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## Predictors of Enteric Pathogens in the Domestic Environment from Human and Animal Sources in Rural Bangladesh

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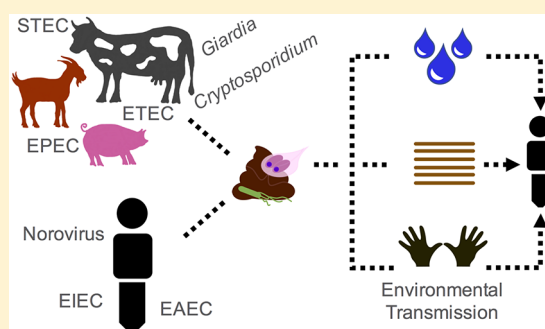
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### Supporting Information

**ABSTRACT:** Fecal indicator organisms are measured to indicate the presence of fecal pollution, yet the association between indicators and pathogens varies by context. The goal of this study was to empirically evaluate the relationships between indicator *Escherichia coli*, microbial source tracking markers, select enteric pathogen genes, and potential sources of enteric pathogens in 600 rural Bangladeshi households. We measured indicators and pathogen genes in stored drinking water, soil, and on mother and child hands. Additionally, survey and observational data on sanitation and domestic hygiene practices were collected. Log<sub>10</sub> concentrations of indicator *E. coli* were positively associated with the prevalence of pathogenic *E. coli* genes in all sample types. Given the current need to rely on indicators to assess fecal contamination in the field, it is significant that in this study context indicator *E. coli* concentrations, measured by IDEXX Colilert-18, provided quantitative information on the presence of pathogenic *E. coli* in different sample types. There were no significant associations between the human fecal marker (HumM2) and human-specific pathogens in any environmental sample type. There was an increase in the prevalence of *Giardia lamblia* genes, any *E. coli* virulence gene, and the specific *E. coli* virulence genes *stx1/2* with every log<sub>10</sub> increase in the concentration of the animal fecal marker (BacCow) on mothers' hands. Thus, domestic animals were important contributors to enteric pathogens in these households.



## INTRODUCTION

In low- and middle-income countries, diarrheal illness is a leading cause of morbidity and mortality.<sup>1</sup> In Bangladesh, 6% of the 129 000 deaths in children under five in 2013 were attributed to diarrheal diseases.<sup>2</sup> Diarrheal illness results from exposure to fecal pathogens which can be transmitted from feces to a new human host through a variety of environmental pathways, including fingers, fields, flies, fluids, and food, described in the F-diagram.<sup>3</sup> Recent additions to the F-diagram stress the importance of animal hosts by expanding fecal sources to include feces from livestock, free-roaming animals, and synanthropic rodents.<sup>4</sup> While it is well-known that enteric pathogens are transmitted through these pathways, few studies have measured pathogens to characterize exposure from different animal reservoirs.

There is a high potential for zoonotic enteric disease transmission in low- and middle-income countries where animal husbandry is a primary source of income.<sup>5</sup> For example, in Bangladesh raising livestock such as cows, goats, and chickens results in animals roaming freely within the home environment.<sup>6</sup> Close proximity to domesticated animals can lead to human exposure to livestock feces. Many pathogens can be transmitted from animal feces to human hosts and result in diarrheal illnesses.<sup>5</sup> Of the five most common etiological agents of moderate to severe diarrhea in children 0–11 months in

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Bangladesh, two (*Cryptosporidium* and *Campylobacter*) are known to have important animal reservoirs.<sup>7,8</sup>

