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Author manuscript

Environ Sci Technol Lett. Author manuscript; available in PMC 2020 June 30.

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Published in final edited form as:

Environ Sci Technol Lett. 2016 November 8; 3(11): 393–398.

Occurrence of Host-Associated Fecal Markers on Child Hands, Household Soil, and Drinking Water in Rural Bangladeshi Households

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Abstract

We evaluated whether provision and promotion of improved sanitation hardware (toilets and child feces management tools) reduced rotavirus and human fecal contamination of drinking water, child hands, and soil among rural Bangladeshi compounds enrolled in a cluster-randomized trial. We also measured host-associated genetic markers of ruminant and avian feces. We found evidence of widespread ruminant and avian fecal contamination in the compound environment; non-human fecal marker occurrence scaled with animal ownership. Strategies for controlling non-human fecal waste should be considered when designing interventions to reduce exposure to fecal contamination in low-income settings. Detection of a human-associated fecal marker and rotavirus was rare and unchanged by provision and promotion of improved sanitation to intervention compounds. The sanitation intervention reduced ruminant fecal contamination in drinking water and general (non-host specific) fecal contamination in soil but overall had limited effects on reducing fecal contamination in the household environment.

INTRODUCTION

Lack of sanitation access in low-income rural settings has been linked to diarrheal illness⁽¹⁾ and impaired growth in children under 5 years old.⁽²⁾ Recent evidence has also connected unsanitary living conditions to child environmental enteropathy.⁽³⁾

Microbial contamination on hands,(4–10) water,(7, 11–13) soil,(14, 15) and household floors(4) has been documented in areas with poor sanitation. However, the majority of studies has measured microbial contamination using fecal indicator bacteria (FIB) such as *Escherichia coli* (EC) and enterococci (ENT). FIB are found in feces of many animal hosts and even in natural reservoirs.(16–18) There have been far fewer studies that measure microbial contamination using enteric pathogens or host-associated genetic markers of fecal contamination.

Previous intervention trials have investigated whether provision of sanitation hardware has improved water quality and hand contamination as measured by FIB.(2, 19, 20) The results of those studies have been equivocal with most showing no change in contamination, which could indicate limited effects of the sanitation intervention on environmental contamination (although two of these trials had low rates of latrine adoption). Another possible explanation for the equivocal results is that the outcomes of interest (FIB) are not primarily from human feces but rather from animal feces.(21) In this case, proper disposal of human feces through latrine provision may not necessarily reduce overall FIB contamination.

This study evaluated whether provision and promotion of improved sanitation to rural Bangladeshi compounds reduced the incidence of a human-associated fecal genetic marker and rotavirus RNA in stored drinking water, in courtyard soil, and on child hands. In addition, we assessed the occurrence of ruminant and avian-associated fecal genetic markers to infer whether animals contribute to microbial contamination in the domestic environment.

Materials and Methods

Environmental sample collection was nested within the WASH Benefits trial in rural Bangladesh,(22) a randomized controlled trial designed to measure the effect of improved water quality, sanitation, hand washing, and nutritional interventions on child diarrhea and growth. We collected environmental samples (stored drinking water, soil, and child hand rinse) from a subset of compounds in the control and sanitation arms of the study; 497 compounds (249 from the control arm and 248 from the sanitation arm) were included.

A compound consisted of 3–10 households comprised of typically blood relatives, with a shared courtyard. Each compound had at least one child under 5 years of age. Groups of adjacent compounds were assigned to clusters, and clusters were randomly assigned to the sanitation versus control arm within geographical strata. In sanitation arm compounds, each household lacking a hygienic latrine was provided with a concrete ring-based dual-pit latrine that had a slab, a water seal, and a superstructure for privacy. The sanitation intervention also included a potty for young children and a metal scoop for removal of child and animal feces from the environment and their safe disposal in the latrine. A behavior change program encouraged regular use of hardware components through weekly compound visits throughout the study. The sanitation hardware and behavior intervention were designed after a 2 year pilot test with documented high user uptake of the selected interventions.(22) The control arm received no hardware or behavior change intervention.

