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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)

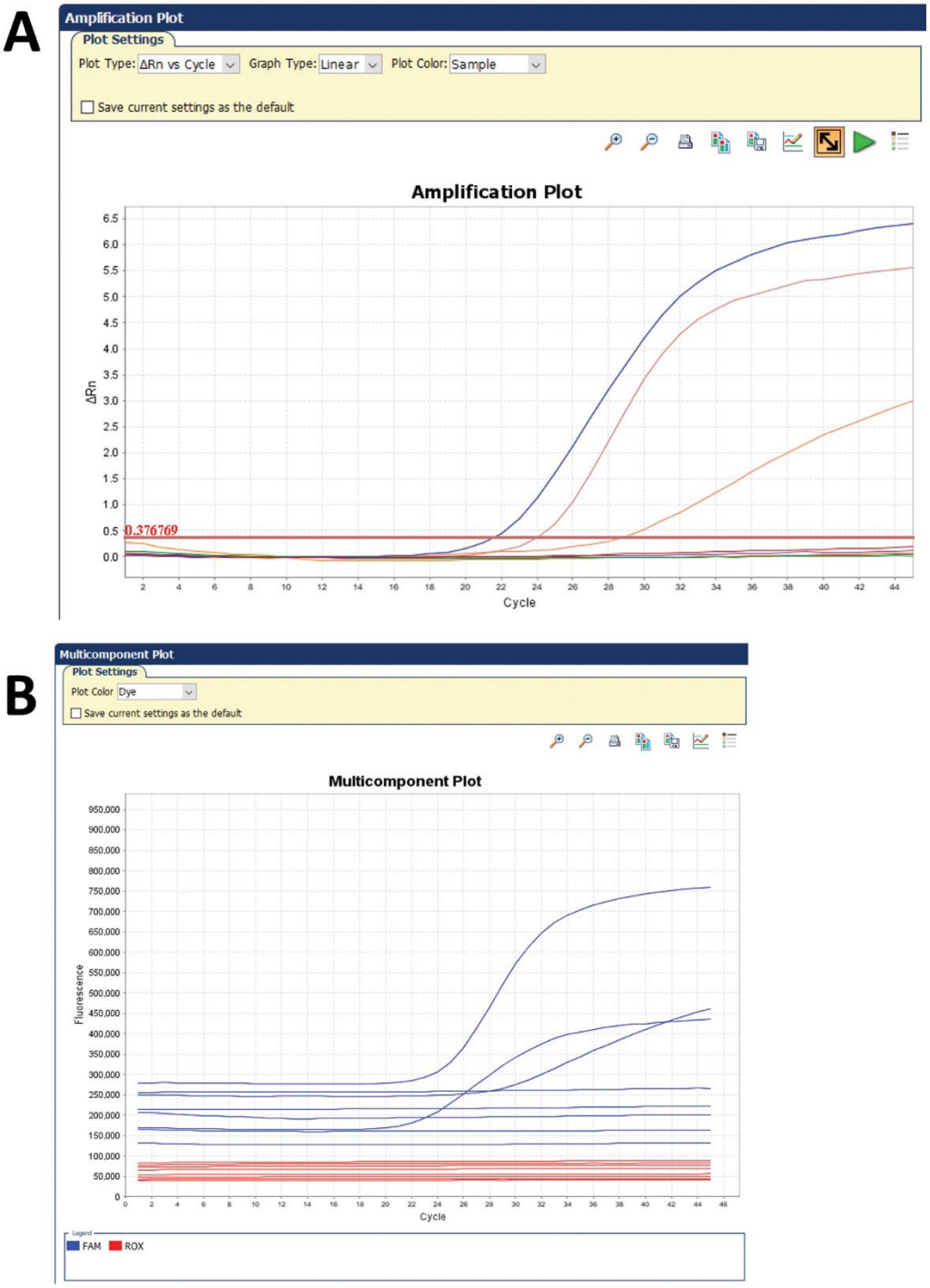
Appendix Table 5. Decay constants for different preservation buffers

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

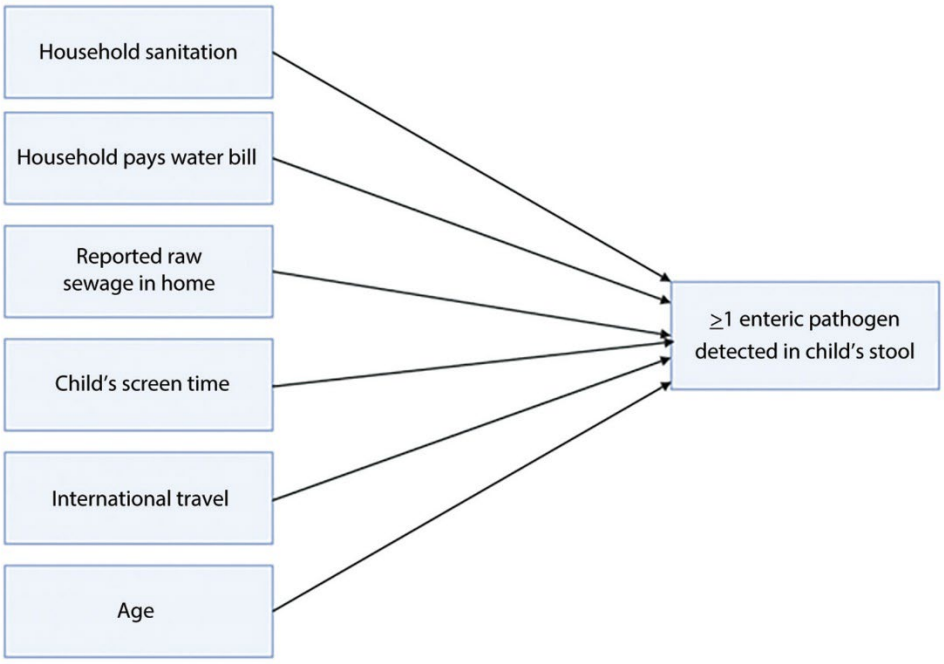
Appendix Table 6. Comparison with Swedish Children

