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Impact of an Urban Sanitation Intervention on Enteric Pathogen Detection in Soils

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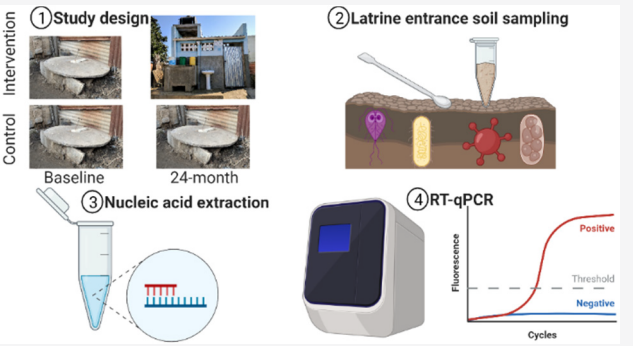
ABSTRACT: Environmental fecal contamination is common in many low-income cities, contributing to a high burden of enteric infections and associated negative sequelae. To evaluate the impact of a shared onsite sanitation intervention in Maputo, Mozambique on enteric pathogens in the domestic environment, we collected 179 soil samples at shared latrine entrances from intervention (n = 49) and control (n = 51) compounds during baseline (preintervention) and after 24 months (postintervention) as part of the Maputo Sanitation Trial. We tested soils for the presence of nucleic acids associated with 18 enteric pathogens using a multiplex reverse transcription qPCR platform. We detected at least one pathogen-associated gene target in 91% (163/179) of soils and a median of 3 (IQR = 1, 5) pathogens. Using a difference-in-difference analysis and adjusting for compound population, visibly wet soil, sun exposure, wealth, temperature, animal presence, and visible feces, we estimate the intervention reduced the probability of detecting ≥1 pathogen gene by 15% (adjusted prevalence ratio, aPR = 0.85; 95% CI: 0.70, 1.0) and the total number of pathogens by 35% (aPR = 0.65; 0.44, 0.95) in soil 24 months following the intervention. These results suggest that the intervention reduced the presence of some fecal contamination in the domestic environment, but pathogen detection remained prevalent 24 months following the introduction of new latrines.



KEYWORDS: *onsite sanitation, urban slum, latrines, fecal sludge management, exposure*

INTRODUCTION

Onsite sanitation systems are designed to sequester human feces away from human contact and prevent the transport of fecal-oral pathogens through well-defined transmission pathways.[1] Large-scale, rigorous randomized controlled trials (RCTs) of onsite sanitation systems, including sanitation alone and combinations of water, sanitation, and hygiene (WASH) interventions, have found mixed effects on health outcomes, such as diarrhea and child growth.[2−7] Assessing the impact of WASH interventions on enteric pathogens in the environment can improve our understanding of pathogen transmission from an infected individual to a new host via the environment, a core intermediate outcome of these trials. Such data may help explain why some WASH interventions observed improved health outcomes and others did not.[8]

There is a growing body of literature that indicates soils contaminated by feces in public and domestic environments pose infection risks.[9−13] In health impact trials that assess improved onsite sanitation systems, soils are assessed to measure how effectively the intervention sequestered human feces.[14−18] Latrines and septic tanks are useful barriers against the transport of human feces into the environment. However, enteric pathogens may still move into soils through open defecation,[19] unhygienic pit emptying,[20,21] fecally contaminated greywater,[22,23] improper disposal of children's feces or anal cleansing materials,[24,25] latrine flooding,[20,26,27] animal feces,[28−30] or subsurface transport from unlined pits.[31−33] Domestic soils contaminated by enteric pathogens can pose infection risks beyond incidental[34] and direct[35] soil ingestion: contaminated soil may be transported to hands, food, fomites, or household stored water.[36] For these reasons, soils may be a useful matrix to assess the impact of onsite sanitation interventions.

The detection of enteric pathogens via molecular methods is being increasingly used to assess the impact of WASH interventions on the transport of these pathogens through the environment.[37−39] Molecular detection of pathogens offers additional insights, as health impact studies have historically relied on fecal indicator bacteria (FIB) as a proxy for enteric

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pathogens for reasons of cost, capacity, and feasibility.[17,36,40−42] However, a 2016 meta-analysis[43] found that improved sanitation had no effect on the presence of FIB in the environment, possibly, because these indicators are often pervasive in low-income settings[15,16,36,44−46] and common FIB, like *E. coli*, may be naturalized in the environment.[47−49]

The Maputo Sanitation (MapSan) Trial was the first rigorous controlled before-and-after trial to evaluate the effect of an urban onsite sanitation intervention on child health.[24,50,51] We conducted the trial in low-income, informal neighborhoods in Maputo, Mozambique, where WASH conditions are poor and the burden of enteric disease is high.[20,24,44,52] Water and Sanitation for the Urban Poor (WSUP, a nongovernmental organization) delivered the intervention to compounds composed of household clusters that shared sanitation and courtyard space. Control compounds were concurrently enrolled from the same or adjacent neighborhoods as intervention compounds and continued using existing shared sanitation infrastructure. Detailed descriptions of the inclusion criteria for intervention and control compounds are described elsewhere.[20,24]

The intervention was built inside the compound boundary and was part of the households' living environment. WSUP replaced shared onsite sanitation systems in poor condition with pour-flush toilets that included septic tanks and soak-away pits. There were two versions of the intervention: shared latrines, serving 15−20 individuals, and community sanitation blocks for compounds with >20 residents. Shared latrines became the property of the residents and included a toilet, superstructure, septic tank, and a lined infiltration pit. Community sanitation blocks officially remained the property of the municipality and included the same infrastructure as a shared latrine but contained multiple toilets (one toilet per 20 people), a new piped water connection with a water storage tank, sink pedestal for handwashing (no running water but the drain was connected to the septic tank), rainwater harvesting tank, cement laundry basin, and community sanitation blocks used by ≥60 residents received a urinal on an external wall of the structure which drained to the septic tank. Compound residents that received community sanitation blocks formed sanitation management committees, which were responsible for maintaining the sanitation infrastructure. The septic tanks in the shared latrines and community sanitation blocks were sized according to the number of users and were designed to be emptied every two years.

A latrine entrance is an ideal soil sampling location to determine the effectiveness of onsite sanitation interventions because it is a standardized location near the fecal waste in the containment chamber.[15,16,53] Soils in low-income Maputo are characterized as coarse to fine sand or silty sand.[54] While the fate and transport of pathogens through soils is dependent on the individual pathogen and environmental conditions,[55] the high porosity of Maputo's sandy soils combined with a high water table in the study area[44] offers potential for pathogen movement.[56] This high risk of fecal contamination suggests we could plausibly observe a reduction in enteric pathogens in soil at latrine entrances if the intervention infrastructure performed better than controls at safely containing fecal wastes.[57] Our study aim was to assess if the intervention reduced the detection of ≥1 pathogen gene, the total number of pathogens, or any individual pathogen in latrine entrance soils from MapSan intervention compounds compared to controls.

MATERIALS AND METHODS

Sample Collection. We prospectively collected latrine entrance soil samples, defined as a location one-meter away from the latrine entrance in the direction of entry or the nearest point not covered by cement, from 49 intervention and 51 control compounds at baseline (preintervention) and from the same compounds 24 months following the intervention, for a total of 200 samples (Text S1). We defined this sample location *a priori* as one that could be standardized across all compounds in the study. Compounds were selected using convenience sampling. Using a spade and ruler, we scooped soil from a 10 cm × 10 cm area to a depth of 1 cm into a Whirl-Pak bag (Nasco, Fort Atkinson, WI). The spade and ruler were sterilized between uses with 10% bleach and 70% ethanol. At the time of sampling, enumerators recorded whether the soil was visibly wet and estimated the daily sun exposure (full sun, partially shaded, full shade).[44] Samples were stored on ice for transport to the Ministry of Health in Maputo, Mozambique, frozen at −20 °C for approximately six months, aliquoted into 2 mL cryovials while working on dry ice, and then stored at −80 °C. During storage at −20 °C, some samples (n = 21) were unable to be evaluated because the permanent marker labeling on some Whirl-Pak bags wore off and some bags burst open. All aliquoted samples (n = 179) were shipped from the Mozambican Ministry of Health in Maputo, Mozambique to Atlanta, GA, USA on dry ice (−80 °C) with temperature monitoring for molecular analysis. We obtained compound observation data and socioeconomic characteristics from the MapSan baseline and 24 month survey data sets, which were collected concurrent to soil samples.[24,58]

Sample Processing. At the Georgia Institute of Technology in Atlanta, GA, USA, we incubated 250 mg of each soil sample at 105 °C for 1 h to determine moisture content[13,59] and then discarded the dry soil. We then extracted total nucleic acids from a separate 1 g (calculated for dry weight) portion of each sample and spiked samples with approximately 10^7 plaque-forming units MS2 (Luminex Corporation, Austin, TX) as an extraction control. Following the manufacturer's protocol, we extracted RNA using the RNeasy PowerSoil Total RNA Kit and DNA using the RNeasy PowerSoil DNA Elution Kit (Qiagen, Hilden, Germany). On each day of extraction (approximately every 5−15 samples), we included one negative extraction control (sterile deionized water). We tested sample extracts for matrix inhibition using the Applied Biosystems Exogenous Internal Positive Control Assay[60] (Applied Biosystems, Waltham, Massachusetts) before downstream molecular analysis (Text S2).

