TITLE

Long-term impacts of an urban sanitation intervention on enteric pathogens in children in Maputo city, Mozambique: study protocol for a cross-sectional follow-up to the Maputo Sanitation (MapSan) trial five years post-intervention

AUTHORS

David Holcomb[1,2], Vanessa Monteiro[3], Drew Capone[1,4], Virgílio António[5], Márcia Chiluvane,[3] Victória Cumbane[3], Nália Ismael[5], Jackie Knee[6], Erin Kowalsky[1], Amanda Lai[1], Yarrow Linden[1], Elly Mataveia[3], Rassul Nalá[7], Gouthami Rao[1], Jorge Ribeiro[3], Oliver Cumming[6], Edna Viegas[3], Joe Brown[1,*]

AFFILIATIONS

1. Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

2. Department of Epidemiology, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

3. Centro de Investigação e Treino em Saúde da Polana Caniço, Instituto Nacional de Saúde, Maputo, Mozambique

4. Department of Environmental and Occupational Health, School of Public Health, Indiana University, Bloomington, Indiana, USA

5. Division of Biotechnology and Genetics, Instituto Nacional de Saúde, Marracuene, Mozambique

6. Department of Disease Control, Faculty of Infectious and Tropical Diseases, London School

of Hygiene and Tropical Medicine, London, UK

7. Division of Parasitology, Instituto Nacional de Saúde, Marracuene, Mozambique

CORRESPONDING AUTHOR

* Joe Brown; 135 Dauer Drive, Chapel Hill, NC, USA, 27599-7435; T: (919) 966-7892; E:

joebrown@unc.edu

ABSTRACT

Introduction

We previously assessed the effect of an onsite sanitation intervention in informal neighborhoods
of urban Maputo, Mozambique on enteric pathogen detection in children after two years of
follow-up (Maputo Sanitation [MapSan] trial, clinicaltrials.gov: NCT02362932). We found
significant reductions in *Shigella* and *Trichuris* prevalence but only among children born after
the intervention was delivered. In this study, we assess the health impacts of the sanitation
intervention after five years among children born into study households post-intervention.

Methods and analysis

We are conducting a cross-sectional household study of enteric pathogen detection in child stool
and the environment in the 16 MapSan study neighborhoods at compounds (household clusters
sharing sanitation and outdoor living space) that received the pour-flush toilet and septic tank
intervention at least five years prior or meet the original criteria for trial control sites. We are
enrolling at least 400 children (ages 29 days-60 months) in each treatment arm. Our primary
outcome is the prevalence of 22 bacterial, protozoan, and soil transmitted helminth (STH) enteric
pathogens in child stool using the pooled odds ratio across the outcome set to assess the
intervention effect. Secondary outcomes include the individual pathogen detection prevalence
and gene copy density of 27 enteric pathogens (including viruses); mean height-for-age (HAZ),
weight-for-age (WAZ), and weight-for-height (WHZ) z-scores; prevalence of stunting,
underweight, and wasting; and the 7-day period-prevalence of caregiver-reported diarrhea. All
analyses are adjusted for pre-specified covariates and examined for effect measure modification
by age. Environmental samples from study households and the public domain are assessed for

pathogens and fecal indicators to explore environmental exposures and monitor disease

transmission.

Ethics and dissemination

Study protocols have been reviewed and approved by human subjects review boards at the

Ministry of Health, Republic of Mozambique and the University of North Carolina at Chapel

Hill. De-identified study data will be deposited at https://osf.io/e7pvk/.

Trial registration number

ISRCTN86084138

KEYWORDS

Enteric infection; diarrheal disease; molecular pathogen detection; environmental microbiology;

wastewater-based epidemiology

STRENGTHS AND LIMITATIONS OF THIS STUDY

- Follow-up to the first controlled health impact trial of an urban on-site sanitation intervention after at least 5 years of intervention conditions. Previous studies have focused on WASH intervention impacts up to 2-3 years after delivery, which may be insufficient time to realize impacts.

- Primary study endpoint is molecular detection of multiple enteric pathogens in child stool, which unambiguously indicates previous exposure to specific sanitation-related pathogens; enteric pathogens are also assessed across multiple environmental compartments to explore environmental exposures and monitor disease transmission.

- Primary outcome is the overall impact of the intervention on enteric pathogen exposure using a novel pooled estimate of the treatment effect across a pre-specified set of enteric pathogens.

- As an observational evaluation of an existing intervention, sample size is constrained by the number of eligible children residing at study sites and selection bias arising from differential enteric pathogen-related mortality may be present, particularly among older age groups.