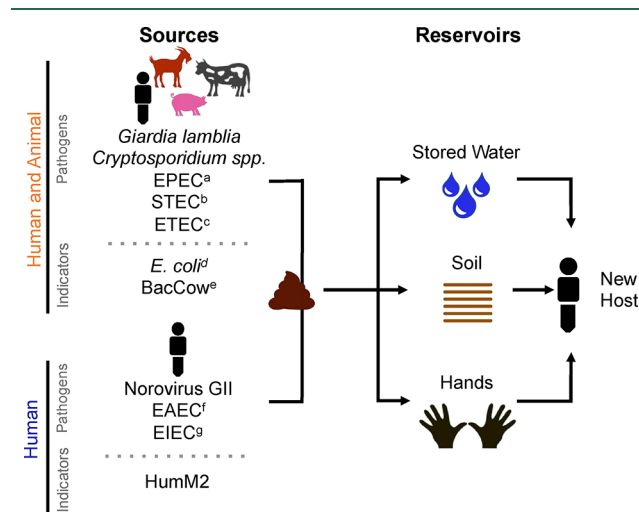
Measuring enteric pathogens in the environment can help identify reservoirs and potential exposure pathways, which can better inform design of strategies to reduce human exposure. However, there are many different fecal pathogens from humans and animals capable of causing disease, making it infeasible to measure them all, especially given that most are difficult to measure in the environment due to their low concentrations in environmental matrices and costly and complex methods of detection.<sup>9</sup> Therefore, fecal indicators are commonly used to indicate the presence of fecal contamination, which may contain pathogens.<sup>9</sup> Thermotolerant coliforms and *Escherichia coli* are recommended indicators of drinking water quality by the World Health Organization.<sup>10</sup> Other fecal indicators such as *Bacteroidales* are used for identifying sources of fecal contamination through microbial source tracking (MST).<sup>11</sup> However, the degree to which indicators and pathogens co-occur in environmental samples varies depending on their concentrations in the original fecal source (pathogen concentrations depend on the infection status of the human or animal, whereas indicator organism concentrations are expected to remain more stable) and the relative transport and die-off/growth rates of organisms once they are in the environment. Additionally, correlations between indicator organisms and pathogens in natural environments, such as soil and surface waters, are impacted by the concentrations of naturally occurring *E. coli* in the environment, often referred to as “naturalized” *E. coli*. Thus, the relationship of indicator organisms and specific pathogens in or on different environmental matrices (e.g., water, soil, hands, fomites, food) varies spatially and temporally depending on these factors.<sup>12</sup> Nonetheless, a better empirical understanding of the relationship between indicators and pathogens in specific environmental reservoirs and contexts in low- and middle-income countries may improve the ability to estimate human health risk and identify fecal sources and exposure pathways of greatest concern. Furthermore, pinpointing dominant sources and reservoirs will help improve the design of targeted interventions that can reduce exposure to fecal pathogens, mitigating the burden of diarrheal illnesses.<sup>13,14</sup>

The aim of this study was to measure fecal indicators and select human pathogen genes in different reservoirs in the domestic environment to (1) determine the association between concentration of indicator *E. coli* and presence of pathogen genes; and (2) use microbial source tracking and observations of animal feces in compounds to investigate potential human and animal sources of detected pathogen genes.

## MATERIALS AND METHODS

We selected multiple types of microorganisms that are leading causes of diarrheal illness in developing countries and have a range of host specificity between humans and animals. The selected targets were indicators (*E. coli*, human-associated *Bacteroidales*-like gene, and nonhuman animal-associated *Bacteroidales* gene) and pathogen genes (enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), shiga-toxin producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), norovirus GII, *Giardia lamblia*, and *Cryptosporidium* spp.). We measured these targets in different reservoirs in the domestic environment within rural

Bangladeshi compounds: stored water, soil, and mother and child hands (Figure 1).



**Figure 1.** Pathogens associated with human and animal origin in different reservoirs investigated in this paper. <sup>a</sup>Atypical enteropathogenic *E. coli* (EPEC) is transmitted by humans and animals, typical is transmitted by humans only. <sup>b</sup>STEC: Shiga toxin-producing *E. coli*. <sup>c</sup>Enterotoxins found in enterotoxigenic *E. coli* (ETEC) can be from humans and animals but species-specific adhesion factors confer host specificity. <sup>d</sup>Outside of human and animal sources, indicator *E. coli* can also occur naturally in the environment. <sup>e</sup>BacCow is specific to animals. <sup>f</sup>AggR gene in enteroaggregative *E. coli* (EAEC) only found in strains isolated from humans. <sup>g</sup>Enteroinvasive *E. coli* (EIEC) has been found in humans and primates; primates are not relevant to this study.