We assayed extracted nucleic acids from all samples using a custom TaqMan Array Card (TAC) (ThermoFisher Scientific, Waltham, MA) that tested for genes from 18 enteric pathogens in duplicate wells following Liu et al. 2013,[61] including ten bacteria (*Campylobacter jejuni/coli*, *Clostridium difficile* [*tcdA* and *tcdB* gene], Enteroaggregative *E. coli* [EAEC, *aaiC* and *aatA* gene], *Shigella*/Enteroinvasive *E. coli* [EIEC, *ipaH* gene], Enteropathogenic *E. coli* [EPEC, *bfpA* and *eae* gene], Enterotoxigenic *E. coli* [ETEC, heat-labile and heat-stabile enterotoxin genes LT and ST], Shiga-toxin producing *E. coli* [STEC, *stx1* and *stx2*], *Salmonella* spp., *Vibrio cholerae*, and *Yersinia* spp.), four viruses (adenovirus 40/41, astrovirus, norovirus [GI and GII], and rotavirus A), two protozoa (*Entamoeba histolytica* and *Giardia duodenalis*) and two soil-transmitted helminths (*Ascaris lumbricoides*, *Trichuris trichiura*)

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Table 1. Characteristics of MapSan Trial Compounds and Households Selected for Soil Sampling[a]

			Baseline				24 Month Phase			
			Control		Intervention		Control		Intervention	
Characteristic	Level	Metric	N	Summary	N	Summary	N	Summary	N	Summary
Wealth index (0−1)	household	mean (sd)	48	0.47 (0.09)	43	0.46 (0.09)	45	0.44 (0.12)	43	0.40 (0.09)
Compound population	compound	mean (sd)	48	14 (6.4)	43	19 (7.8)	45	13 (7.0)	43	16 (7.9)
Any animal(s) present	compound	n (%)	48	28 (58%)	43	28 (65%)	45	32 (71%)	43	35 (81%)
Cat(s) present	compound	n (%)	48	24 (50%)	43	23 (53%)	45	32 (71%)	43	30 (70%)
Chicken(s) or duck(s) present	compound	n (%)	48	6 (13%)	43	7 (16%)	45	4 (8.9%)	43	8 (19%)
Dog(s) present	compound	n (%)	48	3 (6.3%)	43	4 (9.3%)	45	9 (20%)	43	10 (23%)
Other animal(s) present	compound	n (%)	48	1 (2.1%)	43	2 (4.7%)	45	1 (2.2%)	43	0 (0%)
Visible human or animal feces	compound	n (%)	48	22 (46%)	43	22 (51%)	45	4 (8.9%)	43	4 (9.3%)
Visibly wet soil	sample	n (%)	48	37 (77%)	43	34 (79%)	45	37 (82%)	43	34 (79%)
Partially shaded soil	sample	n (%)	48	24 (50%)	43	13 (30%)	45	30 (67%)	43	28 (65%)
Fully shaded soil	sample	n (%)	48	14 (29%)	43	20 (47%)	45	10 (22%)	43	9 (21%)
Temperature (°F)	date	mean (sd)	48	72 (4.5)	43	70 (4.3)	45	72 (4.7)	43	73 (5.3)
No useable sanitation infrastructure	compound	n (%)	48	3 (6.3%)	43	4 (9.3%)	45	0 (0%)	43	0 (0%)
Pit latrine with slab	compound	n (%)	48	27 (56%)	43	14 (14%)	45	18 (40%)	43	0 (0%)
Pit latrine without slab	compound	n (%)	48	16 (33%)	43	24 (56%)	45	14 (31%)	43	0 (0%)
Pour-flush toilet (nonintervention)	compound	n (%)	48	2 (4.2%)	43	1 (2.2%)	45	13 (29%)	43	0 (0%)
Intervention infrastructure	compound	n (%)	48	0 (0%)	43	0 (0%)	45	0 (0%)	43	43 (100%)

[a]Note: Wealth index created using the 2013 Simple Poverty Scorecard© for Mozambique.

(Text S3, Table S1, Table S2).[62] We combined and then added a mixture of 25 μL of RNA eluant, 25 μL of DNA eluant, and 50 μL of Mastermix (qScript XLT 1-Step RT-qPCR ToughMix Low-ROX, Quantabio, Beverly, MA) into each TAC port. We included a positive and negative control on each TAC. The positive control was a plasmid that included all assay gene sequences, and the negative control was either an extract from a negative extraction control or sterile water.[63] We performed one-step reverse transcription qPCR on each TAC using a QuantStudio 7 (Thermo Fisher Scientific, Waltham, MA) with the following thermocycling conditions: 45 °C for 10 min and 94 °C for 10 min, followed by 45 cycles of 94 °C for 30 s and 60 °C for 1 min, with a ramp rate of 1 °C/second between each step. We visually compared exponential curves and multicomponent plots with the positive control plots to validate positive amplification;[12] positive amplification in one or both duplicate wells below a quantification cycle (Cq) of 40 was called as a positive for a target (Text S3).[62,64] In addition, we ran a 5-fold dilution series of positive control material as a standard curve to determine the linearity and efficiency of each assay (Table S3).

Data Analysis. We analyzed data in R version 4.0.0 (R Foundation for Statistical Computing, Vienna, Austria). We used a difference-in-difference (DID)[65] approach to assess the impact of the intervention, our exposure variable, on our outcomes compared to the control group. DID is a quasi-experimental method that uses longitudinal data from intervention and control groups. Fundamental to the DID method is the parallel trend assumption, which assumes that the initial difference between the two groups remains constant over time. Our outcomes included the detection (i.e., binary presence/absence) of ≥1 of the enteric pathogen genes measured, the total number of pathogens detected out of 18, and each individual pathogen (Table S4). We used generalized estimating equations (GEE)[66] to fit unadjusted and adjusted Poisson regression models with robust standard errors, with an exchangeable correlation structure. We accounted for clustering between compounds across the two study phases because the intervention was implemented at the compound level.[67]

To generate adjusted estimates, we selected nine covariates from the MapSan baseline and 24 month data sets based on their biological plausibility to impact the transport[57] or persistence[68] of pathogens in the domestic environment and previously reported associations in the literature[36,44] (Table S4). We used the same nine covariates to adjust all DID models: compound population (a 10-person increase in compound population), wealth (one-quartile increase in wealth index[69]), soil moisture (assessed visually at the time of sampling), sun exposure status (estimated at the time of sampling; full sun, partially shaded, shaded[44]), the mean-centered average air temperature in Fahrenheit for the day of and day preceding sample collection (i.e., two-day average), a binary variable for the presence of cats, a binary variable for the presence of dogs, a binary variable for the presence of chickens or ducks, and a binary variable for the presence of visible animal or human feces in the compound (Table S4).

To estimate the intervention's effect, we used the interaction of dummy variables representing treatment status (intervention vs control) and trial phase (baseline or 24 month). Consequently, we present the effect estimates from our DID analysis as ratio measures (ratio of prevalence ratios, PR) instead of absolute differences. We fit separate GEE models to measure the association between intervention status and the detection of ≥1 pathogen gene and the total number of pathogens detected among the 18 targets we identified *a priori* (Table S4). Likewise, we fit DID models to estimate the intervention's impact for each individual pathogen assessed, but we excluded any pathogen not detected in at least 5% of control and intervention samples during both phases.