- As a cross-sectional study, there is potential for confounding bias in our estimates of the intervention impacts on child health; in particular, the presence of the intervention itself may have influenced the desirability of the intervention sites and thus the socioeconomic status of their residents, which may be associated with increased pathogen exposures.

INTRODUCTION

There is a high burden of childhood enteric infections associated with poor environmental
conditions, with multiple enteric pathogens frequently detected in stool within the first year of
life [1–3]. This is also the period with the highest incidence of diarrhea, which remains a leading
cause of child mortality in low- and middle-income countries (LMIC) and has long been
associated with stunted growth [4–6]. However, many diarrheal episodes are not attributable to
infectious etiologies [3,7], while the etiology of attributable diarrhea varies widely by setting and
only certain diarrheal pathogens are consistently implicated in reduced linear growth [2,8,9]. Far
more prevalent is asymptomatic enteric pathogen shedding in stool [3,7,10–14], which may be
more strongly associated with poor growth than diarrheal illness [2,15–17], potentially by
contributing to intestinal inflammation, gut permeability, and nutrient malabsorption in a
condition known as environmental enteric dysfunction (EED) [18–21]. In addition to the diverse
negative impacts of stunting [22], specific adverse health outcomes associated with enteric
infection and EED include delayed cognitive development [23–25] and reduced oral vaccine
efficacy [26,27].

Water, sanitation, and hygiene (WASH) interventions aim to prevent diarrhea and improve child
growth by interrupting fecal-oral pathogen transmission [28]. However, recent rigorous WASH
intervention trials have demonstrated inconsistent and often limited impacts on child health [29–
35]. Methodological limitations of such distal outcome measures notwithstanding [36,37], these
findings are consistent with the interventions insufficiently interrupting environmental
transmission and exposure to enteric pathogens [38]. Combined WASH interventions with high
fidelity and adherence reduced stool-based detection of *Giardia*, hookworm, and possibly

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Ascaris among 30 month-old children (but not younger children) and enteric viruses in rural

Bangladesh [10,39,40], *Ascaris* in rural Kenya [41], and the number of co-detected parasites in

rural Zimbabwe [11], but not bacterial pathogens or those most associated with stunting in any

setting [2]. The sanitation-only intervention arm in rural Bangladesh reduced *Trichuris*

prevalence in child stool [39] and may have reduced pathogenic *Escherichia coli* on child hands

in more crowded households [42], while a sanitation intervention in rural Cambodia did not

impact any pathogens measured in child stool [35]. Viewed collectively, a clear picture emerges

of pervasive childhood polymicrobial exposures that were not meaningfully prevented by low-

cost WASH interventions [43–45].

The preceding trials were all conducted in rural settings, but rapid urbanization has led to

exceptional growth of densely populated informal settlements that lack basic services and present

unique health challenges [46,47]. We have previously investigated whether an onsite sanitation

intervention delivered in low-income neighborhoods of urban Maputo, Mozambique reduced

enteric pathogen prevalence in child stool [48]. Although we similarly found no evidence of an

effect on combined prevalence of pre-specified enteric pathogens in our primary analyses [34],

additional evidence suggests that the sanitation intervention may have reduced exposures to

enteric pathogens in the environment. The intervention was delivered with high fidelity and was

widely used by intervention households after two years [49]. Exploratory sub-group analyses

indicated that 24 months after the intervention, *Shigella* prevalence was halved (-51%; 95%

confidence interval [CI]: -15%, -72%) and *Trichuris* prevalence reduced by three-quarters (-

76%; 95% CI: -40%, -90%) among children born into the study compounds after the intervention

was implemented, relative to children born into the control compounds after baseline [34].

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Furthermore, overall pathogen prevalence, pathogen counts, *E. coli* gene copy density, and the individual prevalence of *Ascaris* and pathogenic *E. coli* were all significantly reduced in soil at the intervention latrine entrance [50,51], suggesting the intervention effectively contained human excreta. While animals have been implicated as major sources of pathogen exposure in other settings [52], only companion animals were frequently present at study households and a locally validated indicator of poultry fecal contamination (the most commonly observed non-companion animal type) was rarely detected in household environments [53]. Conversely, indicators of human fecal contamination were widespread [51] and the two pathogens most impacted by the intervention—*Shigella* and *Trichuris*—are considered anthroponotic [54], suggesting that human excreta was a primary source of enteric pathogens in our dense, urban setting and that human-associated pathogens were impacted by sustained exposure to the intervention.

