Black Belt of Alabama. Straight pipe sanitation (direct discharge of fecal wastes into the environment near households) was not associated with increased risk for stool pathogen detection compared with conventional sewerage. However, our

Table 3. Risk factors for detection of ≥1 enteric pathogen in stool specimens of children in a study conducted in Lowndes, Wilcox, and Perry Counties, Alabama, USA, January 2019–December 2021*

Variable	Reference	Exposure	RR (95% CI)	aRR (95% CI)
Pay a water bill	Yes	No	1.8 (1.2–2.5)	1.7 (1.1–2.5)
Sanitation	Sewer connection	Cesspit	NA	NA
		Other	3.4 (0.57–20)	5.2 (0.88–30)
		Septic tank	0.89 (0.61–1.3)	0.95 (0.64–1.4)
		Straight pipe	0.95 (0.55–1.6)	0.95 (0.55–1.7)
Child's screen time	<2 h	2–4 h	0.74 (0.48–1.1)	0.79 (0.51–1.2)
		>4 h	0.74 (0.48–1.1)	0.73 (0.47–1.1)
Child's sex	Male	Female	0.89 (0.65–1.2)	0.89 (0.65–1.2)
International travel in past year	No	Yes	0.89 (0.32–2.5)	0.93 (0.34–2.5)
Raw sewage in home or yard in past year	No	Yes	1.1 (0.68–1.9)	1.1 (0.66–2.0)
Child's age	<5 y	5–10 y	0.71 (0.40–1.3)	0.76 (0.41–1.4)
		>10 y	0.82 (0.47–1.4)	0.90 (0.49–1.6)

*Unadjusted RRs are from bivariate models, whereas aRRs are from full model including all covariates. aRR, adjusted risk ratio; RR, risk ratio.

finding that well water consumption was associated with an increased risk for enteric pathogen detection implicates poor sanitation in this geographic area as a possible contributor to groundwater contamination. Soils that are high in clay content undergo shrinking as they desiccate and swelling as they moisten (*3*). Those conditions may lead to fecal waste transport from failing septic tanks and straight pipe discharges through soils to the water table (*3*,*38*), resulting in exposures through drinking water. Previous work in the Black Belt observed an increased concentration of fecal contamination in well water compared with piped municipal water. In a cross-sectional study of randomly selected households in Hale County (bordering Perry County in the Black Belt), 20% of private wells were positive for fecal coliforms, compared with 8% of public water system specimens (*12*). Other studies from the region have reported fecal contamination of water supplies, possibly linked to widespread sanitation deficits (*11*,*39*,*40*).

We used detection of pathogens in stool as a proxy for carriage and as an unambiguous indicator of previous exposure (*41*), a suitable measure given the role of water and sanitation infrastructure in limiting exposures to many of the pathogens we assessed. It is important to note that detecting a pathogen in stool does not necessarily indicate the person experienced symptomatic or asymptomatic infection. For example, detecting *C. difficile* by PCR does not guarantee the presence of *C. difficile* toxin, and infection without the presence of this toxin may not result in diarrheal disease (*42*). Further, the relationship between carriage, infection, and disease is highly host- and pathogen-specific (*43*). Evidence from an international multisite study on the etiology of diarrhea in children posited that the detection of enteroaggregative *E. coli* at low concentrations in stool appeared to be protective against diarrhea, whereas detection of pathogens such as *Helicobacter pylori*, *Shigella*, and norovirus were strongly associated with diarrhea (*43*). Important microbiome-mediated interactions between and among pathogens are possible, and host responses can vary.

Compared with data for children in low- and middle-income countries, the 26% combined prevalence of enteric pathogens we observed is dramatically lower than what has been previously reported (*29*,*43*). Few studies have screened populations for multiple enteric pathogens in high-income countries outside of clinical settings or from asymptomatic populations. A study of 438 children in daycare centers in Uppsala, Sweden, from 2016 tested for 21 different enteric pathogens using PCR and detected ≥1 pathogen in stool specimens from 3.7% of children (*44*). The pathogens they detected most frequently were *C. difficile* (2.5%), adenovirus 40/41 (1.6%), *Campylobacter* (0.7%), and norovirus (0.7%) (Appendix Table 6). A 2001 study of 1,091 asymptomatic children and adults in Australia assessed 28 pathogens and detected ≥1 pathogen in 2.6% of stool specimens, including *Giardia* (1.6%), *Salmonella* (0.4%), *Cryptosporidium* (0.4%), and adenovirus (0.1%) (*45*). Prevalence of ≥1 pathogen was higher for children <10 years of age (4.6%) compared with children 10–20 years of age (0.6%) and adults >20 years of age (1.2%). *Blastocystis hominis*, which the authors did not consider pathogenic and was not included in the reported 2.6% prevalence, was detected in 6.0% of stool specimens.

Our results indicate substantially higher prevalence of gut pathogens compared with those studies. However, we detected some individual pathogens less frequently than in other similar studies in the United States. Among infants in Denver, Colorado, USA, in 1990, an estimated 16% of those attending daycare and 9% of those not enrolled had *Giardia duodenalis* detected in stool specimens (*46*). In 1991, the prevalence of *Cryptosporidium* was 3% and *G. duodenalis* 7% among children attending daycare centers in Fulton County, Georgia, USA (*47*). Those values are higher than the 0.4% (2/488)

prevalence we observed for *Giardia* and the 0% prevalence for *Cryptosporidium*, although the Colorado and Georgia studies took place more than 30 years ago in different settings and populations. More recently, Tisdale et al. (*48*) used the TAC platform to screen adults traveling internationally from the United States and Germany to low- and middle-income countries for 22 pathogens. Similar to our results, they detected ≥1 pathogen in stool specimens from 21% of asymptomatic controls.