Microbial Source Tracking (MST) Marker Validation

We conducted a MST marker validation study to choose the most sensitive and specific MST markers for use in the study setting. MST marker validation has been completed in a handful of large studies,(23–26) but local testing is recommended before implementation in new geographic settings.(24, 27)

Fecal samples were collected from 20 chickens, 20 ducks, 20 cows, 20 goats, and 15 humans in the study communities. Three or four individual fecal specimens of the same animal species were combined at equal masses to form a 2.0 g composite (Table S1); the result was five composite samples per species. The composite samples were made into fecal slurries, and EC and ENT were enumerated in the slurries using defined substrate assays (IDEXX, Westbrook, MN). For quantitative polymerase chain reaction (QPCR) analysis, 2 mL of slurry was filtered through membrane filters seated in disposable filter funnels. Further details are given in the Supporting Information.

DNA was extracted from filters using a modified MoBio (Carlsbad, CA) PowerWater RNA Isolation Kit.(5) For each extraction batch (10–20 samples), an extraction blank control was included. DNA was used as a template in QPCRs for three human-associated (BacHum,(28) HumM2,(26) and HF183Taqman(29)) MST markers, three ruminant-associated (BacCow,(28) BacR,(30) and Rum2Bac(31)) MST markers, and one avian-associated (Avian GFD(32)) MST marker. These markers were chosen because they performed well in previous studies.(23, 24, 33) The sensitivity and specificity of the markers were determined using metrics described previously.(4, 23) The most sensitive and specific host-associated MST markers were selected to analyze the environmental samples (see the Supporting Information for more details).

Environmental Sampling and Survey

Environmental sampling occurred from November 2013 to March 2014. At each enrolled compound, field staff collected a stored drinking water sample, a hand rinse from one child under 5 years old,(7) and a soil sample from the compound's courtyard where the youngest child under 5 years old had most recently played or spent time according to a compound resident. Respondents were asked how many ruminant and avian (e.g., chickens, ducks, and geese) species were owned by the compound.

Samples were preserved on ice, and processing begun within 12 h. Samples were analyzed for EC and fecal coliform (FC) using IDEXX defined substrate assays. Aliquots of the water and hand rinse samples were membrane filtered to collect nucleic acids from bacteria and viruses and the filters archived using the same technique that was used for the fecal slurries. In addition, laboratory process control blanks were processed (see the Supporting Information). Soil was archived in centrifuge tubes and stored at –80 °C. Filters and soil were tested for rotavirus RNA,(34) general Bacteroidales DNA (GenBac3),(35) and select host-associated MST markers. GenBac3 targets fecal bacteria in the Bacteroidetes class.(26, 35)

DNA and RNA were co-extracted from the water and hand rinse filters using the same method as for the fecal samples. DNA and RNA were co-extracted from soil samples using a protocol developed in this study (further described in the Supporting Information). The recoveries of bacterial DNA and viral RNA from soil using the extraction method are estimated to be 50 and 10%, respectively, while recoveries from filters were previously estimated to be 7 and 17%, respectively.(36)

The nucleic acid extracts from environmental samples were assessed for substances that inhibit QPCR using a spike and dilute method;(37) results informed the dilution level of extract to run during QPCR. All samples were run in duplicate. Each QPCR plate included a standard curve run in triplicate as well as triplicate no-template controls. Environmental samples were scored as positive if at least one of the two replicates amplified, even if the concentration was below the lower limit of quantification (LLOQ). Lowest detectable concentration (LDC) and LLOQ values were calculated by converting one copy (cp) per reaction and 10 cp per reaction (the most dilute standard that consistently amplified), respectively, to appropriate units (cp per 100 mL, cp per two hands, and cp per gram of dry soil). Linear regression using respective instrument run specific standard data was used to estimate molecular marker concentrations. The Supporting Information contains further details.

To compare the occurrence of MST markers between the control and sanitation groups, we used logistic regression for binary outcomes and linear regression for continuous outcomes; all models included indicator variables for each pair-matched cluster (minus a reference cluster) as well as robust standard errors to account for geographic clustering of observations.(22)

Results and Discussion

Quality Assurance and Control

HumM2 and Avian-GFD were not detected in no-template controls, or extraction or laboratory processing blanks. BacR was detected at levels below the LLOQ in two of 78 extraction blanks (these were co-extracted with hand rinse samples). GenBac3 was detected in 50% of the extraction blanks and 41 of 111 laboratory processing blanks, with most of these below or near the LLOQ (see the Supporting Information). This cross contamination is likely a result of the extraordinarily high levels of GenBac3 present in the samples and has been observed by others.(23) The LDC and LLOQ values for the environmental samples were 50 and 500 cp/100 mL of water, 125 and 1250 cp/two hands, and approximately 400 and 4000 cp/g of soil (exact value depended on moisture content), respectively.