Enteric pathogen carriage is highly age-dependent [8,12], but we were previously limited by the timing of our follow-up survey (24 months post-intervention) to children under two years of age when assessing impacts on those born into the intervention. Furthermore, it has been suggested that the typically short (1-2 year) follow-up periods often used in WASH evaluations may be inadequate for any longer-term benefits to manifest, potentially contributing to the limited observed effects [43]. Accordingly, we are conducting a cross-sectional follow-up study to better understand the long-term impacts of the sanitation intervention five years after it was implemented on child enteric pathogen exposures and shedding, diarrheal disease, and growth among children born into study households post-intervention.

STUDY OBJECTIVES

Primary objective

To measure the long-term effect of a shared, onsite urban sanitation intervention on the pooled prevalence of pre-specified enteric pathogen targets detected in children's stool at least 60 months post-intervention.

Hypotheses

H1. The risk of stool-based enteric pathogen detection among children 29 days-60 months old is reduced for children born into households that previously received the sanitation intervention.

H2. Children born into households that previously received the sanitation intervention experience delayed exposure to enteric pathogens relative to comparably aged children from non-intervention households, reflected in a greater reduction in the risk of enteric pathogen detection among younger age groups and attenuated reduction in risk among older children.

Secondary objectives

1. To measure the effect of a shared, onsite urban sanitation intervention program on the individual prevalence and density of pre-specified enteric pathogen targets detected in children's stool and environmental samples at least 60 months post-intervention.

2. To measure the effect of a shared, onsite urban sanitation intervention program on child growth and the prevalence of caregiver-reported diarrhea in children at least 60 months post intervention.

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METHODS AND ANALYSIS

Study setting

This household-based study is being conducted in 11 *bairros* (neighborhoods) in the

Nhlamankulu District and 5 *bairros* in the *KaMaxaquene* District of Maputo city, Mozambique.

Households in these densely populated, low-income neighborhoods typically cluster into self-

defined compounds delineated by a wall or boundary that share outdoor living space and

sanitation facilities. Study activities with participating households are conducted primarily in the

compound shared outdoor space by staff from the *Centro de Investigação e Treino em Saúde da*

Polana Caniço (Polana Caniço Health Research and Training Center; CISPOC) in Maputo,

Mozambique, from which the study is implemented and managed.

Study design

Urban sanitation intervention

The nongovernmental organization (NGO) Water and Sanitation for the Urban Poor (WSUP)

implemented a sanitation intervention in 2015-2016 at approximately 300 compounds in

informal urban neighborhoods of Maputo under a larger program led by the Water and Sanitation

Programme (WSP) of the World Bank [48]. In these compounds, ranging between three and

twenty-five households each, existing unhygienic shared latrines were replaced with pour-flush

toilets and a septic tank with a soak-away pit for the liquid effluent. Two intervention designs

employing the same sanitation technology were implemented, with approximately 50 communal

sanitation blocks (CSBs) and 250 shared latrines (SLs) constructed across 11 neighborhoods that

exhibited diversity across density and other key characteristics (susceptibility to flooding,

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relative poverty, access to water and sanitation infrastructure) [12,53,55]. SLs served compounds with fewer than 21 residents and provided a single cabin with the intervention toilet, while CSBs provided an additional cabin for every 20 compound residents and other amenities including rainwater harvesting, municipal water connections and storage, and washing, bathing and laundry facilities [34,56].

Original controlled before-and-after study

To evaluate the impact of the WSUP sanitation intervention on child health, we conducted the Maputo Sanitation (MapSan) trial, a controlled before-and-after study of enteric pathogen detection in child stool (clinicaltrials.gov: NCT02362932) [48]. WSUP selected intervention compounds using the following criteria: (1) residents shared sanitation in poor condition as determined by an engineer; (2) the compound was located in the pre-defined implementation neighborhoods; (3) there were no fewer than 12 residents; (4) residents were willing to contribute financially to construction costs; (5) sufficient space was available for construction of the new facility; (6) the compound was accessible for transportation of construction materials and tank-emptying activities; (7) the compound had access to a legal piped water supply; and (8) the groundwater level was deep enough for construction of a septic tank. Control compounds were selected according to criteria 1, 3, 4, and 7 from the 11 neighborhoods where the intervention was implemented and 5 additional neighborhoods with comparable characteristics [12].