We reviewed current literature to determine which of the selected pathogens had the potential to originate from both human and animal reservoirs or only human reservoirs. Pathogens associated with both human and animal feces include *Cryptosporidium*, *Giardia lamblia*, EPEC, STEC, and ETEC.<sup>15–17</sup> Humans serve as a reservoir of typical EPEC, while animals such as pigs and chickens can serve as reservoirs of atypical EPEC.<sup>17,18</sup> Though typical and atypical EPEC can be differentiated by the presence of the gene *bfpA*, this study only measured *eaeA* and will therefore not distinguish between these strains.<sup>19</sup> STEC is characterized by the presence of shiga toxin genes (*stx1* or *stx2*) and is known to come from ruminants.<sup>17</sup> Enterohemorrhagic *E. coli* (EHEC) is a common type of STEC that can also carry the *eaeA* gene found in EPEC.<sup>17,20</sup> ETEC is found in both humans and animals but species-specific adhesion factors confer host specificity.<sup>8</sup> We were unable to differentiate ETEC with human adhesion factors in this study because the enterotoxins we measured, *st1b* and *lt1*, can be found in both humans and animals.<sup>21–24</sup> Previous studies have isolated *Giardia lamblia* and *Cryptosporidium* spp. in many fecal sources and identified closely related isolates from humans and animals.<sup>15,25</sup> On the other hand, norovirus GII, EAEC, and EIEC are associated with primarily human sources, largely based on genetic analysis of pathogens in human and animal fecal samples.<sup>17,26,27</sup> While EAEC is also found in animals, the gene we detected, *aggR*, has been identified in EAEC isolated from humans but not animals.<sup>27,28</sup> EIEC has also been found in primates, which are unlikely to be important contributors to fecal pollution in the study communities.<sup>17</sup> It should be noted that the associations

between pathogens and possible hosts in Figure 1 are based on current scientific understanding and evidence. Zoonotic pathogen transmission is difficult to demonstrate, and we are limited by the methods that have been employed and the pathogens, virulence genes, locations, and animal hosts that have been investigated.

**Study Setting and Design.** 600 households were randomly selected from those enrolled in the sanitation and control arms (300 households per arm) of the WASH Benefits randomized controlled trial in rural Bangladesh, described elsewhere.<sup>29</sup> One study household was selected per compound and compounds were usually relatives living in adjacent households surrounding a central courtyard. Compounds were clustered in groups of eight to reduce spillover effects and for the ease of community promoters delivering the interventions. Clusters were geographically pair-matched and then randomly allocated to different intervention arms.<sup>30</sup> Study households were from the rural Gazipur, Mymensingh, Tangail, and Kishoreganj districts. WASH Benefits enrolled and followed households with a pregnant woman. All study households contained a young child (aged 9–44 months) at the time of our visit. In this same subset of households, another analysis in preparation was conducted to investigate the impact of the sanitation intervention on the prevalence of pathogen genes and microbial source tracker markers.<sup>31</sup> Another related study investigated how the presence of domestic animals impacted concentrations of indicator *E. coli* in reservoirs in a subset of households from the control arm.<sup>32</sup>

**Data Collection.** During household visits between March and October 2015, trained field staff from the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) collected environmental samples, interviewed the female caregiver of young children regarding household practices on handling of animal feces, and observed the number and type of animal fecal piles in compound courtyards. Field surveyors also recorded the number and type of animals owned by the household as reported by the caregiver.