Ethics. The study protocol was approved by the Comité Nacional de Bioética para a Saúde (CNBS), Ministério da Saúde (333/CNBS/14), the Research Ethics Committee of the London School of Hygiene and Tropical Medicine (reference # 8345), and the Institutional Review Board of the Georgia Institute of Technology (protocol # H15160). The overall trial was preregistered at ClinicalTrials.gov (NCT02362932), but we did not preregister this environmental analysis.

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RESULTS

Matched Samples. We analyzed latrine entrance soils collected at baseline from 48 control compounds and 43 intervention compounds, and soils collected at the 24 month phase from 45 control and 43 intervention compounds (Table S5). We did not analyze 12 intervention samples and nine control samples because they were either lost or damaged during storage. This resulted in some samples collected at either phase not having a matched sample from the same compound from the earlier or later phase. Among the 93 control samples analyzed, 42 compounds had samples from both phases (n = 84), six baseline samples did not have a matched 24 month phase sample, and three 24 month samples did not have a matched baseline sample. Among the 86 intervention samples analyzed, 41 compounds had samples from both phases (n = 82), two baseline samples did not have a matched 24 month phase sample, and two 24 month samples did not have a matched baseline sample. There was a mean of 788 days between the collection of matched control samples (sd = 36, min = 733, max = 860) and a mean of 789 days between matched intervention samples (sd = 56, min = 731, max = 953). Control and intervention samples were collected approximately during the same period of the year (Figure S1).

Compound Characteristics. Control and intervention compounds had similar wealth indices at baseline (mean = 0.47 [sd = 0.09] and mean = 0.46 [sd = 0.09], respectively, p = 0.49) but control compounds had higher wealth indices at the 24 month phase (mean = 0.46 [sd = 0.12] and mean = 0.40 [sd = 0.09], respectively, p = 0.05) (Table 1). The number of residents in the intervention compounds was greater than those in the control compounds at baseline (mean = 19 [sd = 7.8] and mean = 14 [sd = 6.4], respectively, p = 0.004) and at the 24 month phase (mean = 16 [sd = 7.9] and mean = 13 [sd = 7.0], respectively, p = 0.02) (Table 1).

Reported or observed animal ownership was high across trial arms during both phases (Table 1). Most compounds had at least one animal at baseline (62% [56/91]) including cats (50% [24/48] control, 53% [23/43] intervention), chickens or ducks (13% [6/48] control, 16% [7/43] intervention), and dogs (6.3% [3/48] control, 9.3% [4/43] intervention). Three-quarters of compounds had at least one animal 24 months post intervention (76% [67/88]): cats were most common (71% [32/45] control, 70% intervention [30/43]) followed by dogs (20% [9/45] control, 23% [10/43] intervention), and chickens or ducks (8.9% [4/45] control, 19% [8/43] intervention).

At the baseline, seven compounds had no useable sanitation infrastructure (6.3% [3/48] control, 9.3% [4/43] intervention) and three compounds had pour-flush sanitation (4.2% [2/48] control, 2.3% [1/43] intervention) (Table 1). Control compounds more often had pit latrines with slabs (56%, [27/48]) than those without slabs (33%, [16/48]), compared to intervention compounds, which more often had pit latrines without slabs (56%, [24/43]) than those with slabs (33%, [14/43]) (p = 0.09). At the 24 month phase, most control compounds had a pit latrine (with slab 40%, [18/45]; without slab 31%, [14/45]), but some (29%, [13/45]) had independently upgraded their pit latrines to pour-flush toilets. All intervention compounds (100%, [43/43]) still had the intervention sanitation infrastructure at the 24 month phase.

Laboratory Controls. We did not observe inhibition in any sample (Text S2). We observed positive amplification for all assays using our positive controls (n = 32). We did not observe positive amplification for any assay in our extraction controls (n = 16) nor any no template controls (n = 16) below a Cq of 40. Although we did observe positive amplification for EPEC (*eae* gene) in two no template controls above a Cq of 40.

All Pathogens. We detected at least one pathogen-associated gene in 91% (163/179) of latrine entrance soils, genes from two or more pathogens in 75% (134/179), and a mean of 3.3 out of 18 measured pathogen targets (IQR = 4). The four most frequently detected pathogens were *Ascaris lumbricoides* (62%, [111/179]), EAEC (46%, [82/179]), *Giardia duodenalis* (36%, [64/179]), and astrovirus (26%, [47/179]). We found evidence that the intervention reduced the detection of ≥1 pathogen gene in latrine entrance soils by 15% (aPR = 0.85, 95% CI [0.70, 1.0]) and the total number of pathogens by 35% (aPR = 0.65, 95% CI [0.44, 0.95]) (Table 2). The mean Cq values of detected pathogen genes were similar across trial arms and phases (Table S6).

Table 2. Detection of Pathogens at Baseline and 24-Months

Detection	Baseline Detection	24 month Detection	Unadjusted BL-24 M DID estimate	Adjusted BL-24 M DID estimate
≥1 pathogen gene				
control	0.88 (42/48)	0.96 (43/45)	0.82 (0.68, 1.0) p = 0.05	0.85 (0.70, 1.0) p = 0.11
intervention	0.95 (41/43)	0.86 (37/43)		
Total pathogen detects (out of 18)	Mean (IQR)	Mean (IQR)		
control	3.5 (4)	3.8 (3)	0.67 (0.45, 1.0) p = 0.05	0.65 (0.44, 0.95) p = 0.03
intervention	3.5 (3)	2.5 (2.5)		

Note: DID: difference-in-difference. BL: baseline. 24M: 24 month. IQR: Interquartile range.

There was a consistent trend among all individual pathogens except for astrovirus: the adjusted point estimates for nine of the ten most frequently detected suggest the intervention reduced the prevalence of these pathogens in soils compared to controls (Table 3). Among these nine pathogens, we observed a significant reduction in *Ascaris lumbricoides* (aPR = 0.62, 95% CI [0.39, 0.98]), EAEC (aPR = 0.51, 95% CI [0.27, 0.94]), and EPEC (aPR = 0.20 95% CI [0.05, 0.82]).

DISCUSSION

We found evidence that the onsite shared urban sanitation intervention evaluated in the MapSan trial was somewhat protective against the detection of ≥1 pathogen gene and against the total number of pathogens in latrine entrance soils although the confidence intervals for the detection of ≥1 pathogen gene included one, meaning that there may have been no true effect of the intervention on this outcome. The adjusted estimates for nine of the ten most common pathogens suggest the intervention reduced their presence in soils compared to controls (DID estimates = 0.20−0.95), and pathogen-specific effect estimates from adjusted models indicated a significant reduction in *Ascaris lumbricoides*, EAEC, and EPEC. This suggests that intervention septic tanks may have better sequestered or inactivated these

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Table 3. Detection of Individual Pathogens at Baseline and 24-Month[a]