The MapSan trial recruited an open cohort at three time-points: baseline (pre-intervention), 12 months post-intervention, and 24 months post-intervention; children were eligible to participate if older than 1 month at the time of enrollment and under 48 months at baseline. We enrolled

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intervention and control compounds concurrently to limit any differential effects of seasonality

or other secular trends on the outcomes. We found no evidence that the sanitation intervention

reduced the combined prevalence of 12 bacterial and protozoan enteric pathogens (the pre-

specified primary outcome), the individual prevalence of any single bacterial, protozoan, STH, or

viral pathogen, or the period prevalence of caregiver-reported diarrhea 12 and 24 months post-

intervention [34]. However, exploratory analyses indicated the intervention may have been

protective against bacterial and soil-transmitted helminth infections among the cohort who were

born into the intervention and may have reduced the spread of some pathogens into latrine

entrance soils.

Long-term cross-sectional follow-up study

We are revisiting MapSan trial compounds at least five years after the sanitation intervention to

conduct a cross-sectional survey of children who were born after the intervention was

implemented. Due to substantial population turnover [34], both intervention and control

compounds are being identified using previously collected geolocation data and their locations

confirmed by study staff during site identification visits. Any compounds identified in the study

neighborhoods during the course of these mapping efforts that have received the sanitation

intervention (built by WSUP to the same specifications) or match the original enrollment criteria

for MapSan control compounds are also invited to participate, irrespective of previous

participation in the MapSan trial. As an existing intervention, neither staff nor participants can be

blinded to intervention status. We anticipate enrollment to continue for approximately one year

and are again enrolling intervention and control compounds concurrently to limit any differential

effects of seasonality and other secular trends across the enrollment period.

Patient and public involvement

Patients and/or the public were not involved in the design, conduct, reporting, or dissemination plans of this research.

Eligibility criteria

We attempt to enroll all eligible children in each compound that either previously participated in the MapSan trial, has previously received an intervention latrine, or meets the original enrollment criteria for MapSan control compounds [34]. Participant inclusion criteria include:

1. Child aged 29 days-60 months old

2. Born into and residing in a compound enrolled in the five-year follow-up study; in intervention compounds, the child must have been born following the delivery of the sanitation intervention

3. Has continuously resided in the study compound for the preceding 6 months, or since birth if under 6 months of age

4. Has a parent or guardian who is able to understand and complete the written informed consent process and allow their child to participate

Children are excluded if they have any caregiver-indicated medical condition or disability that precludes participation in the study.

Participant enrollment

Enrollment is conducted by trained study staff in either Portuguese or Changana, according to the respondent's preference, with written materials provided in Portuguese. We first obtain

verbal consent from the compound leader to approach households in the compound for

enrollment. We then seek written, informed consent to participate from the parent or guardian of

each eligible child. Participation is entirely voluntary; guardians may decline their child's

participation for any reason and can withdraw their child at any point. We began enrolling

participants on 28 March 2022.

Household visits and procedures

After locating study compounds, the household of a participating child is visited twice, typically

on consecutive days. On the first day, trained study staff conduct written consent procedures;

administer compound, household, and child questionnaires; record child anthropometry

measures; collect environmental samples; and request the child's caregiver to retain a sample of

the child's stool. The household is revisited the following day to collect a stool sample from the

child and complete environmental sample collection. If the stool sample is unavailable, the study

staff coordinate an additional visit to retrieve the stool. In the event that 7 days pass since the

initial visit without collection of a stool sample, a qualified nurse visits the child to obtain a

rectal swab.

After collection of the stool sample, deworming is offered to all household members >1 year old

who have not been dewormed in the past year, unless pregnant or breastfeeding. Deworming

consultation and medication provision is conducted by Ministry of Health staff following the

national guidelines for deworming procedures. Deworming is offered in-kind to all household

members and leverages the household interaction to provide an important public health service.

Besides deworming, this study offers no direct benefit to children participating in this study. No

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incentives are provided to study participants, but we provide 50 meticais (approximately US$1) of mobile phone credit on the caregiver's preferred network for each child to compensate for the costs incurred in communicating with the study team to arrange household visits. Households from which we collect food and/or large-volume water samples are reimbursed 50 additional meticais to offset these expenses.

Environmental sample collection

We are sampling environmental compartments at a randomly selected subset of 100 intervention and 100 control compounds to represent compound- and household-level exposures [38]. At the entrance to the compound latrine we collect soil, flies, and a large volume air sample, as well as fecal sludge from the latrine or septic tank and any animal feces observed in the shared outdoor space [50,51,57,58]. One household is randomly selected among those households with children enrolled in the child health study, from which we collect swabs of flooring at the household entrance, flies in cooking area, prepared child's food, stored drinking water, and water from the household's primary source [53,59,60].