MST Marker Validation

We evaluated MST marker sensitivity and specificity using both a binary and a quantitative assessment approach(23) (Figure S1 and Table S2). In brief, the binary approach assesses the percent of target and nontarget fecal samples where the MST marker was and was not detected to calculate the sensitivity and specificity, respectively; a cutoff of 80% was used to define good performance.(23) The quantitative approach takes into account the

concentration of marker detected in tested feces and requires higher marker concentrations in target versus nontarget feces (see the Supporting Information).

Of the three tested human-associated MST markers, HumM2 was specific via the quantitative assessment approach and sensitive via the binary assessment approach. The other human MST markers (HF183 and BacHum) were not specific by either assessment approach. Previous work in urban Bangladesh (Dhaka) found that none of these three human-associated markers was specific(4) and that an assay not tested herein (HF183 SYBR) was sensitive and specific.(38) A large method evaluation study in the United States found that HF183 and HumM2 were sensitive and specific but that BacHum lacked specificity.(23) The different outcomes of these validation studies underscore the need for local validation before human-associated MST assays are applied to environmental samples. (27)

Of the three ruminant-associated MST markers, BacR performed the best; it was the only marker that was sensitive and specific as determined by both assessment approaches. The high specificity of ruminant-associated assays has been observed in multiple geographic settings.(24)

In the study presented here, the avian-associated marker was specific via the quantitative assessment approach and sensitive via the binary approach. Tested in the United States and Australia, the marker performed with good sensitivity and specificity.(32, 39) In another study conducted in urban Bangladesh, it was neither sensitive nor specific.(4)

Given their good sensitivity and specificity in our study area (rural Bangladesh), we used HumM2, BacR, and avian-GFD markers to assess the presence of human, ruminant, and avian fecal contamination in the environmental samples, respectively.

Environmental Fecal Contamination

Across all compounds, EC and FC concentrations were on the order of 10 MPN/two hands, 10 MPN/100 mL of stored water, and 105 MPN/g of soil. GenBac3 concentrations were on the order of 10^6 cp/two hands, 10^4 cp/100 mL of water, and 10^6 cp/g of soil (Tables S3 and S4).

The number of samples positive for each MST marker and rotavirus is reported in aggregate and by study arm (Table 1 and Table S4). HumM2 and rotavirus were present in 0–9% of samples from the three environmental matrices. Mattioli et al.(5) found similar rotavirus prevalence (3–9% of samples tested) in hand rinses and stored water in Tanzania. A previous study in Bangladesh found a prevalence of rotavirus in tubewell water (40%) substantially higher than what we found in stored drinking water (0.6%).(40) The difference may be due to a number of factors, including different detection limits; Ferguson et al.(40) filtered 2–8 L of tubewell water, while we processed 100 mL of stored water.

Percent positive also shown by sanitation arm (N = 248) vs control (N = 249). The p value indicates whether the presence of a marker is associated with the study arm as indicated by logistic regression with robust standard errors.

If we exclude observations with positive extraction blanks, the total number of positive samples was 258 (56.7%); the difference between arms remained insignificant ($p = 0.154$).

BacR was prevalent in all sample matrices; 22% of water samples and more than 50% of soil and hand rinse samples were positive. The high prevalence of ruminant fecal contamination may be due to the use of cow dung for domestic fuel. Avian-GFD was present in 9% of water, 16% of hand rinse, and 33% of soil samples.

Ruminant-associated and avian GFD markers were more frequently detected than human markers in the compounds (z -test; $p < 0.05$ for all matrices). This suggests that ruminant and avian species contribute general indicators of fecal pollution such as EC, ENT, and GenBac3 to the compound environment. The low occurrence of the human-associated marker in human feces relative to the occurrence of non-human-associated markers in non-human feces (Figure S1) confounds the direct comparison of MST marker prevalence data and may contribute to the reduced detection of human-associated markers relative to other markers. (41) Although it is difficult to compare across sample types because of different units of measure, all MST markers assessed in this study were more prevalent in soil than in water or hand rinses. Results suggest ingestion of animal fecal matter is probable when children intentionally or accidentally consume soil.(42)

Effect of Sanitation Intervention on Marker Occurrence

Mean GenBac3 concentrations were reduced in soil (mean difference = 0.2 log unit; $p = 0.02$) in sanitation compared to control compounds, suggesting the intervention reduced levels of general fecal contamination in compound soil, albeit modestly. GenBac3 in hand rinse samples ($p = 0.35$) and water samples ($p = 0.33$) was not different between sanitation and control compounds (Table S3). Results did not change after accounting for the low levels of GenBac3 contamination (see the Supporting Information).