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

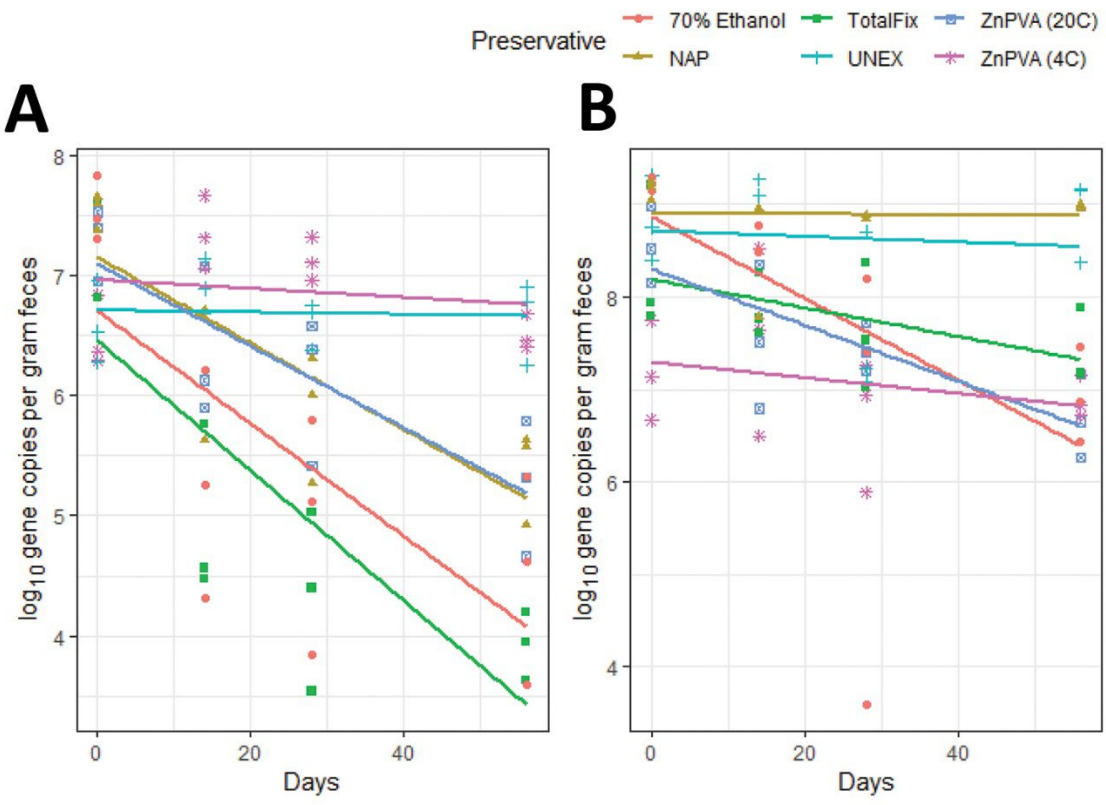
Type	Pathogen	Prevalence in rural Alabama	Prevalence among Swedish Children in Daycare (*11*)
	Enterocytozoon bieneusi	0% (0/488)	Not assessed
	Encephalitozoon intestinalis	0% (0/488)	Not assessed
Protozoa	*Balantidium coli*	0.6% (3/488)	Not assessed
	Acanthamoeba spp.	0.4% (2/488)	Not assessed
	Giardia 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



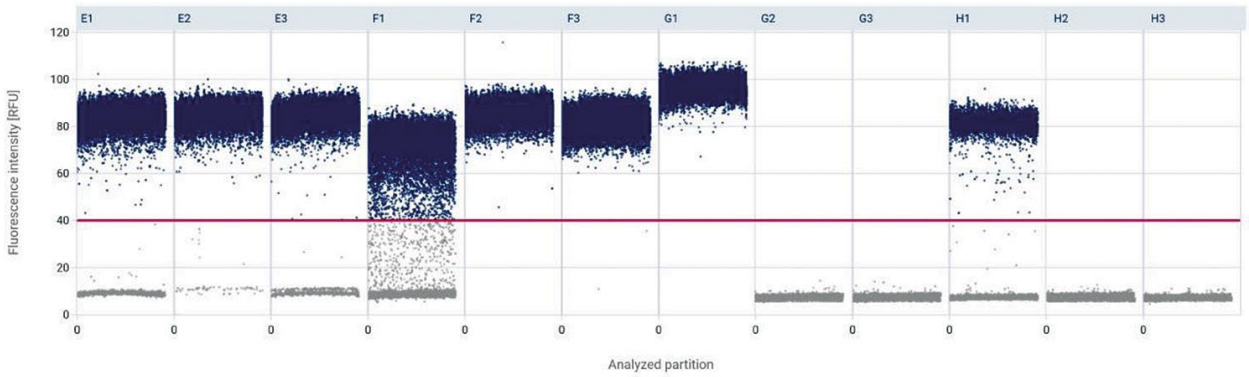
Appendix Figure 1. Amplification and multicomponent plots.



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.