One limitation of this study is that logistical constraints did not enable analysis of fresh specimens. Transport and storage conditions (time, temperature, and transport media) can influence recovery of pathogen-associated nucleic acids, potentially lowering the sensitivity of molecular assays we used and possibly leading to false-negative results if DNA or RNA fell below our detection limits. Although we attempted to reduce time-to-analysis and to optimize storage conditions to preserve the stability of DNA and RNA, some loss of signal is unavoidable. We assessed Zn-PVA's performance in preserving nucleic acids in spiked controls (Appendix). In addition, we had missing data in our surveys because of logistical difficulties imposed by the COVID-19 pandemic, such as the need for participants to complete surveys at home and mail them separately from specimens. In addition, some missing data may have been the result of hesitancy to share sanitary conditions because straight pipe discharge of domestic wastewater (*8*) is illegal in the study area (*7*). To mitigate the effect of this missing data, we used MICE and obtained similar results by using this imputation approach compared with analysis on the complete dataset. Further, we were unable to conduct household visits to confirm water and sanitation infrastructure characteristics, including those that may be additional important risk factors for exposure to key pathogens, including wastewater discharges, water source characteristics, soil types, and other environmental variables.

In conclusion, our results suggest that children in households in this region that are reliant on domestic wells may experience increased risks for enteric pathogen exposure compared with children in households with water supplied by utilities. Elevated levels of fecal contamination in groundwater (*12*) could be related to documented deficiencies in rural sanitation in the region, and water as a proximal exposure pathway merits further exploration. New models for infrastructure delivery and management may help expand services, given the limitations of the current paradigm of each household being fully responsible for waste management despite the potential for collective impacts on public health.

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About the Author

Dr. Capone is an assistant professor at Indiana University–Bloomington. He uses tools from engineering, epidemiology, and environmental microbiology in the study of public health solutions for underserved communities.

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Risk Factors for Enteric Pathogen Exposure among Children in Black Belt Region of Alabama, USA

Appendix

Zn-PVA Validation

The recovery of *Giardia duodenalis* and *Shigella sonnei* from stool were assessed using different preservative conditions over a period of 8 weeks. First, canine stools collected form a local shelter. Then, an aliquot of each sample was mixed 1:1 into five preservation buffers, which included Zn-PVA (ProtocolTM Parasitology System, Thermo Scientific, Middletown, VA), Total-FixTM (Medical Chemical Corp, Torrance, CA), Universal Extraction (UNEX) buffer (*1*), Nucleic Acid Preservation (NAP) buffer (*2*), and 70% ethanol (Fisher Scientific, Hampton, NH). During mixing, we spiked each aliquot with ≈10^6 *Giardia duodenalis* cysts and 10^8 *Shigella sonnei* cells (BEI Resources, Manassas, VA). Stool preservative mixtures were stored at ambient temperatures, except Zn-PVA which we assessed at ambient and at 4°C because samples were shipped at ambient conditions but stored at 4°C in the lab. Nucleic acids were extracted from the aliquots using the same protocol as for children's stools immediately upon aliquot preparation and then intermittently over a period of 8 weeks. Finally, gene targets for the two pathogens were quantified using digital PCR (dPCR) to determine the temporal reduction in DNA recovery.

The two PCR assays used were adapted and optimized for dPCR using *Giardia duodenalis* (*3*) and *Shigella sonnei* (*4*) assays published for real-time PCR. Assays were validated and optimized using the QIAcuity Four Digital PCR system (QIAcuity 4, Qiagen, Hilden, Germany). Positive control materials were custom gBlocks (IDT, Coralville, IA) containing each assay's target sequence. PCR reactions were made by combining 2 µL of template with 38 µL of mastermix (Probe PCR Master Mix, Qiagen, Hilden, Germany) and run using 26k 24-well Nanoplates (Qiagen, Hilden, Germany). The Thermocycling conditions used

were 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Partition fluorescence was measured using preset imaging settings in relative fluorescence units (RFU). Six negative process controls (preservative only) were extracted corresponding to each preservative on days 0 and 28, and from one negative extraction control (water) on each extraction day. One negative PCR control (water) and one positive control was run on each dPCR plate. All negative controls tested negative. Extracts were stored at −80°C until analysis. Thresholding was performed manually by selecting the mid-point between the positive and negative bands in the QIAcuity Software Suite (Qiagen, Hilden, Germany).

Data analysis was performed in Excel (Microsoft, Seattle, Washington) to convert gene copies per µL into gene copies per gram of stool and calculate the mean $\log_{10}$ gene copies and differences in those values over time.

Results

We observed heterogenous results for the decay of *Giardia* and *Shigella* DNA in the five preservation buffers (Appendix Table 2, Appendix Figure 3). For recovery of DNA from Giardia cysts, UNEX performed best, followed by ZnPVA at 4°C. Whereas for the recovery of DNA from Shigella cells, NAP performed best, followed by UNEX. For both pathogens ZnPVA at 4°C outperformed ZnPVA at ambient conditions. There was typically a 2-week gap from sample collection to receipt at the lab (median = 14 days, IQR = 11, 21) and DNA was extracted approximately 2 weeks later (median = 15 days, IQR = 8, 28). For a hypothetical sample stored at ambient for 14 days and at 4°C for 15 days, this suggests a 0.53 $\log_{10}$ decrease in the *Giardia* concentration and a 0.55 $\log_{10}$ decrease in the *Shigella* concentration would have occurred.