We are also collecting environmental samples from the public domain, including wastewater, open surface water, soils in the vicinity of solid and fecal waste disposal locations, and wastewater effluent from hospitals treating COVID-19 patients. These samples are not linked to specific individuals in any way and are used to conduct environmental surveillance of pathogens circulating in the community [57,61].

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Study outcomes

Enteric pathogen detection in stool

Stool-based molecular detection is performed for 27 enteric pathogens commonly implicated in

both symptomatic and asymptomatic childhood infections globally, including those identified at

the Global Enteric Multicenter Study (GEMS) study site in Manhiça, Mozambique

[12,37,57,62]. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) is

conducted by custom TaqMan Array Card (TAC; Thermo Fisher, Carlsbad, CA, USA) to

simultaneously quantify genetic targets corresponding to 13 bacterial pathogens (*Aeromonas*

spp.; *Campylobacter jejuni/coli*; *Clostridioides difficile*; *E. coli* O157; enteroaggregative *E. coli*

(EAEC); enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); Shiga toxin-

producing *E. coli* (STEC); enteroinvasive *E. coli* (EIEC)/*Shigella* spp.; *Helicobacter pylori*;

Plesiomonas shigelloides; *Salmonella enterica*; *Vibrio cholerae*), 4 protozoan parasites

(*Cryptosporidium* spp.; *Cyclospora cayetanensis*; *Entamoeba histolytica; Giardia* spp.), 5 soil

transmitted helminths (*Ascaris lumbricoides*; *Ancylostoma duodenale*; *Necator americanus*;

Strongyloides stercolaris; *Trichuris trichiura*), and 5 enteric viruses (adenovirus 40/41;

astrovirus; norovirus GI/GII; rotavirus; sapovirus) [63]. We also include the respiratory virus

SARS-CoV-2 on the custom TAC to support surveillance through fecal waste streams [64,65].

Our primary outcome is the prevalence of a pre-specified subset of 22 enteric pathogens; as in

the original MapSan study, we exclude enteric viruses from the primary outcome due to the

potential for direct contact transmission, which is unlikely to be impacted by the intervention

[34,48,66]. Because we anticipate a combined prevalence (detection of at least one pathogen in a

given stool sample) near 100%, and the intervention could plausibly increase the prevalence of

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some pathogens while reducing others, we do not define a composite prevalence outcome
[34,67]. Rather, we use mixed effects models to analyze all pathogens concurrently, thereby
estimating the pooled effect of the intervention on enteric pathogen prevalence across the
outcome set [68,69].

Secondary outcomes include the individual prevalence of all 27 pre-specified enteric pathogens,
including *Shigella* spp. and *T. trichiura*, the two pathogens most impacted at 24 months among
children born after the intervention was delivered [34]. A similar mixed effects model is used to
analyze all pathogens concurrently to estimate separate treatment effects on the prevalence of
each pathogen while accounting for correlations between outcomes, which alleviates concerns
about *post-hoc* multiple comparisons adjustments [10,68]. Additional secondary outcomes
include the continuous gene copy density in stool of the 22 primary outcome pathogens, for
which a pooled treatment effect on the mean gene copy density is estimated, as well as the
individual gene copy densities for all 27 enteric pathogens assessed on the TAC. Recognizing the
potential challenges to interpretation due to pervasive exposure, persistent infection and rapid re-
infection, and possible protective effects [70–73], we will also repeat the primary outcome
analysis excluding *Giardia* from the outcome set.

Anthropometry

Child weight and recumbent length (child age < 24 months) or standing height (24-60 months)
are assessed according to standard World Health Organization (WHO) protocols and transformed
to age-adjusted z-scores using WHO reference populations to obtain height-for-age (HAZ),
weight-for-age (WAZ), and weight-for-height (WHZ) z-scores [74,75]. Secondary outcomes

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include continuous HAZ, WAZ, and WHZ, as well as prevalence of binary growth outcomes

stunting (HAZ < -2), underweight (WAZ < -2), and wasting (WHZ < -2) [35].

Caregiver reported illness

Caregiver surveys are administered to ascertain child diarrheal disease, defined as the passage of

three or more loose or watery stools in a 24-hour period, or any bloody stool, in the past 7 days

[76]. We also assess two caregiver-reported negative control outcomes for each child: the 7-day

period-prevalence of bruises, scrapes, or abrasions and the 7-day period-prevalence of toothache

[77]. We do not expect the intervention to impact either child bruising or toothache prevalence,

so significant differences in these outcomes by treatment arm would suggest possible bias in our

caregiver-reported outcomes.