Pathogen	Baseline Detection	24 month Detection	Unadjusted BL-24 M DID estimate[b]	Adjusted BL-24 M DID estimate[b]
Ascaris lumbricoides				
control	0.65 (31/48)	0.76 (34/45)	0.64 (0.40, 1.0) p = 0.06	0.62 (0.39, 0.98) p = 0.04
intervention	0.63 (27/43)	0.44 (19/43)		
Enteroaggregative *E. coli*				
control	0.42 (20/48)	0.53 (24/45)	0.57 (0.30, 1.1) p = 0.08	0.51 (0.27, 0.94) p = 0.03
intervention	0.51 (22/43)	0.37 (16/43)		
Giardia duodenalis				
control	0.42 (20/48)	0.38 (17/45)	0.76 (0.34, 1.7) p = 0.50	0.85 (0.37, 1.9) p = 0.69
intervention	0.37 (16/43)	0.26 (11/43)		
Shigella/Enteroinvasive *E. coli*				
control	0.33 (16/48)	0.33 (15/45)	0.58 (0.17, 1.9) p = 0.37	0.59 (0.18, 1.9) p = 0.38
intervention	0.16 (7/43)	0.09 (4/43)		
Enterotoxigenic *E. coli*				
control	0.25 (12/48)	0.33 (15/45)	0.45 (0.17, 1.2) p = 0.10	0.44 (0.17, 1.1) p = 0.09
intervention	0.35 (15/43)	0.21 (9/43)		
adenovirus 40/41				
control	0.23 (11/48)	0.33 (15/45)	0.34 (0.08, 1.5) p = 0.19	0.32 (0.07, 1.5) p = 0.14
intervention	0.14 (6/43)	0.07 (3/43)		
astrovirus				
control	0.23 (11/48)	0.27 (12/45)	1.3 (0.51, 3.1) p = 0.62	1.6 (0.60, 4.0) p = 0.36
intervention	0.23 (10/43)	0.33 (14/43)		
Enteropathogenic *E. coli*				
control	0.15 (7/48)	0.24 (11/45)	0.20 (0.05, 0.88) p = 0.02	0.20 (0.05, 0.82) p = 0.03
intervention	0.21 (9/43)	0.07 (3/43)		
Trichuris trichiura				
control	0.31 (15/48)	0.18 (8/45)	1.0 (0.33, 3.2) p = 0.96	0.95 (0.32, 2.9) p = 0.93
intervention	0.28 (12/43)	0.16 (7/43)		
Clostridium difficile				
control	0.13 (6/48)	0.16 (7/45)	0.68 (0.17, 2.8) p = 0.59	0.70 (0.16, 3.0) p = 0.62
intervention	0.16 (7/43)	0.14 (6/43)		
Salmonella spp.				
control	0.02 (1/48)	0.09 (4/45)	NA	
intervention	0.05 (2/43)	0.05 (2/43)		
Shiga-toxin producing *E. coli*				
control	0.02 (1/48)	0.07 (3/45)	NA	
intervention	0 (0/43)	0.02 (1/43)		
Campylobacter jejuni/coli				
control	0.15 (7/48)	0.04 (2/45)	NA	
intervention	0.09 (4/43)	0.09 (4/43)		
Yersinia spp.				
control	0.02 (1/48)	0.04 (2/45)	NA	
intervention	0.05 (2/43)	0.05 (2/43)		
norovirus GI/GII				
control	0.06 (3/48)	0.02 (1/45)	NA	
intervention	0.07 (3/43)	0.02 (1/43)		
rotavirus A				
control	0.08 (4/48)	0 (0/45)	NA	
intervention	0.14 (6/43)	0.09 (4/43)		
Entamoeba histolytica				
control	0.02 (1/48)	0 (0/45)	NA	
intervention	0.02 (1/43)	0.02 (1/43)		
Vibrio cholerae				
control	0 (0/48)	0 (0/45)	NA	
intervention	0 (0/43)	0 (0/43)		

[a]Sorted by detection in control soils at the 24 month phase. Note: DID: difference-in-difference. [b]We did not calculate DID estimates for pathogens with <5% detection.

pathogens, which are passed in stool, in comparison with controls.

Seven of the ten pathogens we most frequently detected in soils were measured in child stools via multiplex end-point

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PCR or microscopy as part of the MapSan trial. These seven include *Ascaris lumbricoides*, *Giardia duodenalis*, *Shigella*/EIEC, ETEC, adenovirus 40/41, *Trichuris trichiura*, and *Clostridium difficile*, while EAEC, EPEC, and astrovirus were not measured in stools. At baseline, *Shigella*/EIEC (44%) and *Trichuris trichiura* (37%), generally thought to be transmitted human-to-human, were the second and third most common pathogens detected in child stool,[24,50] following *Giardia* (51%) which can be zoonotic.[70] Given the high prevalence of these anthroponotic enteric pathogens in stools and the lack of a zoonotic reservoir for *Shigella*/EIEC and *Trichuris trichiura*,[71,72] the trial may have had greater power to observe an effect on *Shigella*/EIEC and *Trichuris trichiura* compared with other pathogens. For children born into study compounds before the 24 month visit, the intervention reduced the detection of *Shigella*/EIEC in children's stools by 51% and *Trichuris trichiura* by 76%.[58] Results from soils in this study differ from trial findings in stools: we observed a 41% reduction in the point estimate for *Shigella*/EIEC detection but the wide confidence interval indicates this result was not significant, and we identified no difference with respect to detection of *Trichuris trichiura*. This absence of impact on *Trichuris trichiura* in soils may have been due to limited power from infrequent detection; we did observe a reduction in the other STH assessed, *Ascaris lumbricoides*, which was the most frequently detected individual pathogen in soils. The MapSan trial found the sanitation intervention reduced the detection of *Ascaris lumbricoides* by 32% among children born into study compounds before the 24 month visit, but the confidence interval extended above one indicating the intervention may have had no true effect or may even have increased children's risk of infection.[58] Overall, the protective trend we observed in soils, therefore, is consistent with the enteric infection data for children born into trial compounds. This may suggest that the intervention reduced the transport of pathogens to latrine entrance soils and subsequently contributed to a reduction in children's exposures, but our small sample size and the resulting uncertainty of point estimates suggest results should be interpreted with caution.

In comparison to other recent large-scale, rigorous trials of onsite sanitation improvements in rural Bangladesh (pour flush to double-pit latrine),[2] rural Kenya (single unlined pit latrine with plastic slab and hole-lid),[3] and rural Zimbabwe (ventilated improved pit latrine),[4] we evaluated a more sophisticated intervention that included site-specific engineered septic tanks and subsurface discharge of aqueous effluent to a soakaway pit,[24,73] and it is the only recent controlled health impact trial of onsite sanitation to take place in an urban setting. In the early 2000s, Barreto et al. observed health benefits from household sewerage connections in urban Brazil in an uncontrolled trial.[74,75] However, the scope, complexity, and cost of that intervention make it an imperfect point of comparison.

The WASH Benefits Trial (WASH-B) evaluated the impacts of single and combined water, sanitation, and handwashing intervention arms in rural Bangladesh and Kenya. In Bangladesh, a molecular analysis of household entrance soils, hand rinses, and stored water from the sanitation arm found no significant reductions in enteric pathogens (EAEC, EPEC, STEC, *Shigella*/EIEC, ETEC, norovirus, *Cryptosporidium* spp., *Giardia duodenalis*) or microbial source tracking markers (HumM2, BacCow).[38] The combined WASH arm and individual water treatment arm observed a reduction in *E. coli* prevalence and concentration in stored drinking water; the individual water treatment and handwashing arms reduced *E. coli* prevalence and concentration in food. WASH-B trial arms in Bangladesh did not observe reductions in *E. coli* in courtyard soil, ambient waters, child hands, or sentinel objects.[76,77] Likewise, WASH-B Kenya found the individual water treatment arm and combined WASH arm reduced culturable *E. coli* in stored drinking water, but not along other transmission pathways.[18] The Sanitation, Hygiene, Infant Nutrition Efficacy Project (SHINE) trial in rural Zimbabwe has not yet published the results from a substudy on environmental fecal contamination. In separate analyses of environmental samples collected during MapSan baseline[15] and the 24 month phase,[13,21,44] we found widespread fecal contamination in soils and other environmental compartments. At the 12-month MapSan trial phase, Holcomb et al. 2021 found the intervention reduced *E. coli* gene densities by more than 1-$\log_{10}$ in latrine entrance soils but observed no reduction in culturable *E. coli* or human microbial source tracking markers.[78] Our study is the first controlled evaluation of an urban onsite sanitation intervention to show a decrease in the detection of enteric pathogens, via molecular methods, in soils from the domestic living environment.