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Appendix Table 1. TAC performance

Target	Target Gene	y-intercept	R^2	Efficiency	95% limit of detection †	Reference
enteric 16S	16S	38.9	0.998	101%	0.60	(*3*)
Acanthamoeba spp.	18S rRNA	37.8	1.000	97%	23	(*5*)
Adenovirus 40/41*	Fiber gene	NA	0.670	NA	NA	(*3*)
astrovirus	Capsid	37.5	0.998	87%	6.2	(*3*)
Balantidium coli	ITS-1	37.9	1.000	97%	2.2	(*6*)
Blastocystis spp.	18S rRNA	40.6	0.997	100%	2.2	(*3*)
Cystoisospora belli	18S rRNA	37.8	0.999	99%	6.2	(*3*)
Cyclospora cayetanensi	18S rRNA	37.2	0.998	99%	2.2	(*3*)
Campylobacter jejuni/coli	*cadF*	38.3	0.999	99%	21	(*3*)
Clostridioides difficile	*tcdB*	37.5	0.999	96%	6.2	(*3*)
Cryptosporidium spp.	18S rRNA	38.0	0.999	97%	0.6	(*3*)
DNA control (phocine herpes virus)	*gB*	37.0	0.998	100%	6.2	(*3*)
Enterocytozoon bieneusi	ITS	37.2	0.999	102%	4.8	(*3*)
E. coli O157:H7	*rfbE*	38.0	1.000	95%	2.2	(*3*)
Encephalitozoon intestinalis	SSU rRNA	38.5	0.999	98%	2.2	(*3*)
Enterobius vermicularis	5S	38.6	0.999	95%	72	(*7*)
EAEC (aaiC)	*aaiC*	38.2	0.999	96%	6.2	(*3*)
EAEC (aatA)	*aatA*	37.7	0.998	96%	23	(*3*)
Entamoeba hystolytica	18S rRNA	38.0	0.996	102%	6.2	(*3*)
Entamoeba spp.	18S rRNA	37.3	0.974	104%	21	(*3*)
EPEC (typical)	*bfpA*	37.5	0.999	98%	6.2	(*3*)
EPEC (atypical)	*eae*	37.6	0.999	98%	2.2	(*3*)
ETEC (LT)	*LT*	47.6	0.990	94%	291	(*3*)
ETEC (STh)	*STh*	38.8	0.999	98%	6.2	(*3*)
ETEC (STp)	*STp*	37.3	0.999	99%	2.2	(*3*)
Giardia spp.	18S rRNA	37.9	1.000	96%	6.2	(*3*)
Helicobacter pylori	*ureC*	37.7	0.998	97%	6.2	(*3*)
hepatitis A virus*	NCR	NA	0.840	132%	NA	(*8*)
Shigella/EIEC	*ipaH*	37.5	0.999	99%	23	(*3*)
MS2 (RNA control)	*MS2g1*	37.5	0.999	90%	1.0	(*3*)
Norovirus GII	ORF1–2	37.0	0.999	92%	23	(*3*)
Norovirus GI	ORF1–2	35.9	0.997	93%	23	(*3*)
Plesiomonas shigelloides	*gyrB*	38.2	1.000	96%	23	(*3*)
rotavirus	NSP3	38.0	0.998	91%	6.2	(*3*)
Salmonella spp.	*invA*	38.4	1.000	96%	2.2	(*3*)
Sapovirus I/II/IV	RdRp	38.2	0.998	88%	2.2	(*3*)
Sapovirus V	RdRp	36.7	0.999	91%	2.2	(*3*)
SARS-CoV-2	N1	36.2	0.995	92%	6.2	(*9*)
STEC (stx1)	*stx1*	39.9	1.000	97%	72	(*3*)
STEC (stx2)	*stx2*	38.3	0.967	98%	96	(*3*)
Yersinia enterocolitica	*lytA*	38.3	0.998	94%	2.2	(*3*)

*Excluded due to poor standard curve performance
†Stokdyk *et al*. 2016 (*10*); units are gene copies per reaction.