**Sample Collection.** We collected approximately 720 stored drinking water samples, 720 soil samples, 720 mother hand rinses, and 360 child hand rinses from 600 study households. We sampled 176 of households in the control and 156 of households in the sanitation arms twice, approximately four months apart to assess the impact of season. Hand rinse samples were collected by mothers placing their left-hand into a sterile Whirlpak bag (Nasco, Modesto, CA) filled with 250 mL of distilled water. The hand was massaged from the outside of the bag for 15 s, followed by 15 s of shaking. The same procedure was repeated with the right-hand in the same bag. To collect child hand rinse samples, respondents placed their child's hand into a separate Whirlpak bag and followed the same procedure. Soil samples were collected from a 30 × 30 cm<sup>2</sup> area as close to the house entrance as possible by scraping the top layer of soil within a stencil into a sterile Whirlpak bag using a sterile disposable plastic scoop. The sample area was scraped both vertically and horizontally. Stored water samples were collected by asking mothers to provide a glass of water as they would give it to their child under five. The provided water was poured into a sterile Whirlpak bag. All samples were transported to the icddr,b field laboratory on ice and processed within 12 h of collection.

**Indicator *E. coli* Enumeration and Detection of Pathogen Genes.** *E. coli* were enumerated (100 mL sample volumes) using IDEXX Colilert-18 (IDEXX Laboratories,

Westbrook, Maine). Trays were incubated at 44.5 °C for 18 h.<sup>33</sup> To prepare for IDEXX analysis, hand rinse samples were diluted 1:2 with sterile distilled water in Whirlpak bags, whereas stored water was analyzed undiluted. Twenty g of soil was homogenized with 200 mL of distilled water and diluted 1:10<sup>4</sup>. Soil was dried at 110 °C for 24 h to determine moisture content. Each lab technician processed one blank (sterile distilled water) for *E. coli* enumeration per day.

Pathogenic *E. coli* genes were identified using previously published methods.<sup>20,34</sup> All samples positive for *E. coli* as determined by IDEXX Colilert-18 were archived for subsequent pathogenic *E. coli* gene analysis. Broth from up to 20 positive large wells was aseptically extracted from IDEXX trays, composited, and centrifuged. Pellets were treated with 10× the pellet volume (≈0.1 mL) of RNAlater (Qiagen, Germantown, MD), stored at −80 °C, and transported to UC Berkeley at room temperature. Each lab technician analyzed one lab blank per week for the archiving process by archiving wells from IDEXX trays incubated with sterile distilled water. Pellets were stored at −80 °C upon arrival at UC Berkeley. DNA was extracted from bacteria pellets using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD). Multiplex reactions were used to detect seven *E. coli* virulence genes indicative of five possible pathotypes of *E. coli*: EAEC (*aggR*), EPEC or EHEC (*eaeA*), STEC (*stx1/stx2*), EIEC (*ipaH*), ETEC (*lt1/st1b*) (Supporting Information (SI) Table S1). PCR products were run on 2% agarose gels at 110 V for 30 min. Additional information on DNA extraction and PCR protocols is available in the SI.

**Filtration and Nucleic Acid Extraction for qPCR Targets.** Laboratory technicians preprocessed samples in the field laboratory by filtering 50 mL of hand rinse samples and up to 500 mL of stored water (range: 100–500 mL) through a 0.45 μm HA filter (Millipore, Burlington, MA) to capture bacteria and viruses. 0.5 mL of 2.5 M MgCl<sub>2</sub> was added to every 50 mL of sample to increase retention of viruses.<sup>35</sup> Filters were treated with 0.5 mL of RNAlater, stored at −80 °C and transported to UC Berkeley at room temperature. Once per day, each lab technician processed a lab blank by filtering 5 mL of sterile distilled water with 0.5 mL of MgCl<sub>2</sub>, followed by the addition of RNAlater. Five grams of soil were weighed and stored at −80 °C until transport at room temperature to UC Berkeley in accordance with a USDA soil import permit (PPQ 525). Filter and soil samples were stored at −80 °C at UC Berkeley. DNA and RNA were extracted from filters and 0.25 g of soil samples using modified Mobio PowerWater and PowerViral (both now Qiagen, Germantown, MD) protocols, described in previous studies and in the SI.<sup>36,37</sup> An extraction blank was included in each batch of 23 samples. Extraction efficiencies for DNA and RNA were determined in a subset of samples using *Pseudomonas syringae* pv. *phaseolicola* (pph6) and MS2 (SI Table S2).