The intervention may have reduced the presence of enteric pathogens in soils compared with that in controls because the intervention may have better sequestered or treated fecal material than control latrines. In high-income countries, properly designed, constructed, and maintained septic tank systems have been demonstrated to be efficient and economic alternatives to public sewage disposal systems.[79] Although some pathogen die-off will occur in pit latrines, the primary purpose of pit latrines is to sequester human feces and reduce exposures, and they are not designed to achieve a specific level of pathogen reduction.[80] Design features of the intervention septic tanks may have resulted in better treatment of fecal wastes than control systems. Intervention septic tanks contained inlet and outlet pipes configured to maximize detention time, baffles to direct incoming waste downward, t-pipes to ensure sequestration of solids and floatable materials, and a sealed containment chamber to promote anaerobic treatment of stored solids and nonsettleable materials. In addition, the intervention septic tank systems represented an upgrade to a more permanent sanitation infrastructure. The construction included masonry block walls, a concrete floor, masonry block lined septic tank, masonry block lined soakaway pit, tin roof, and a water seal squat pan.[20,24,53,73] These features may have acted as a physical barrier that prevented the contamination of soils by enteric pathogens. At the 24 month phase, most control compounds used a pit latrine with or without a slab and therefore lacked similar physical barriers such as a water seal. In addition, the control compounds that did upgrade to pour flush sanitation may not have used the same rigorous design criteria as intervention septic tanks.[50]

Pit latrines in low-income Maputo are often covered when full and rebuilt, or the fecal sludge is emptied and buried or dumped nearby.[20] The intervention included programming to encourage hygienic pit emptying and provided equipment and training to local organizations to offer hygienic emptying services.[73] During the 24 month phase, only 5.6% of intervention compounds had emptied their sanitation systems in the previous year, compared to 30% of controls.[20] In addition, intervention compounds were 3.8 more likely to have their onsite systems emptied hygienically than control

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compounds.[20] Less frequent emptying would have been beneficial for two reasons. First, longer residence times would likely have resulted in greater pathogen die-off.[80] Second, less frequent emptying would have created fewer opportunities for environmental fecal contamination to occur and hygienic emptying may have reduced the quantity of fecal sludge that contaminated soils during emptying. In addition, intervention systems contained a drain for bathing, which may have prevented fecally contaminated graywater from flowing into nearby soils, and the concrete floors were likely easier to clean than control systems with dirt floors.[56]

Although our findings suggest that some pathogens appeared to be reduced by the latrine improvements, it is likely that the potential for exposure remains high in this setting.[13] While we detected some individual pathogens, such as *Ascaris lumbricoides*, EAEC, *Shigella*/EIEC and EPEC, in intervention soils less frequently compared to controls during the 24 month phase, we also detected one or more enteric pathogens in 86% of intervention latrine entrance soils two years postintervention. Fecal waste from children unable to use the latrines was not addressed by the intervention.[28,81] At the 24 month follow-up, 29% (289/980) of children reported defecating into a latrine, 29% (281/980) defecated into a child potty which was emptied into a latrine, 20% (192/980) used disposable diapers that were disposed with solid waste, 7.3% defecated on the ground (72/980), and 2.7% (26/980) defecated into diapers that were washed and reused (Table S7). In addition, the intervention did not address animal feces. While we adjusted for animals in our DID estimates, many animals are not penned in this setting and may defecate outside of their respective compounds, which was not accounted for in our analysis.[30] Live chickens are also commonly purchased and stored in the compound for consumption.[82] We may not have adequately captured this intermittent chicken ownership in our cross-sectional surveys.

The similar reduction in pathogen detection in soils and child stools may be informative about exposures. At two years postintervention in the MapSan cohort, children born into study compounds were 1−24 months old, while children born previously and enrolled at baseline were 25−73 months old.[58] Considering the consistent reduction in the detection of pathogens observed in soils and stools from children 1−24 months old, the dominant exposure pathways for these younger children may be inside the compound or soil ingestion may have represented a more important transmission pathway for these children.[83] Older children are more mobile than younger children, and their potential exposures outside of study compounds may explain why the intervention did not reduce the prevalence of pathogen carriage among them.

Our study had several limitations, including a relatively small sample size that was not intended to observe small reductions in pathogen detection. Nevertheless, in high burden settings, sanitation interventions may need to achieve a large reduction in environmental fecal contamination both within households and in the larger community to reduce exposure risks and yield improved health outcomes.[84] Further, intervention compounds had lower wealth indices and higher compound populations 24 months post intervention compared to control. This may suggest we underestimated changes due to sanitation improvements, but we adjusted for these in our regression analyses and did not observe substantial differences between unadjusted and adjusted point estimates that would indicate confounding. In addition, we assessed gene targets via molecular assays, which may not be 100% sensitive or specific,[61,85,86] that can be effected by target specific inhibitors[87] and did not assess pathogen viability or infectivity.

There is substantial evidence that city-wide upgrades to sewerage infrastructure improve health outcomes.[74,75,88] However, the high capital and maintenance costs,[89] and water usage requirements[90] of such improvements suggest they are currently impractical for many LMICs. Until sewerage becomes widely feasible in high-burden settings, onsite sanitation systems remain necessary to achieve safely managed sanitation in many urban areas. The results of this study, and other rigorous environmental impact evaluations of onsite sanitation interventions,[18,38,77] suggest that fecal contamination is transported into the environment through multiple complex pathways that may vary among settings.[91] In urban Maputo and in similar settings with poor sanitation infrastructure, widespread environmental fecal contamination, and a high burden of enteric infection, other, more transformative interventions interrupting multiple transmission pathways may need to accompany improvements to onsite sanitation infrastructure. These improvements likely require an integrated and incremental approach that might include legal protections (e.g., land tenure),[92] contact control interventions (e.g., hardscape cleanable flooring),[13,93,94] public infrastructure (e.g., drainage, and improvements in quality, quantity, and access to water),[95] and public services (e.g., education, hygienic fecal sludge and solid waste management).[20,96,97] Such improvements may reduce the transport of enteric pathogens into the environment through site-specific pathways and subsequently reduce children's infection risks.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c02168.

Compound enrollment at baseline, test for matrix inhibition, custom TaqMan Array Card (TAC), assays used on the custom TAC, interpretation of gene targets on the TAC, standard curve, description of variables and their respective sources, soils samples matched at baseline and 24 month trial periods, histogram of dates that latrine entrance soils were collected, mean Cq values, child feces disposal at 24 month phase (PDF)

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Notes

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IMPACT OF AN URBAN SANITATION INTERVENTION ON ENTERIC PATHOGEN DETECTION IN SOILS

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Supplemental Information

Text S1. Compound enrollment at baseline

At study baseline we intended to enroll an equal number of control and intervention compounds for soil sample collection. We mistakenly collected soil samples from more control compounds (n = 51) compared to compounds which received an intervention after the baseline visit (n=49) due to a miscommunication between the implementing organization, the research team, and the field staff.