The sanitation intervention did not significantly affect the frequency of HumM2 or rotavirus detection in stored water, soil, hands, or any compound sample relative to the control (Table 1). Latrine access was high in both treatment and control compounds after the intervention; however, the treatment group had greater access to improved latrines (98% vs 66%) and to latrines with safe drainage (99% vs 73%) (Table S5). Our study documents a limited effect of improving and promoting basic latrines and sanitation practices on human fecal contamination in the household environment.

Association between Non-human MST Marker Occurrence, Sanitation Intervention, and Animal Ownership

Ownership of ruminant and avian species was common among compounds; 78% of compounds owned at least one ruminant (median of 3). BacR occurrence was twice as likely [odds ratio (OR) = 2.4; $p < 0.001$] in hand rinses from compounds that owned at least one ruminant than in samples from compounds without ruminants; in soil samples, BacR occurrence was 4 times greater among ruminant owners (OR = 4.1; $p < 0.001$). This analysis for BacR in hand rinses (as well as all other BacR hand rinse analyses) was repeated excluding samples co-extracted with a positive extraction blank, and the results were

unchanged. A study in Dhaka, Bangladesh, also found that owning ruminants was associated with the presence of BacR in households.(4)

Ninety-four percent of the households owned at least one avian species (median of 10). Avian-GFD marker occurrence frequency was greater in soil (OR = 1.6; $p = 0.025$), in hand rinses (OR = 1.5; $p = 0.077$), and in stored water (OR = 1.8; $p = 0.082$) from compounds that owned 10 avian species than in the same samples from compounds that owned <10.

Ruminant marker BacR was more likely detected in stored water in the control versus sanitation compounds (Table 1), although the proportion of compounds owning ruminants was not different between the two groups (χ^2 test; $p > 0.05$). This result could be explained by the use of the provided sanitary scoops by the sanitation compounds to reduce the amount of ruminant feces in the environment that eventually entered the water supply.

Although exposure to non-human feces is considered a lower risk than exposure to the same amount of human feces,(43, 44) there is evidence that non-human feces can still present a substantial health risk. A recent systematic review reported a positive association between diarrheal illness and domestic animal husbandry.(45) A study in rural India reported similar magnitudes of increased risk of diarrhea associated with domestic animal contamination compared to human contamination in the household environment.(19) Non-human feces can harbor a number of zoonotic organisms, including bacteria (toxigenic *E. coli*, *Campylobacter*, and *Salmonella*) and protozoa (*Cryptosporidium*), that cause diarrheal illness.(46) Notably, three of the four pathogens that cause most cases of severe to moderate child diarrhea in low-income countries are zoonotic.(46) Biomarkers of environmental enteropathy have also been linked to children sleeping in the proximity of animals.(47) Future interventions to reduce fecal contamination in the household environment must consider control of animal feces, particularly in settings where domestic animal ownership is prevalent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

Information has been subjected to U.S. Environmental Protection Agency (USEPA) peer and administrative review and has been approved for external publication. This research was financially supported by the World Bank and in part by Grant OPPGD759 from the Bill & Melinda Gates Foundation to the University of California, Berkeley. We thank Leanne Unicomb, Masud Parvez, and Solaiman Abeer.

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Table 1.

Number Positive (# pos) and Percent Positive (%) for All Households (N = 497 for most) of the Human-Associated, Ruminant-Associated, and Avian-Associated Markers, and Rotavirus RNA in Water, Hand Rinse (hands), and Soil Samplesa

sample type	all # pos	all %	sanitation # pos	sanitation %	control # pos	control %	p value
soil avian	165	33.3	81	32.8	84	33.7	0.992
water avian	46	9.3	19	7.7	27	11.0	0.263
hands avian	80	16.2	40	16.2	40	16.3	0.809
soil ruminant	331	66.7	163	66.0	168	67.5	0.974
water ruminant	108	21.9	42	17.0	66	26.8	0.004
hands ruminantb	267	54.2	131	53.0	136	55.3	0.465
soil human	44	8.9	21	8.5	23	9.2	0.842
water human	0	0.0	0	0.0	0	0.0	na
hands human	12	2.4	7	2.8	5	2.0	0.158
soil rotavirus	7	1.4	5	2.0	2	0.8	0.229
water rotavirus	3	0.6	2	0.8	1	0.4	0.978
hands rotavirus	30	6.1	16	6.5	14	5.7	0.817