**qPCR Assays.** Samples were analyzed for norovirus GII, *Giardia lamblia*, *Cryptosporidium* spp. genes, and microbial source tracking markers using quantitative PCR (SI Table S3). Specifically, to detect human fecal contamination we used the HumM2 assay which targets a gene for a hypothetical protein in human-associated *Bacteroidales*-like microorganisms.<sup>38,39</sup> We obtained a research license (#864-15) from the U.S. Environmental Protection Agency to use the patented HumM2 assay. To detect nonhuman, animal-associated fecal contamination we used the BacCow assay which, although designed to target the 16S rRNA of *Bacteroidales* associated with ruminants, has



Table 1. Units, Limits of Detection, and Mean Concentrations in Hand Rinse, Stored Water, and Soil Samples

	child hands	mother hands	stored water	soil
sample matrix unit	2 hands	2 hands	100 mL	1 g dry weight
<i>E. coli</i> lower limit of detection <sup>a</sup>	5	5	1	1000–1880 <sup>d</sup>
<i>E. coli</i> upper limit of detection <sup>a</sup>	12 098	12 098	2420	$2.42 \times 10^6$ – $4.55 \times 10^{6d}$
<i>n</i> < lower limit of detection	100	183	93	39
<i>n</i> > upper limit of detection	7	17	26	97
log <sub>10</sub> mean (stdev) concentration of <i>E. coli</i> <sup>a</sup>	1.38 (0.92)	1.47 (1.02)	1.15 (1.05)	5.14 (1.06)
BacCow lower limit of detection <sup>b</sup>	285	285	8.1–40.5 <sup>c</sup>	130–244 <sup>d</sup>
BacCow lower limit of quantification <sup>b</sup>	2500	2500	100–500 <sup>c</sup>	2000–3760 <sup>d</sup>
<i>n</i> < lower limit of detection	25	9	242	97
<i>n</i> < lower limit of quantification	218	79	414	281
log <sub>10</sub> mean (stdev) concentration BacCow <sup>b</sup>	3.84 (0.87)	3.67 (0.85)	1.25 (0.64)	3.34 (1.26)

<sup>a</sup>In units of MPN/sample matrix. <sup>b</sup>In units of gene copies/sample matrix. <sup>c</sup>Corresponds to volumes of 100 and 500 mL filtered. <sup>d</sup>Corresponds to soil moisture contents ranging from 0 to 88%.

been shown to detect *Bacteroidales* in cows, ducks, goats, and chickens but not humans in rural Bangladesh.<sup>36,40</sup> The microbial source tracking markers were previously evaluated for sensitivity and specificity in rural Bangladesh.<sup>36</sup> BacCow was sensitive but not specific to ruminant feces, and HumM2 performed the best out of all tested human-associated assays (HumM2, HF183, and BacHum), although it also amplified in the feces of chickens and goats.<sup>36</sup>

Approximately 100 stored water, soil, and mother and child hand rinse samples were processed for norovirus GII, *Giardia*, and *Cryptosporidium* genes. Among this subset, <1% of soil and stored water samples were positive for norovirus and *Giardia* genes, whereas 3% of mother hands and 6% of child hands were positive for norovirus, and 1% of mother hands and 6% of child hands were positive for the *Giardia* gene. Therefore, we decided to analyze only hand rinse samples for norovirus and *Giardia* genes. Less than 1% of all environmental sample types were positive for the *Cryptosporidium* gene; consequently, we did not continue to analyze for the *Cryptosporidium* gene in any sample type.