Pathogen detection in environmental matrices

Molecular detection of selected pathogens and fecal source tracking (FST) markers is performed

for environmental samples from both the private (compound and household) and public domains

using a second custom TAC [50,57,78,79]. A subset of the enteric pathogens assessed in stool is

included on the environmental TAC (adenovirus 40/41; astrovirus; norovirus GI/GII; rotavirus;

sapovirus; *Aeromonas* spp.; *C. jejuni*/*coli*; *C. difficile*; *E. coli* O157; EAEC; EPEC; ETEC;

STEC; EIEC/*Shigella* spp.; *H. pylori*; *S. enterica*; *V. cholerae; Cryptosporidium* spp.; *E.*

histolytica; Giardia spp.; *A. lumbricoides*; *A. duodenale*; *N. americanus*; *T. trichiura*), as well as

select environmental and zoonotic pathogens (*Leptospira* spp; *Toxocara* spp.) [54,80,81], other

human pathogens detectable in feces (SARS-CoV-2, Zika virus, HIV proviral DNA;

Plasmodium spp.; *Mycobacterium tuberculosis*) [82–85], FST markers (human, poultry, and

canine mitochondrial DNA; avian 16S rRNA) [86,87], and general bacterial and anthropogenic

pollution/antimicrobial resistance markers (bacterial 16S rRNA; class 1 integron-integrase gene

intI1) [88,89]. We also culture fecal indicator bacteria (total coliforms and *E. coli*) using the

IDEXX Colilert-18 and Quanti-Tray 2000 system [87,90]. We conduct ongoing genomic

surveillance of SARS-CoV-2 viral lineages in wastewater samples using amplicon-based

Illumina next generation sequencing [91].

Statistical analysis

Enteric pathogen outcomes

We will use mixed effects models with varying slopes and intercepts to estimate the weighted-

average intervention effect across all pathogens included in the model, in essence treating each

pathogen as a separate study of the intervention effect on enteric pathogen detection. Additional

random effects will be included to account for clustering by compound and child. The prevalence

odds ratio (POR) for children living in intervention compounds, relative to children in control

compounds, will be estimated as the measure of effect for binary enteric pathogen detection

outcomes, including the primary outcome and secondary individual pathogen detection

outcomes. Prevalence differences (PD) between children in intervention and control compounds

will also be estimated from the posterior predictive distribution at representative values of other

model covariates [92]. Mean differences in standard deviation-scaled gene copy density will be

estimated as the measure of effect for continuous enteric pathogen outcomes, with non-detects

considered true zeros and censoring used to create a zero class (as in Tobit regression) [93].

Effect estimates will be summarized using the mean to represent the expected effect size and the

central 95% probability interval to capture the range of effect sizes compatible with the data (the

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95% CI). Parameters with 95% CIs that exclude the null will be considered significant, although

the magnitude and uncertainty of parameter estimates will also be considered holistically in

evaluating evidence for clinically meaningful effects [94].

Growth and caregiver reported outcomes

The effects of the intervention on mean HAZ, WAZ, and WHZ; the prevalence of stunting,

underweight, and wasting; and the period-prevalence of caregiver-reported diarrhea and negative

control outcomes (bruising, scrapes, and abrasions; toothache) will be analyzed separately as

secondary outcomes using generalized estimating equations (GEE) and robust standard errors

with exchangeable correlation structure and clustering by compound (the level at which the

sanitation intervention was delivered) [34,35,95]. The estimated difference in age-adjusted z-

scores by treatment assignment will be used as the measure of effect for continuous

anthropometry outcomes. The prevalence ratio (PR) will be estimated by Poisson regression for

binary growth status and caregiver-reported outcomes. We will not adjust for multiple

comparisons [31,96].

Covariates and effect measure modification

As a cross-sectional study of an existing intervention, all analyses will be adjusted for a set of

covariates selected *a priori* as potential confounders of the sanitation-enteric pathogen carriage

relationship [97]. The adjustment set will include child age and sex, caregiver's education, and

household wealth index [34,98]; records missing covariate data will be excluded from primary

analyses. Analyses will be repeated with missing covariate data imputed by multivariate

imputation using chained equations (MICE) as a sensitivity analysis [34,99]. Additional

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covariates will be considered in exploratory adjusted analyses [10,11]. The specific enteric

pathogens detected are expected to be strongly related to child age [8,34]. We will examine

effect measure modification of the primary and secondary outcomes stratifying by age group (1-

11 months, 12-23 months, and 24-60 months) [3,100].