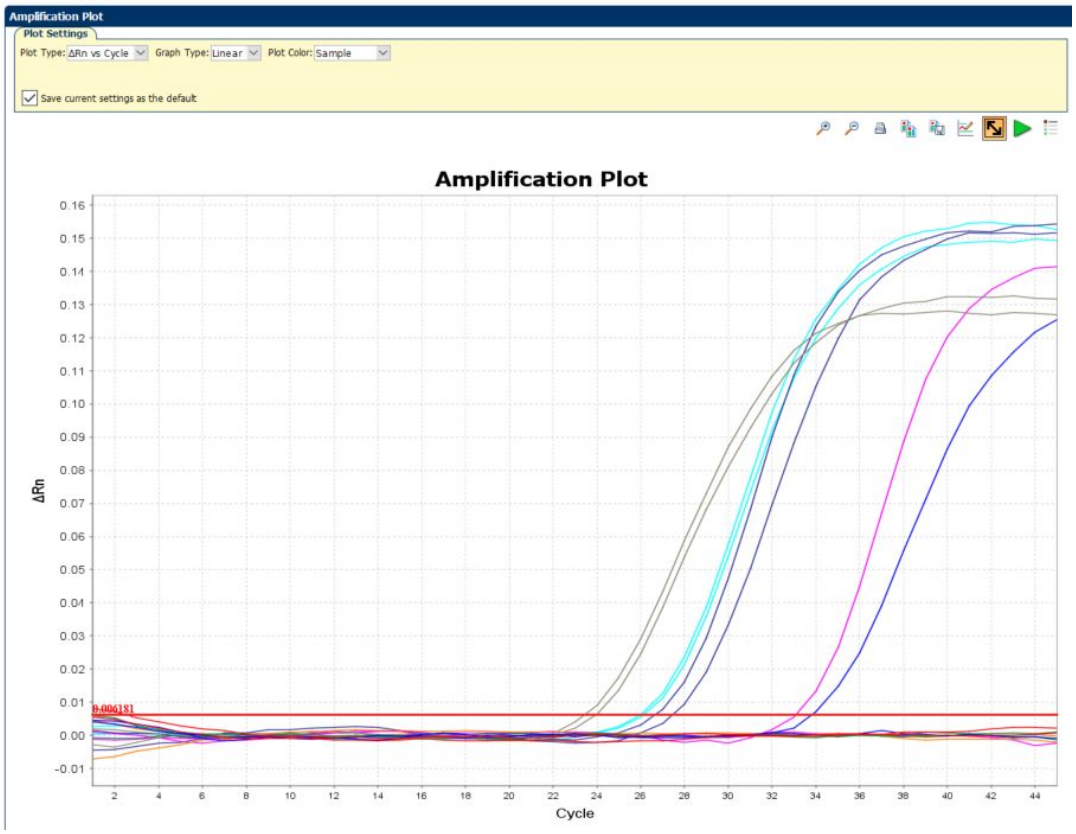
Text S2. Test for Matrix Inhibition

We used the TaqMan™ Exogenous Internal Positive Control Assay to test soils samples for matrix inhibition. Following the manufacturer's protocol, we spiked in the internal positive control (IPC) to qPCR assays containing IPC specific primers and probes, TaqMan Universal PCR Mastermix, and nucleic acid extract from each soil sample. This assay is not designed for normalization, but it is designed to determine if a sample exhibits PCR inhibition. A negative call for the IPC suggests the presence of PCR inhibition and positive call for the IPC suggests an absence of PCR inhibition. We ran this assay on an Applied Biosystems 7500 real-time PCR system with manufacturer recommended thermocycling conditions: two minutes at 50°C, ten minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds followed by 60°C for one minute. We observed positive amplification of the IPC in all assays suggesting an absence of PCR inhibition in our samples. The lack of PCR inhibition may be a result of the mass of soil we extracted from. The RNeasy PowerSoil Total RNA Kit and RNeasy PowerSoil DNA Elution Kit are designed for extraction from up to two grams of soil, but we only extracted from one gram of dry weight soil. This relatively low mass of soil used may have reduced the potential for inhibitors in our extracts.

Text S3. Custom TaqMan Array Card (TAC)

We purchased custom TACs produced by Thermo Fisher Scientific (Waltham, MA). TAC is a 384-well array card with 8 ports for loading samples and each 1-µL well contains dried-down primers and hydrolysis probes for the detection of defined targets.

For analysis, we mixed 25 µL of DNA template and 25 µL of RNA template (0.5 µL total template per reaction well) with 50 µL of qScript XLT 1-Step RT-qPCR ToughMix (Quantabio, Beverly, MA), then filled ports 2-7 with the combined 100 µL. In total we tested 6 samples per card, using the first port as a negative control and the last port as a positive control, for which we used individual aliquots of our combined positive control material (gene targets inserted into plasmids) (IDT, Coralville, IA). Combined positive controls were developed using methods from Kodani *et al.* 2012. Following the manufacturer's instructions, we centrifuged each card twice at 1,200 rpm for one minute, sealed the card, trimmed the loading ports, and loaded the card into a QuantStudio 7 (Thermo Fisher Scientific, Waltham, MA). All positive controls amplified as expected (typically ~ Cq = 28-30 depending on the assay) and we detected MS2 in all samples. Using the Quantstudio Real-Time PCR software (Thermo Fisher Scientific, Waltham, MA) we plotted ΔRn (y-axis) vs. cycle number (x-axis) for each assay and set the threshold (on the y-axis) at the point where the positive control began exponential amplification (Figure S1). Among 16 extraction controls and 16 no template controls we observed no amplification for any target below a Cq of 40.



Text S3 Figure 1: Amplification plot in Quantstudio Real-Time PCR Software. The threshold, which determines the Cq value, is the red horizontal line and was set manually by the user. All assays were run in duplicate wells, and amplification in either well was called positive.

Using the extraction methods described in the manuscript, the 0.25 µL of DNA or RNA template in each reaction well on TAC represents a 400-fold dilution from the starting gram of soil.

Equation S1: $$dilution\ factor = \frac{0.25\ \mu L\ template\ per\ well}{100\ \mu L\ total} \times 1\ gram\ dry\ weight\ soil = \frac{1}{400}$$

Table S1. Assays used on the custom TAC

Target	Assay reference
Bacteria	
Campylobacter coli	Cunningham, S. A.; Sloan, L. M.; Nyre, L. M.; Vetter, E. A.; Mandrekar, J.; Patel, R. Three-Hour Molecular Detection of Campylobacter, Salmonella, Yersinia, and Shigella Species in Feces with Accuracy as High as That of Culture. *J. Clin. Microbiol.* **2010**, *48* (8), 2929–2933.
Campylobacter jejuni	Cunningham, S. A.; Sloan, L. M.; Nyre, L. M.; Vetter, E. A.; Mandrekar, J.; Patel, R. Three-Hour Molecular Detection of Campylobacter, Salmonella, Yersinia, and Shigella Species in Feces with Accuracy as High as That of Culture. *J. Clin. Microbiol.* **2010**, *48* (8), 2929–2933.
Clostridium difficile (*tcdA*)	Houser, B. A.; Hattel, A. L.; Jayarao, B. M. Real-Time Multiplex Polymerase Chain Reaction Assay for Rapid Detection of Clostridium Difficile Toxin-Encoding Strains. *Foodborne Pathog. Dis.* **2010**, *7* (6), 719–726.
Clostridium difficile (*tcdB*)	Houser, B. A.; Hattel, A. L.; Jayarao, B. M. Real-Time Multiplex Polymerase Chain Reaction Assay for Rapid Detection of Clostridium Difficile Toxin-Encoding Strains. *Foodborne Pathog. Dis.* **2010**, *7* (6), 719–726.
E. coli / Shigella (*ipaH* gene)	Thiem, V. D.; Sethabutr, O.; Seidlein, L. von; Tung, T. Van; Canh, D. G.; Chien, B. T.; Tho, L. H.; Lee, H.; Houng, H.-S.; Hale, T. L.; et al. Detection of Shigella by a PCR Assay Targeting the IpaH Gene Suggests Increased Prevalence of Shigellosis in Nha Trang, Vietnam. *J. Clin. Microbiol.* **2004**, *42* (5), 2031–2035.
EAEC (*aaiC* gene)	Liu, J.; Gratz, J.; Amour, C.; Kibiki, G.; Becker, S.; Janaki, L.; Verweij, J. J.; Taniuchi, M.; Sobuz, S. U.; Haque, R.; et al. A Laboratory-Developed Taqman Array Card for Simultaneous Detection of 19 Enteropathogens. *J. Clin. Microbiol.* **2013**, *51* (2), 472–480.
EAEC (*aatA* gene)	Boisen, N.; Struve, C.; Scheutz, F.; Krogfelt, K. A.; Nataro, J. P. New Adhesin of Enteroaggregative Escherichia Coli Related to the Afa/Dr/AAF Family. *Infect. Immun.* **2008**, *76* (7), 3281–3292.
EPEC (*bfpA* gene)	Liu, J.; Gratz, J.; Amour, C.; Kibiki, G.; Becker, S.; Janaki, L.; Verweij, J. J.; Taniuchi, M.; Sobuz, S. U.; Haque, R.; et al. A Laboratory-Developed Taqman Array Card for Simultaneous Detection of 19 Enteropathogens. *J. Clin. Microbiol.* **2013**, *51* (2), 472–480.
EPEC (*eae* gene)	Liu, J.; Gratz, J.; Amour, C.; Kibiki, G.; Becker, S.; Janaki, L.; Verweij, J. J.; Taniuchi, M.; Sobuz, S. U.; Haque, R.; et al. A Laboratory-Developed Taqman Array Card for Simultaneous Detection of 19 Enteropathogens. *J. Clin. Microbiol.* **2013**, *51* (2), 472–480.
ETEC-LT	Hidaka, A.; Hokyo, T.; Arikawa, K.; Fujihara, S.; Ogasawara, J.; Hase, A.; Hara-Kudo, Y.; Nishikawa, Y. Multiplex Real-Time PCR for Exhaustive Detection of Diarrhoeagenic *Escherichia Coli*. *J. Appl. Microbiol.* **2009**, *106* (2), 410–420.
ETEC-ST	Liu, J.; Gratz, J.; Amour, C.; Kibiki, G.; Becker, S.; Janaki, L.; Verweij, J. J.; Taniuchi, M.; Sobuz, S. U.; Haque, R.; et al. A Laboratory-Developed Taqman Array Card for Simultaneous Detection of 19 Enteropathogens. *J. Clin. Microbiol.* **2013**, *51* (2), 472–480.