All samples were run in triplicate on a 96-well plate (Applied Biosystems, Foster City, CA) on a StepOnePlus thermal cycler (Applied Biosystems, Foster City, CA). Each run contained a standard curve and three no template controls. A subset of samples were tested for inhibition using the spike-and-dilute method.<sup>41</sup> We did not dilute any samples for any of the pathogen and indicator assays based on our results of inhibition testing (SI Tables S4 and S5). Standard curves for each assay were determined by pooling all results and using a linear mixed effects model (SI Table S6).<sup>42</sup> We used the curves from the linear mixed effects models to determine Ct values in samples for all assays. Assays and qPCR methods are described in more detail in the SI.

**Data Analysis.** Samples below the limit of detection of *E. coli* (Table 1) were set to 0.5 MPN, and those above the limit of quantification were set to 2420 MPN. Samples were considered positive for qPCR targets if at least one of three replicates amplified, even if amplification was below the limit of quantification (BLOQ). The limit of quantification (LOQ) was 10 gene copies (the lowest point on the pooled standard curve). The limit of detection (LOD) was determined based on the lowest gene copy that amplified in at least one of three replicates in each sample type. Samples below the limit of detection (BLOD) of BacCow were assigned half the LOD, and samples below the LOQ were assigned the midpoint

between the LOD and LOQ. The LOD of BacCow was 285 gene copies (gc)/2 hands, 8.1–40.5 gc/100 mL stored water (depending on the volume of water filtered), and 130–244 gc/g dry soil weight (depending on the soil moisture content) (Table 1). The LOQ of BacCow was 2500 gc/2 hands, 100–500 gc/100 mL stored water, and 2000–3760 gc/g dry soil weight. No samples were above the upper limit of quantification for BacCow (defined by the highest point on the pooled standard curve).

Most samples did not amplify within the quantifiable range for norovirus GII, *G. lamblia*, and HumM2 (LOD reported in SI). For binary statistical analyses on pathogen/source tracker marker presence as the dependent variable, all samples with positive lab, extraction, or archiving blanks were omitted from the analysis according to the date processed and lab technician. For BacCow, the blanks that amplified were BLOQ; samples that amplified in the same region (BLOQ) were treated as BLOD. Additional details on positive blanks and limits of detection are available in the SI.

Data were analyzed using R (version 3.5.0).<sup>43</sup> The association between indicators and pathogens was assessed using generalized linear models, with a Poisson distribution and log link to estimate prevalence ratios. Robust standard errors were used to account for the trial's clustered study design.<sup>29</sup> Analyses for pathogenic *E. coli* were conducted for genes individually and for the presence of any of the seven genes detected (any ECVG). Co-occurring genes (SI Table S7) were not accounted for in our linear models. The input values for *E. coli* and BacCow concentrations were log<sub>10</sub>-transformed. Adjusted models controlled for the effects of month of sample collection and study arm. Conditional probabilities (positive predictive value (PPV), negative predictive value (NPV), sensitivity, and specificity) to assess the ability of indicators to correctly predict pathogen gene presence were calculated for binary data and for continuous data by binning continuous data at a specified cutoff point (SI Figure S1). We investigated threshold trends in stored water associated with the WHO water quality guidelines by evaluating PPV, NPV, sensitivity, and specificity of pathogen presence and indicator *E. coli* concentrations above cutoff values.