Independently upgraded controls

We anticipate some of the control compounds may have independently upgraded their sanitation

facilities to conditions comparable to the intervention. Control compounds with sanitation

facilities observed to possess cleanable, intact hardscape slabs; pour-flush or water-sealed toilets;

a functional ventilation pipe; and a fixed superstructure with sturdy walls and a secure door that

ensure privacy during use are considered to have independently upgraded to conditions

comparable to the intervention [56]. Children living in control compounds with independently

upgraded latrines are enrolled but will be excluded from the main analyses of the intervention

effects. Two sets of subgroup analyses will instead be conducted that include all participants: one

in which children in independently upgraded controls are considered as part of the control (non-

intervention) arm, and again considered as part of the intervention arm. We will compare

parameter estimates from the three sets of analyses to investigate whether the sanitation

improvements independently available in the study communities are comparable to the full

sanitation intervention package assessed in the MapSan trial in terms of child health impacts.

Eligible children residing in any compound that has received a WSUP intervention latrine will be

considered part of the intervention arm in primary analyses. We record the current conditions of

intervention facilities but will not exclude or otherwise adjust for either upgraded or degraded

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sanitation facilities in intervention compounds in order to evaluate the long-term impacts of the intervention following extended use.

Minimum detectable effect size

The number of participants will be constrained by the number of compounds in the study neighborhoods that have received the sanitation intervention or meet the eligibility requirements for MapSan control compounds, most of which were previously enrolled in the MapSan trial. At the 24 month follow-up, an average of 2.5 children per compound were enrolled from 408 compounds [34]. Compound-level intra-class correlation coefficients (ICCs) were generally less than 0.1 for individual pathogens, corresponding to cluster variances of ~0.05. We calculate the minimum detectable effect size (MDES) on individual pathogen prevalence with 80% power, 5% significance level, and 0.05 compound cluster variance for a conservative scenario with 200 compounds per treatment arm and 2 children enrolled per compound (for 800 children total, 400 per arm), a moderate scenario of 220 compounds per treatment arm and 2.5 children enrolled per compound (550 children per arm, 1100 total), and a maximal scenario of 300 compounds per arm, 2.5 children per compound (750 children per arm, 1500 total) [101]. Across all scenarios, a minimum baseline (untreated) prevalence of 6-8% is required to reach 80% power for even the largest theoretical effect (nearly 100% reduction). The minimum detectable relative reduction in prevalence decreases (that is, smaller effect sizes are more readily detected) as pathogen prevalence increases towards 100% (Figure 1). The difference between the scenarios on the multiplicative scale is relatively minor, with the relative reduction MDES largely driven by pathogen baseline prevalence. A pathogen with baseline prevalence below 15% must have its prevalence halved (PR < 0.5) in order to attain 80% power, while a 25% reduction is detectable

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with 80% power for baseline prevalence of 34-46% under the maximal and conservative

scenarios, respectively. We expect the simultaneous consideration of multiple pathogens to

reduce the MDES for the primary outcome pooled intervention effect by effectively increasing

the sample size. Because this pooled effect is dependent on the prevalence of each pathogen

considered and the correlations between them, we will conduct simulation analyses to

characterize plausible MDES ranges for the pooled primary outcome treatment effect [67,101].

LIMITATIONS

As an observational, cross-sectional evaluation of an existing intervention, this study faces a number of limitations that may impact the generalizability of our findings. By design, all of the study participants will have been born after the intervention was implemented in order to evaluate intervention impacts among individuals across a range of ages who have been exposed to intervention for their entire lives. However, there is potential for confounding bias in our estimates of the intervention impacts on child health, particularly concerning socioeconomic factors that may be associated with increased pathogen exposures. While non-randomized, the criteria for intervention and control compounds differed only by engineering considerations that we believe to be independent of the outcome. However, in the years since the intervention was implemented, the presence of the intervention itself may have influenced the desirability of the intervention sites and thus the socioeconomic status (SES) of their residents, particularly in light of the high population turnover previously observed after only 1-2 years. All analyses will be adjusted for a location-specific wealth index to account for potential differential SES between treatment arms [98], in addition to other pre-specified covariates associated with enteric pathogen exposure that may plausibly be related to treatment status, but the possibility of unmeasured and insufficiently controlled confounding remains.