Appendix Table 2. MIQE Checklist

Item to check	Importance	Checklist
Experimental design		
Definition of experimental and control groups	E	Cross-sectional study with no intervention or control group
Number within each group	E	Stools from 488 children were analyzed
Assay carried out by core lab or investigator's lab?	D	Investigator's lab
Sample		
Description	E	150 mg of stool preserved 1:1 in ZnPVA (75mg of stool and 75mg of preservative)
Volume/mass of sample processed	D	150 mg
Microdissection or macrodissection	E	Not applicable
Processing procedure	E	Shipped at ambient, and stored at 4C
If frozen - how and how quickly?	E	Not frozen
If fixed - with what, how quickly?	E	Preserved in ZnPVA at the time of stool passage
Sample storage conditions and duration (especially for FFPE samples)	E	Median 14 d from collection to analysis. Median 15 d from receipt to DNA extraction.
Nucleic acid extraction		
Procedure and/or instrumentation	E	See methods section
Name of kit and details of any modifications	E	QIAamp 96 Virus QIAcube HT Kit automated on a QIAcube HT
Source of additional reagents used	D	Precellys SK38 bead beating tubes (Bertin Technologies, Rockville, MD)
Details of DNase or RNase treatment	E	Not applicable
Contamination assessment (DNA or RNA)	E	At least one extraction negative control was included during each day of extractions
Nucleic acid quantification	E	Qubit 1X HS dsDNA Kit
Instrument and method	E	Qubit 4 Fluorometer
RNA integrity method/instrument	E	Not measured
Inhibition testing (Cq dilutions, spike or other)	E	Monitored amplification of spiked controls
REVERSE TRANSCRIPTION		
Complete reaction conditions	E	One-step reverse transcription
Amount of RNA and reaction volume	E	Reaction volume = 1.5 µL
Priming oligonucleotide (if using GSP) and concentration	E	Proprietary
Reverse transcription and concentration	E	ArrayScript Reverse transcription
Temperature and time	E	45°C for 20 min
Manufacturer of reagents and catalog numbers	D	Applied Biosystems, AgPath-ID One-Step RT-PCR Reagents, Catalog number: 4387391
qPCR target information		
If multiplex, efficiency and LOD of each assay.	E	Appendix Table 1
Location of amplicon	D	Appendix Table 1
In silico specificity screen (BLAST, etc)	E	We BLASTed all assays to confirm specificity before ordering the custom TAC.
qPCR oligonucleotides		
Primer sequences	E	Appendix Table 2
Probe sequences	D**	Appendix Table 2
Location and identity of any modifications	E	No modifications
Manufacturer of oligonucleotides	D	ThermoFisher Scientific
qPCR protocol		
Complete reaction conditions	E	45°C for 20 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min
Reaction volume and amount of cDNA/DNA	E	40 µL of template with 60 µL of AgPath-ID One-Step RT-PCR Reagents
Primer, (probe), Mg++ and dNTP concentrations	E	All assays contained the same concentrations of primers (900 nmol/L) and probe (250 nmol/L). The Mg2+ and dNTP concentrations are not listed in the in the User Guide.
Polymerase identity and concentration	E	AmpliTaq Gold polymerase
Buffer/kit identity and manufacturer	E	AgPath-ID One-Step RT-PCR Reagents
Additives (SYBR Green I, DMSO, etc.)	E	No additives
Manufacturer of plates/tubes and catalog number	D	ThermoFisher Scientific
Complete thermocycling parameters	E	45°C for 20 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min
Reaction setup (manual/robotic)	D	Manual set-up in a disinfected dead air box (10% bleach with fifteen minutes of contact time, UV for fifteen minutes, and a final cleaning step with 70% ethanol)
Manufacturer of qPCR instrument	E	ThermoFisher Scientfic

Item to check	Importance	Checklist
qPCR validation		
Evidence of optimisation (from gradients)	D	See Liu *et al*. 2016 (*3*)
Specificity (gel, sequence, melt, or digest)	E	See Liu *et al*. 2016 (*3*)
Standard curves with slope and y-intercept	E	Appendix Table 1
PCR efficiency calculated from slope	E	Appendix Table 1
r2 of standard curve	E	Appendix Table 1
Evidence for limit of detection	E	Appendix Table 1
Data analysis		
qPCR analysis program (source, version)	E	QuantStudio Real-Time PCR Software V1.2 CDC
Cq method determination	E	Manual thresholding
Results of NTCs	E	We observed no amplification before at Ct of 40 in our two PCR negative controls. Among the 12 negative extraction controls, we observed no amplification before a Ct of 40.
Justification of number and choice of reference genes	E	
Description of normalization method	E	Normalized to mass of stool ZnPVA mixture extracted from (150mg)
Number and concordance of biologic replicates	D	See results section.
Number and stage (RT or qPCR) of technical replicates	E	See results section.
Statistical methods for result significance	E	See methods section
Software (source, version)	E	R Studio V2.2.2

Appendix Table 3. Primer and probe sequences

Pathogen	Primer or probe sequence (5′ - 3′)
Astrovirus	Fwd: CAGTTGCTTGCTGCGTTCA
	Rev: CTTGCTAGCCATCACACTTCT
	Probe: CACAGAAGAGCAACTCCATCGC
Norovirus GI	Fwd: CGYTGGATGCGNTTYCATGA
	Rev: CTTAGACGCCATCATCATTYAC
	Probe: TGGACAGGAGATCGC
Norovirus GII	Fwd: CARGARBCNATGTTYAGRTGGATGAG
	Rev: TCGACGCCATCTTCATTCACA
	Probe: TGGGAGGGCGATCGCAATCT
Sapovirus (I, II, IV)	Fwd: GAYCAGGCTCTCGCYACCTAC
	Rev: CCCTCCATYTCAAACACTA
	Probe: CYTGGTTCATAGGTGGTRCAG
Sapovirus V	Fwd: TTTGAACAAGCTGTGGCATGCTAC
	Rev: CCCTCCATYTCAAACACTA
	Probe: CAGCTGGTACATTGGTGGCAC
Adenovirus 40/41	Fwd: AACTTTCTCTCTTAATAGACGCC
	Rev: AGGGGGCTAGAAAACAAAA
	Probe: CTGACACGGGCACTCT
Rotavirus	Fwd: ACCATCTWCACRTRACCCTCTATGAG
	Rev: GGTCACATAACGCCCCTATAGC
	Probe: AGTTAAAAGCTAACACTGTCAAA
Campylobacter jejuni or *coli*	Fwd: CTGCTAAACCATAGAAATAAAATTTCTCAC
	Rev: CTTTGAAGGTAATTTAGATATGGATAATCG
	Probe: CATTTTGACGATTTTTGGCTTGA
C. difficile	Fwd: GGTATTACCTAATGCTCCAAATAG
	Rev: TTTGTGCCATCATTTTCTAAGC
	Probe: CCTGGTGTCCATCCTGTTTC
EAEC (aaiC)	Fwd: ATTGTCCTCAGGCATTTCAC
	Rev: ACGACACCCCTGATAAACAA
	Probe: TAGTGCATACTCATCATTTAAG
EAEC (aatA)	Fwd: CTGGCGAAAGACTGTATCAT
	Rev: TTTTGCTTCATAAGCCGATAGA
	Probe: TGGTTCTCATCTATTACAGACAGC
STEC (stx1)	Fwd: ACTTCTCGACTGCAAAGACGTATG
	Rev: ACAAATTATCCCCTGWGCCACTATC
	Probe: CTCTGCAATAGGTACTCC
STEC (stx2)	Fwd: CCACATCGGTGTCTGTTATTAACC
	Rev: GGTCAAAACGCGCCTGATAG
	Probe TTGCTGTGGATATACGAGG