Salmonella	Liu, J.; Gratz, J.; Amour, C.; Kibiki, G.; Becker, S.; Janaki, L.; Verweij, J. J.; Taniuchi, M.; Sobuz, S. U.; Haque, R.; et al. A Laboratory-Developed Taqman Array Card for Simultaneous Detection of 19 Enteropathogens. *J. Clin. Microbiol.* **2013**, *51* (2), 472–480.
Shiga-like toxin 1 (*stx1*)	Liu, J.; Gratz, J.; Amour, C.; Kibiki, G.; Becker, S.; Janaki, L.; Verweij, J. J.; Taniuchi, M.; Sobuz, S. U.; Haque, R.; et al. A Laboratory-Developed Taqman Array Card for Simultaneous Detection of 19 Enteropathogens. *J. Clin. Microbiol.* **2013**, *51* (2), 472–480.
Shiga-like toxin 2 (*stx2*)	Hidaka, A.; Hokyo, T.; Arikawa, K.; Fujihara, S.; Ogasawara, J.; Hase, A.; Hara-Kudo, Y.; Nishikawa, Y. Multiplex Real-Time PCR for Exhaustive Detection of Diarrhoeagenic *Escherichia Coli*. *J. Appl. Microbiol.* **2009**, *106* (2), 410–420.
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Viruses	
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Norovirus GI	Jothikumar, N.; Lowther, J. A.; Henshilwood, K.; Lees, D. N.; Hill, V. R.; Vinjé, J. Rapid and Sensitive Detection of Noroviruses by Using TaqMan-Based One-Step Reverse Transcription-PCR Assays and Application to Naturally Contaminated Shellfish Samples. *Appl. Environ. Microbiol.* **2005**, *71* (4), 1870–1875.
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Rotavirus A	Jothikumar, N.; Kang, G.; Hill, V. R. Broadly Reactive TaqMan® Assay for Real-Time RT-PCR Detection of Rotavirus in Clinical and Environmental Samples. *J. Virol. Methods* **2009**, *155* (2), 126–131.
Protozoa	
Entamoeba histolytica	Verweij, J. J.; Blangé, R. A.; Templeton, K.; Schinkel, J.; Brienen, E. A. T.; van Rooyen, M. A. A.; van Lieshout, L.; Polderman, A. M. Simultaneous Detection of Entamoeba Histolytica, Giardia Lamblia, and Cryptosporidium Parvum in Fecal Samples by Using Multiplex Real-Time PCR. *J. Clin. Microbiol.* **2004**, *42* (3), 1220–1223.

Giardia duodenalis	Verweij, J. J.; Blangé, R. A.; Templeton, K.; Schinkel, J.; Brienen, E. A. T.; van Rooyen, M. A. A.; van Lieshout, L.; Polderman, A. M. Simultaneous Detection of Entamoeba Histolytica, Giardia Lamblia, and Cryptosporidium Parvum in Fecal Samples by Using Multiplex Real-Time PCR. *J. Clin. Microbiol.* **2004**, *42* (3), 1220–1223.
Soil-transmitted helminths	
Ascaris lumbricoides	Wiria, A. E.; Prasetyani, M. A.; Hamid, F.; Wammes, L. J.; Lell, B.; Ariawan, I.; Uh, H. W.; Wibowo, H.; Djuardi, Y.; Wahyuni, S.; et al. Does Treatment of Intestinal Helminth Infections Influence Malaria? Background and Methodology of a Longitudinal Study of Clinical, Parasitological and Immunological Parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study). *BMC Infect. Dis.* **2010**, *10* (1), 77.
Trichuris trichiuria	Pilotte, N.; Papaiakovou, M.; Grant, J. R.; Bierwert, L. A.; Llewellyn, S.; McCarthy, J. S.; Williams, S. A. Improved PCR-Based Detection of Soil Transmitted Helminth Infections Using a Next-Generation Sequencing Approach to Assay Design. *PLoS Negl. Trop. Dis.* **2016**, *10* (3), e0004578.

Table S2. Interpretation of gene targets on the TAC

Target	Pathogen Gene Targeted	Interpretation
Bacteria		
Campylobacter coli	*cadF* gene	If either was detected, call as *Campylobacter coli/jejuni* positive
Campylobacter jejuni	*cadF gene*	
Clostridium difficile (*tcdA*)	*tcdA* gene	If either was detected, call as *Clostridium difficile* positive
Clostridium difficile (*tcdB)*	*tcdB gene*	
EIEC */ Shigella* (*ipaH*)	*ipaH* gene	If detected, call as *Shigella*/EIEC positive
EAEC (*aaiC*)	*aaiC* gene	If either was detected, call as EAEC positive
EAEC (*aatA*)	*aatA* gene	
EPEC (*bfpA*)	*bfpA* gene	If either was detected, call as EPEC positive
EPEC (*eae*)	*eae* gene	
ETEC-LT	*LT* gene	If either was detected, call as ETEC positive
ETEC-ST	*STh/STp*	
Salmonella spp.	*invA* gene	If detected, call as *Salmonella spp.* positive
Shiga-like toxin 1 (stx1)	stx_1 gene	If either was detected, call as STEC positive
Shiga-like toxin 2 (stx2)	stx_2 gene	
Vibrio cholerae	*toxR* gene	If detected, call as *Vibrio cholerae* positive
Yersinia spp.	*lysP* gene	If detected, call as *Yersinia spp.* positive
Viruses		
Adenovirus 40/41	*Fiber* gene	If detected, call as Adenovirus 40/41 positive
Astrovirus	*Capsid* gene	If detected, call as Astrovirus positive
Norovirus GI	*ORF1-ORF2* gene	If either was detected, call as Norovirus GI/GII positive
Norovirus GII	*ORF1-ORF2* gene	
Rotavirus A	*NSP3* gene	If detected, call as Rotavirus A positive
Protozoa		
Entamoeba histolytica	*18S*	If detected, call as *Entamoeba histolytica* positive
Giardia duodenalis	*18S*	If detected, call as *Giardia duodenalis* positive

Helminth		
Ascaris lumbricoides	*18S*	If detected, call as *Ascaris lumbricoides* positive
Trichuris trichiura	*ITS1*	If detected, call as *Trichuris trichiuria* positive

Table S3. Standard Curve

Target	Gene Targeted	Linearity	Efficiency	Positive Control Material Source
Bacteria				
Campylobacter coli	*cadF* gene	0.99	1.08	BEI Resources
Campylobacter jejuni	*cadF gene*	0.98	1.15	BEI Resources
Clostridium difficile	*tcdA* gene	1.0	0.97	BEI Resources
Clostridium difficile	*tcdB gene*	1.0	1.01	BEI Resources
EIEC / *Shigella*	*ipaH* gene	1.0	1.01	BEI Resources
EAEC	*aaiC* gene	0.99	0.99	BEI Resources
EAEC	*aatA* gene	0.99	1.15	BEI Resources
EPEC	*bfpA* gene	0.99	1.0	BEI Resources
EPEC	*eae* gene	1.0	1.04	BEI Resources
ETEC-LT	*LT* gene	1.0	0.95	BEI Resources
ETEC-ST	*STh/STp*	1.0	0.93	BEI Resources
Salmonella spp.	*invA* gene	0.99	0.84	BEI Resources
Shiga-like toxin 1	stx_1 gene	1.0	1.09	BEI Resources
Shiga-like toxin 2	stx_2 gene	1.0	1.01	BEI Resources
Vibrio cholerae	*toxR* gene	0.99	1.02	BEI Resources
Yersinia spp.	*lysP* gene	1.0	1.04	BEI Resources
Viruses				
Adenovirus 40/41	*Fiber* gene	0.99	0.81	IDT (g-block)
Astrovirus	*Capsid* gene	0.99	1.07	BEI Resources
Norovirus GI	*ORF1-ORF2* gene	0.96	0.86	IDT (g-block)
Norovirus GII	*ORF1-ORF2* gene	1.0	0.60	IDT (g-block)
Rotavirus A	*NSP3* gene	0.99	0.81	IDT (g-block)
Protozoa				
Entamoeba histolytica	*18S*	0.99	1.09	BEI Resources
Giardia duodenalis	*18S*	1.0	0.97	American Type Culture Collection (ATCC)
Helminth				
Ascaris lumbricoides	*18S*	1.0	0.96	IDT (g-block)
Trichuris trichiura	*ITS1*	0.99	1.09	IDT (g-block)