Because childhood diarrhea is a leading cause of child mortality, selection bias arising from survivor effects may be present in our sample, particularly among older age groups [102]. However, loss to follow-up due to mortality was exceptionally rare in the previous assessment— far less common than emigration, which was not previously differential by treatment arm [34]. Our use of stool-based enteric pathogen detection as an objective primary outcome mitigates the

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potential for measurement bias [37] and we are assessing multiple negative control outcomes to
account for potential response bias in our secondary caregiver-reported outcomes [77]. As a
long-term evaluation of an existing intervention, the potential for exposure measurement error is
low—interventions that have degraded substantially may shift results towards the null, but in so
doing would represent a valid assessment of intervention sustainability [103]. The potential for
exposure misclassification among controls is higher, in that they may have independently
upgraded to sanitation infrastructure comparable to the intervention. We are actively monitoring
this possibility at all study sites; have pre-specified criteria for identifying sites achieving such
conditions; and will conduct sensitivity analyses with these sites excluded, considered as
controls, and considered as equivalent to the intervention to characterize the impact of any such
potential exposure misclassification among controls.

A key challenge is identifying an appropriate set of outcomes against which to evaluate the
intervention; in this regard, we preferred inclusivity given the pathogens likely to be observed in
our study setting, at the risk of unduly shifting our eventual results towards the null. This
challenge persists, regardless of study design, so long as the study aims include assessing the
effect of some condition or intervention on exposure to, or infection by, multiple pathogens that
share a common route of transmission [104].

CONTRIBUTION

Although observational, this study is unique in evaluating sanitation intervention effects at least
five years after the intervention was implemented and up to five years after participants were
borne into the intervention conditions. Previous studies have focused on WASH intervention

impacts up to 2-3 years after delivery [31–35], which may be insufficient time to realize impacts [43]. Furthermore, most previous studies have been conducted in rural settings, while we are investigating the long-term effects of an urban sanitation intervention that is broadly representative of the types of infrastructure improvements likely to be available in the rapidly growing urban informal settlements in coming years. Finally, we use stool-based detection of multiple enteric pathogens as an objective outcome and propose a novel pooled estimate of the treatment effect across a pre-specified outcome set to summarize the overall impact of the intervention on enteric pathogen exposure as the primary trial outcome.

ETHICS AND DISSEMINATION

This study was approved by Comité Nacional de Bioética para a Saúde, Ministério da Saúde de Moçambique (FWA#: 00003139, IRB00002657, 326/CNBS/21; approved: 15 June 2021) and University of North Carolina at Chapel Hill Ethics Committee (IRB#: 21-1119; approved 19 August 2021), and was prospectively registered with ISRCTN on 16 March 2022 (https://doi.org/10.1186/ISRCTN86084138). We pre-specified a statistical analysis plan that was deposited in a permanent online repository (https://osf.io/e7pvk/) prior to commencing enrollment. All protocol modification will be submitted to, and approved by, the respective ethics committees prior to implementation and promptly updated on the ISRCTN registry.

Written, informed consent is obtained from the parent(s)/guardian(s) of each participant. Identifiable information is maintained separately to protect confidentiality and linked to surveys, stool and environmental sample data by unique codes; the linking file to identifiable information will be destroyed upon completion of the study and the study data and remaining biospecimens retained for fully de-identified ancillary analyses. Results will be presented to key stakeholders in Mozambique, including local and national government officials, public utilities, and non-governmental organizations, and published in open access peer-reviewed journals. Upon publication of study results, the underlying individual participant data will be fully de-identified according to the Safe Harbor method and made freely available in the permanent online repository (https://osf.io/e7pvk/).

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AUTHOR CONTRIBUTIONS

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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FIGURE CAPTION

Figure 1. Range of minimum detectable effect sizes (shaded region) for the percent reduction in pathogen prevalence with 80% power, 5% significance level, and 0.05 cluster variance across three sample size scenarios. The upper edge of the shaded area represents a conservative scenario with 800 total participants (2 per compound, 200 compounds per arm) while the lower edge corresponds to a maximal scenario with 1500 total participants (2.5 per compound, 300 compounds per arm). A moderate scenario with 1100 total participants (2.5 per compound, 220 compounds per arm) is represented by the black curve within the shaded area. The vertical lines show the prevalence of a subset of pathogens assessed in control compound children during the 24-month follow-up in the original MapSan trial. Line color indicates the specific pathogen and line pattern reflects the pathogen class.

Minimum Detectable Relative Reduction in Prevalence