Pathogen	Primer or probe sequence (5′ - 3′)
EPEC (eae)	Fwd: CATTGATCAGGATTTTTCTGGTGATA Rev: CTCATGCGGAAATAGCCGTTA Probe: ATACTGGCGAGACTATTTCAA
EPEC (bfpA)	Fwd: TGGTGCTTGCGCTTGCT Rev: CGTTGCGCTCATTACTTCTG Probe: CAGTCTGCGTCTGATTCCAA
ETEC LT	Fwd: TTCCCACCGGATCACCAA Rev: CAACCTTGTGGTGCATGATGA Probe: CTTGGAGAGAAGAACCCT
ETEC ST	Fwd h: GCTAAACCAGYAGRGTCTTCAAAA Fwd p: TGAATCACTTGACTCTTCAAAA Rev h: CCCGGTACARGCAGGATTACAACA Rev p: GGCAGGATTACAACAAAGTT Probe h: TGGTCCTGAAAGCATGAA Probe p: TGAACAACACATTTTACTGCT
EIEC or *Shigella*	Fwd: CCTTTTCCGCGTTCCTTGA Rev: CGGAATCCGGAGGTATTGC Probe: CGCCTTTCCGATACCGTCTCTGCA
Salmonella	Fwd: CTCACCAGGAGATTACAACATGG Rev: AGCTCAGACCAAAAGTGACCATC Probe: CACCGACGGCGAGACCGACTTT
E. coli O157	Fwd: TTTCACACTTATTGGATGGTCTCAA Rev: CGATGAGTTTATCTGCAAGGTGAT Probe: CTCTCTTTCCTCTGCGGTCCT
Cryptosporidium	Fwd: GGGTTGTATTTATTAGATAAAGAACCA Rev: AGGCCAATACCCTACCGTCT Probe: TGACATATCATTCAAGTTTCTGAC
Giardia spp.	Fwd: GACGGCTCAGGACAACGGTT Rev: TTGCCAGCGGTGTCCG Probe: CCCGCGGCGGTCCCTGCTAG
E. histolytica	Fwd: ATTGTCGTGGCATCCTAACTCA Rev: GCGGACGGCTCATTATAACA Probe: TCATTGAATGAATTGGCCATTT
Entamoeba spp.	Fwd: AAACGATGTCAACCAAGGATTG Rev: TCCCCCTGAAGTCCATAAACTC Probe: CCTTGTTCAGAACTTAAAGAGAAA
Blastocystis spp.	Fwd: TGGTCCGRTGAACACTTTGGAT Rev: CCTACGGAAACCTTGTTACGACTTCA Probe: CTTCCTCTAAATGRTAAGATT
16s	Fwd: TGCAAGTCGAACGAAGCACTTTA Rev: GCAGGTTACCCACGCGTTAC Probe: CGCCACTCAGTCACAAA
PhHV	Fwd: GGGCGAATCACAGATTGAATC Rev: GCGGTTCCAAACGTACCAA Probe: TATGTGTCCGCCACCATCT
Yersinia enterocolitica	Fwd: TGATTCACCAGCAGCAATAC Rev: GGCATCATGAAAGGCGG Probe: TGTCGGTTTCTCCTTCCAGG
Heliobacter pylori	Fwd: GACACCAGAAAAAGCGGCTA Rev: AGCGCATGTCTTCGGTTAAA Probe: TCACTAAAGCGTTTTCTACC
Plesiomonas shigelloides	Fwd: CCGCCGTGAAGGCAAAG Rev: GCTACCGGCTCACCCAGAT Probe: CACACCCAAGAATAC
Cyclospora cayetanensi	Fwd: AAAAGCTCGTAGTTGGATTTCTG Rev: AACACCAACGCACGCAGC Probe: AAGGCCGGATGACCACGA
Cystoisospora belli	Fwd: ATATTCCCTGCAGCATGTCTGTTT Rev: CCACACGCGTATTCCAGAGA Probe: CAAGTTCTGCTCACGCGCTTCTGG
Blastocystis spp.	Fwd: TGGTCCGRTGAACACTTTGGAT Rev: CCTACGGAAACCTTGTTACGACTTCA Probe: CTTCCTCTAAATGRTAAGATT
Enterocytozoon bieneusi	Fwd: TGTGTAGGCGTGAGAGTGTATCTG Rev: CATCCAACCATCACGTACCAATC Probe: CACTGCACCCACATCCCTCACCCTT
Encephalitozoon intestinalis	Fwd: CACCAGGTTGATTCTGCCTGAC Rev: CTAGTTAGGCCATTACCCTAACTACCA Probe: CTATCACTGAGCCGTCC