Table S4. Description of variables and their respective sources

	Variable description	**Data source**
Outcome Data		
Presence of ≥1 enteric pathogen gene in latrine entrance soils	Binary detect/non-detect; 1/0	Experimental data
Total number of enteric pathogens detected	Count; from 0 to 18	Experimental data
Presence of *Ascaris lumbricoides*	Binary detect/non-detect; 1/0	Experimental data
Presence of enteroaggregative *E. coli*	Binary detect/non-detect; 1/0	Experimental data
Presence of *Giardia duodenalis*	Binary detect/non-detect; 1/0	Experimental data
Presence of *Shigella*/EIEC	Binary detect/non-detect; 1/0	Experimental data
Presence of enterotoxigenic *E. coli*	Binary detect/non-detect; 1/0	Experimental data
Presence of adenovirus 40/41	Binary detect/non-detect; 1/0	Experimental data
Presence of astrovirus	Binary detect/non-detect; 1/0	Experimental data
Presence of enteropathogenic *E. coli*	Binary detect/non-detect; 1/0	Experimental data
Presence of *Trichuris trichiura*	Binary detect/non-detect; 1/0	Experimental data
Presence of *Clostridium difficile*	Binary detect/non-detect; 1/0	Experimental data
Covariates used in multivariate model selection		
Compound population	Continuous variable: transformed to represent a 10-person increase	Baseline and 24-month datasets
Wealth index	Quartile (1, 2, 3, or 4)	Baseline and 24-month

	derived from a continuous variable (from 0 to 1)	datasets (Calculated using the Simple Poverty Scorecard® Poverty-Assessment Tool: Mozambique)
Visibly wet soil	Wet/dry; 1/0	Observed and recorded by enumerator at time of sampling
Sun exposure status	Factor; complete sun, partially shaded, complete shade	Observed and recorded by enumerator at time of sampling
Average temperature in Fahrenheit during the day of and day before the soil sample was collected (e.g. 2-day average temperature)	Continuous variable, mean centered	Downloaded data from the National Oceanic and Atmospheric Administration's National Centers for Environmental Information (https://www.ncdc.noaa.gov/cdo-web/datatools/findstation)
Baseline and 24-month sanitation infrastructure	Factor; Pit latrine (without slab), pit latrine (with slab), intervention pour-flush toilet, non-intervention pour flush toilet, or unusable latrine (e.g. used neighbor's latrine or reported open defecation)	Baseline and 24-month datasets In addition, we reviewed illustrative photographs of sanitation infrastructure to confirm the sanitation infrastructure present
Dog(s) present	Binary, present / not present; 1/0	Baseline and 24-month datasets
Chicken(s)/duck(s) present	Binary, present / not present; 1/0	Baseline and 24-month datasets
Cat(s) present	Binary, present / not present; 1/0	Baseline and 24-month datasets
Visible feces in the compound (human or animal)	Binary, present / not present; 1/0	Baseline and 24-month datasets

Table S5. Soils samples matched at baseline and 24-month trial periods

Latrine entrance soil samples	**Control**	**Intervention**
Just baseline	6	2
Matched baseline and 24-month	42	41
Just 24-month	3	2
Total baseline	48	45
Total 24-month	43	43

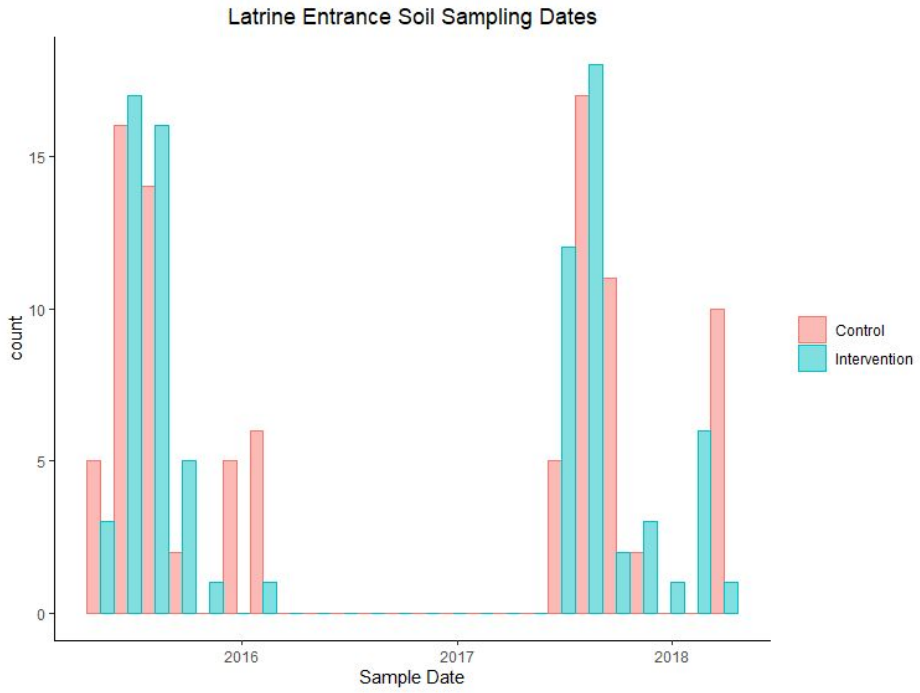

Figure S1. Histogram of dates that latrine entrance soils were collected

Table S6. Mean Cq Values

Pathogen	Baseline Cq Mean (sd, n)	24-month Cq Mean (sd, n)
Ascaris lumbricoides		
control	28.4 (4.6, 31)	29.6 (4.1, 34)
intervention	29.0 (4.4, 27)	28.3 (4.2, 19)
Enteroaggregative *E. coli*		
control	31.9 (2.0, 20)	31.0 (2.8, 24)
intervention	32.7 (1.8, 22)	32.2 (2.6, 16)
Giardia duodenalis		
control	30.9 (2.7, 20)	32.5 (2.7, 17)
intervention	32.9 (2.9, 16)	33.1 (2.7, 11)
Shigella/Enteroinvasive *E. coli*		
control	32.6 (1.3, 16)	32.4 (2.5, 15)
intervention	34.9 (2.5, 7)	33.3 (0.97, 4)
Enterotoxigenic *E. coli*		
control	33.8 (4.0, 12)	33.7 (3.1, 15)
intervention	34.4 (3.4, 15)	33.4 (2.5, 9)
adenovirus 40/41		
control	30.9 (2.8, 11)	31.6 (2.2, 15)
intervention	30.9 (4.6, 6)	31.0 (1.7, 3)
astrovirus		
control	32.8 (4.4, 10)	33.0 (3.2, 12)
intervention	34.3 (4.2, 11)	33.7 (3.7, 14)
Enteropathogenic *E. coli*		
control	31.5 (2.3, 7)	32.0 (2.3, 11)
intervention	31.0 (3.8, 9)	31.2 (2.4, 3)
Trichuris trichiura		
control	27.6 (4.3, 15)	26.8 (4.0, 8)
intervention	28.5 (3.7, 12)	28.5 (5.6, 7)
Clostridium difficile		
control	34.3 (0.87, 6)	33.8 (1.4, 7)
intervention	32.7 (2.8, 7)	34.2 (1.0, 6)
Salmonella spp.		
control	NA	
intervention		
Shiga-toxin producing *E. coli*		
control	NA	
intervention		
Campylobacter jejuni/coli		
control	NA	

intervention		
Yersinia spp.		
control	NA	
intervention		
norovirus GI/GII		
control	NA	
intervention		
rotavirus A		
control	NA	
intervention		
Entamoeba histolytica		
control	NA	
intervention		
Vibrio cholerae		
control	NA	
intervention		

Note: Cq values are the mean of detected samples and non-detects were not included in the calculation. For pathogens with 2 gene targets where both targets were detected, we use the smaller Cq value in the calculation. sd = standard deviation. n = number of samples used to calculate the mean and standard deviation.

Table S7. Child feces disposal at 24-month phase

Feces Disposal	Sub-category	Survey response	
In a diaper		22% (218/980)	
	Diaper is washed and reused		2.7% (26/980)
	Diaper is discarded with solid waste		20% (192/980)
In the latrine		29% (289/980)	
	In the latrine		29% (289/980)
On the ground		7.3% (72/980)	
	Left on the ground		0.3% (3/980)
	Put with the solid waste		0.1% (1/980)
	Put into a soakaway pit		0.1% (1/980)
	Put into the latrine		5.6% (55/980)
	Buried		1.2% (12/980)
Child potty (contents emptied into the latrine)		29% (281/980)	
	Child potty		29% (281/980)
No response		12% (120/980)	
	No response		12% (120/980)