Pathogen	Primer or probe sequence (5′ - 3′)
Balantidium coli	Fwd: TGCAATGTGAATTGCAGAACC
	Rev: TGGTTACGCACACTGAAACAA
	Probe: CTGGTTTAGCCAGTGCCAGTTGC
Acanthamoeba spp.	Fwd: CCCAGATCGTTTACCGTGAA
	Rev: TAAATATTAATGCCCCCAACTATC
	Probe: CTGCCACCGAATACATTAGCATGG
Hepatitis A Virus	Fwd: TCACCGCCGTTTGCCTAG
	Rev: GGAGAGCCCTGGAAGAAAG
	Probe: TTAATTCCTGCAGGTTCAGG
SARS-CoV-2	Fwd: GACCCCAAAATCAGCGAAAT
	Rev: TCTGGTTACTGCCAGTTGAATCTG
	Probe: ACCCCGCATTACGTTTGGTGGACC

Appendix Table 4. Risk factors for ≥1 pathogen detection (using only complete cases, n = 341)

Variable	Reference	Exposure	RR (95% CI)	aRR (95% CI)
Pay a water bill	Yes	No	1.8 (1.3, 2.6)	1.8 (1.3, 2.6)
Sanitation	Sewer connection	Cesspit	NA	NA
		Other	NA	NA
		Septic Tank	0.90 (0.59, 1.4)	0.91 (0.60, 1.4)
		Straight Pipe	0.98 (0.53, 1.8)	0.91 (0.49, 1.7)
Child's Screen Time	<2 h	2–4 h	0.66 (0.42, 1.0)	0.71 (0.45, 1.1)
		>4 h	0.67 (0.43, 1.0)	0.64 (0.41, 1.0)
Gender	Male	Female	0.91 (0.66, 1.3)	0.92 (0.66, 1.3)
International Travel	No	Yes	0.92 (0.34, 2.5)	1.0 (0.37, 2.9)
Raw Sewage	No	Yes	1.2 (0.65, 2.3)	1.2 (0.70, 2.1)
Age	<5 y	5–10 y	0.77 (0.39, 1.5)	1.0 (0.48, 2.1)
		>10 y	0.88 (0.46, 1.7)	1.1 (0.55, 2.4)

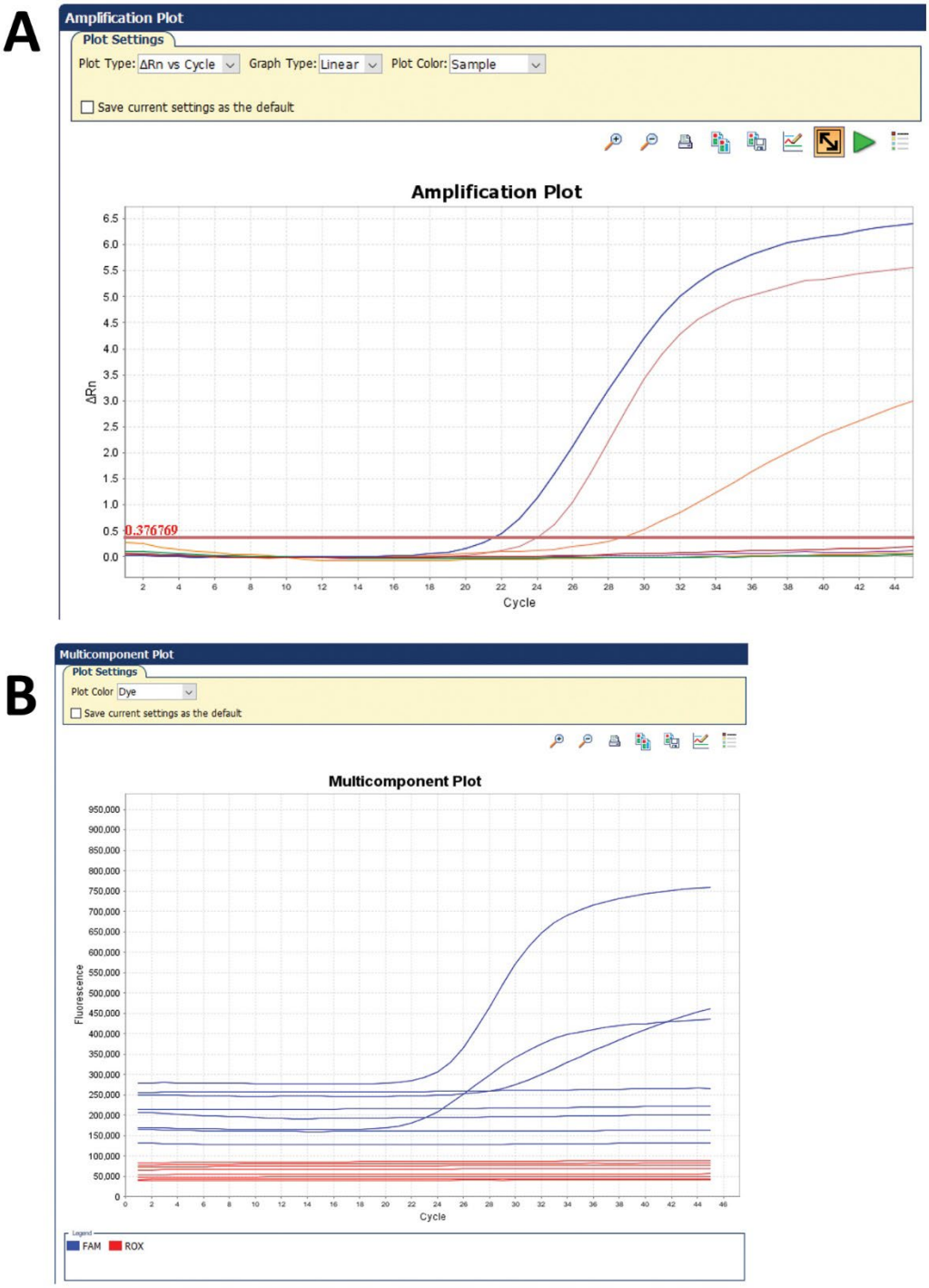
Appendix Table 5. Decay constants for different preservation buffers

Target	Preservative	Log10 decay in DNA concentration per day
Giardia	Zn PVA (4C)	−0.0037
Giardia	Zn PVA (20C)	−0.034
Giardia	UNEX	−0.0008
Giardia	TotalFix	−0.0541
Giardia	NAP	−0.0358
Giardia	70% Ethanol	−0.0469
Shigella	Zn PVA (4C)	−0.0085
Shigella	Zn PVA (20C)	−0.0303
Shigella	UNEX	−0.003
Shigella	TotalFix	−0.0154
Shigella	NAP	−0.0003
Shigella	70% Ethanol	−0.0442

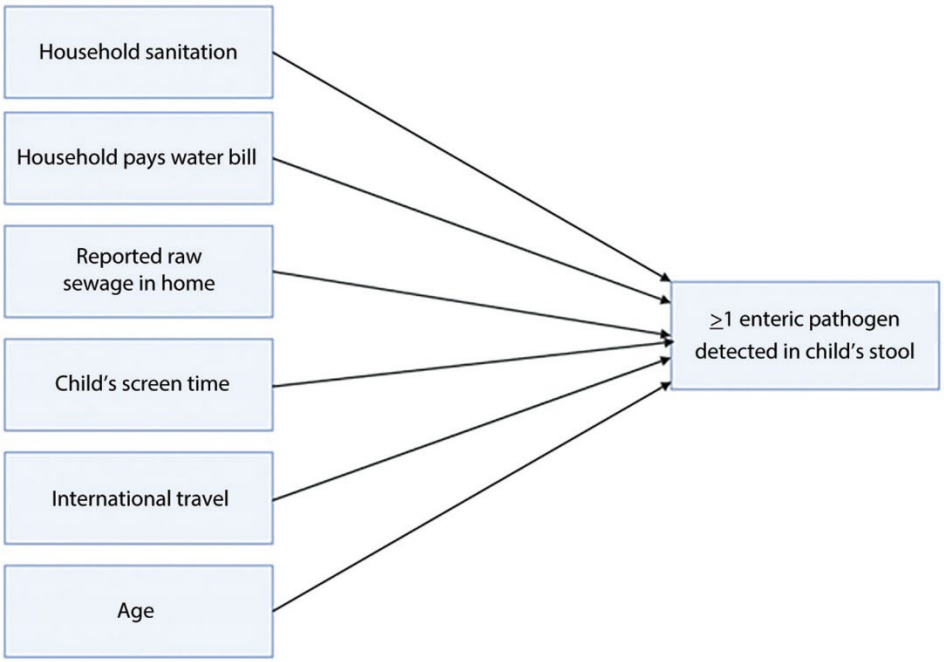
Appendix Table 6. Comparison with Swedish Children

Type	Pathogen	Prevalence in rural Alabama	Prevalence among Swedish Children in Daycare (*11*)
Any	≥1 Pathogen detected	26% (127/488)	
Bacteria	*Clostridioides difficile* (toxin B)	6.6% (32/488)	2.5% (11/438)
	EPEC (atypical)	6.1% (30/488)	Not assessed
	EAEC	3.9% (19/488)	Not assessed
	Helicobacter pylori	2.3% (11/488)	Not assessed
	EPEC (typical)	1.4% (7/488)	Not assessed
	Yersinia enterocolitica	1.0% (5/488)	0% (0/438)
	E. coli O157:H7	0.8% (4/488)	0% (0/438)
	Plesiomonas shigelloides	0.4% (2/488)	Not assessed
	ETEC	0.4% (2/488)	1.4% (6/438)
	Shigella/EIEC	0.2% (1/488)	0% (0/438)
	Salmonella spp.	0.2% (1/488)	0% (0/438)
	STEC	0.2% (1/488)	0% (0/438)
	Campylobacter jejuni/coli	0% (0/488)	0.7% (3/438)
Fungus/Algae	*Blastocystis* spp.	3.7% (18/488)	Not assessed

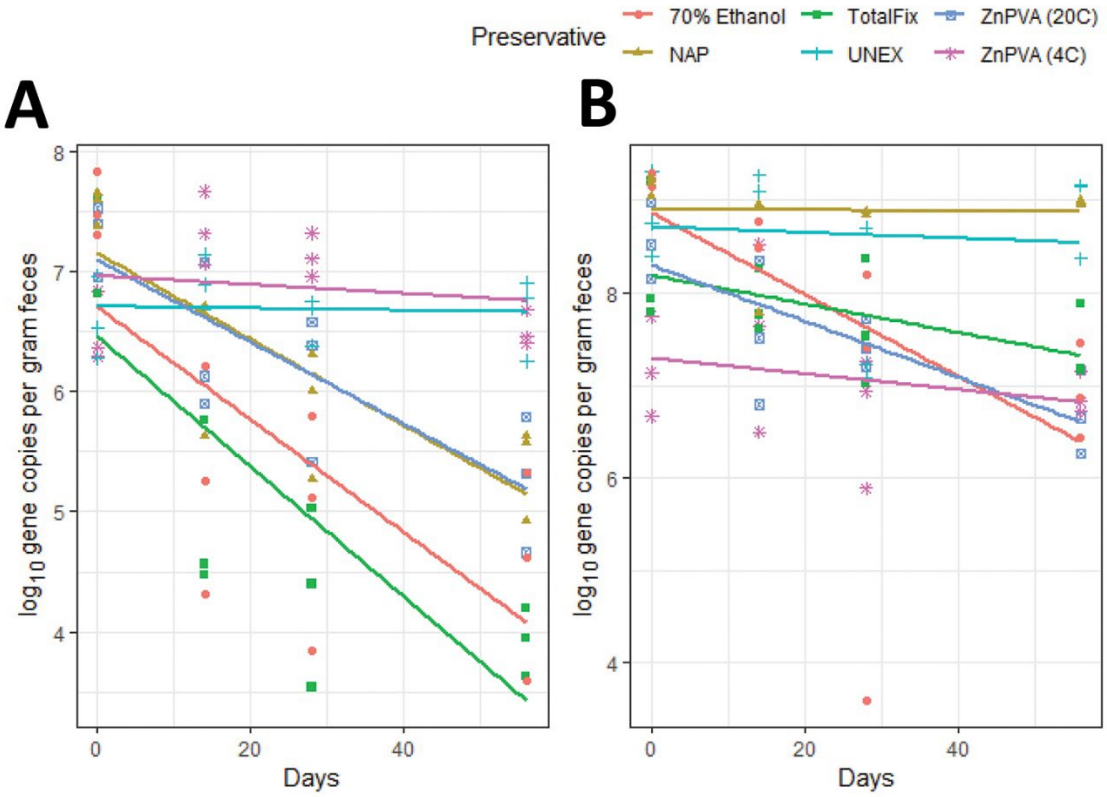
Type	Pathogen	Prevalence in rural Alabama	Prevalence among Swedish Children in Daycare (*11*)
	Enterocytozoon bieneusi	0% (0/488)	Not assessed
	Encephalitozoon intestinalis	0% (0/488)	Not assessed
Protozoa	*Balantidium coli*	0.6% (3/488)	Not assessed
	Acanthamoeba spp.	0.4% (2/488)	Not assessed
	Giardia spp.	0.4% (2/488)	0% (0/438)
	Entamoeba hystolytica	0.2% (1/488)	0% (0/438)
	Cystoisospora belli	0% (0/488)	Not assessed
	Cyclospora cayetanensi	0% (0/488)	Not assessed
	Cryptosporidium spp.	0% (0/488)	0% (0/438)
	Entamoeba spp.	0% (0/488)	Not assessed
Virus	norovirus GI/GII	1.4% (7/488)	0.7% (3/438)
	SARS-CoV-2	0.6% (3/488)	Not assessed
	rotavirus	0.4% (2/488)	0% (0/438)
	sapovirus	0.4% (2/488)	Not assessed
	astrovirus	0.2% (1/488)	Not assessed



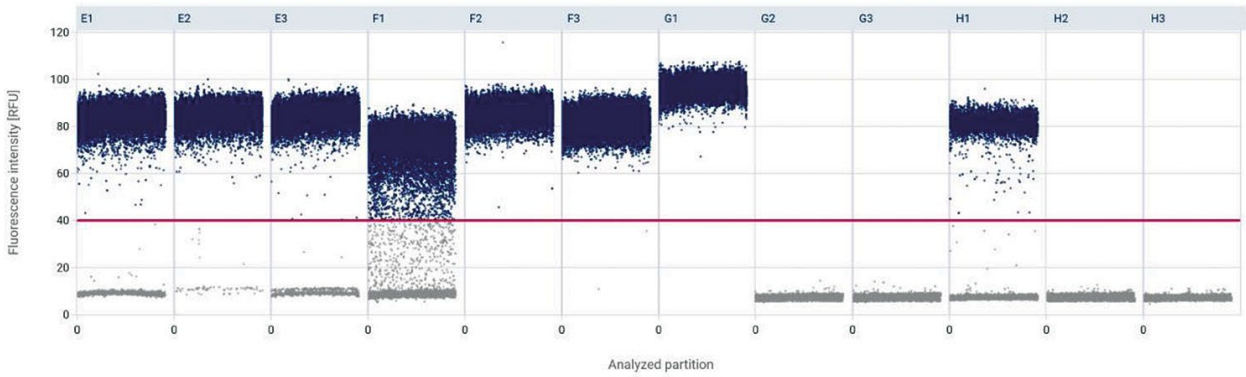
Appendix Figure 1. Amplification and multicomponent plots.



Appendix Figure 2. Acyclic graph.



Appendix Figure 3. Gene copy recovery.



Appendix Figure 4. dPCR 2-D Scatterplot. Wells G2, G3, H2, and H3 were negative extraction controls, well H1 was a PCR positive control; all other wells were samples. Samples that were outside the range of quantification (i.e., F2, F3, and G1) were rerun at a 1:10 dilution.