The association between the presence of animal fecal piles within the compound courtyard and presence of pathogens was also assessed using generalized linear models with a Poisson distribution, a log link, and robust standard errors. We

**Table 2. Percentage of Hand Rinse, Stored Water, and Soil Samples Positive for Culturable *E. coli*, Microbial Source Tracking Markers, Norovirus GII, *Giardia lamblia*, and *E. coli* Virulence Genes**

	percent positive		percent positive
<b>Child Hands (N = 311–373)<sup>a</sup></b>		<b>Mother Hands (N = 603–738)<sup>a</sup></b>	
culturable <i>E. coli</i>	73.2	culturable <i>E. coli</i>	75.2
BacCow	97.5	BacCow	96.5
HumM2	21.9	HumM2	18.0
norovirus GII	4.2	norovirus GII	3.1
<i>Giardia lamblia</i>	4.8	<i>Giardia lamblia</i>	2.3
any <i>E. coli</i> virulence gene	32.4	any <i>E. coli</i> virulence gene	22.1
<i>stx1/stx2</i> (STEC <sup>b</sup> )	15.1	<i>stx1/stx2</i> (STEC)	8.3
<i>eaeA</i> (EPEC <sup>c</sup> /EHEC <sup>d</sup> )	11.4	<i>eaeA</i> (EPEC/EHEC)	7.7
<i>aggR</i> (EAEC <sup>e</sup> )	11.8	<i>aggR</i> (EAEC)	7.7
<i>st1b/l1</i> (ETEC <sup>f</sup> )	8.7	<i>st1b/l1</i> (ETEC)	4.0
<i>ipaH</i> (EIEC <sup>g</sup> )	0.3	<i>ipaH</i> (EIEC)	0.8
<b>Stored Water (N = 652–742)<sup>a</sup></b>		<b>Soil (N = 644–755)<sup>a</sup></b>	
culturable <i>E. coli</i>	87.5	culturable <i>E. coli</i>	94.8
BacCow	66.6	BacCow	86.8
HumM2	2.6	HumM2	20.2
any <i>E. coli</i> virulence gene	37.0	any <i>E. coli</i> virulence gene	60.3
<i>stx1/stx2</i> (STEC)	16.6	<i>stx1/stx2</i> (STEC)	24.8
<i>eaeA</i> (EPEC/EHEC)	14.9	<i>eaeA</i> (EPEC/EHEC)	37.7
<i>aggR</i> (EAEC)	11.0	<i>aggR</i> (EAEC)	15.9
<i>st1b/l1</i> (ETEC)	8.6	<i>st1b/l1</i> (ETEC)	15.3
<i>ipaH</i> (EIEC)	1.2	<i>ipaH</i> (EIEC)	1.2

<sup>a</sup>Sample sizes vary due to the omission of samples corresponding to positive processing blanks. <sup>b</sup>STEC: Shiga toxin-producing *E. coli*. <sup>c</sup>EPEC: Enteropathogenic *E. coli*. <sup>d</sup>EHEC: Enterohemorrhagic *E. coli*. <sup>e</sup>EAEC: Enteroaggregative *E. coli*. <sup>f</sup>ETEC: Enterotoxigenic *E. coli*. <sup>g</sup>EIEC: Enteroinvasive *E. coli*.

assessed the association by binning animal feces into two categories for each animal fecal type. For cow feces, goat/sheep, cow patty, and nonpoultry birds the prevalence ratio represents the prevalence of pathogen genes in households with any visible fecal piles (>0) compared to those with no fecal piles. For chicken/nonchicken poultry feces the prevalence ratio represents the prevalence of pathogen genes in households with >5 fecal piles compared to those with ≤5 fecal piles. Cow patties for cooking were differentiated from cow feces because they were formed into cakes and dried in the sun. Exposures (feces of different animals) were screened against outcomes (pathogen genes) in bivariate models, and those with a *p*-value < 0.2 were included in the final multivariable model. Therefore, models adjusted for the presence of other feces types in the same household if these were associated with the outcome pathogen gene in a bivariate model at the *p*-value < 0.2 level. Final models also controlled for the month of sample collection. We corrected for multiple comparisons (i.e., the same pathogen evaluated in four sample types) using a Bonferroni correction.<sup>44</sup>

**Ethics.** Participants provided written informed consent. The study protocol was approved by human subjects committees at the icddr,b (PR-11063), University of California, Berkeley (2011–09–3652), and Stanford University (25863).

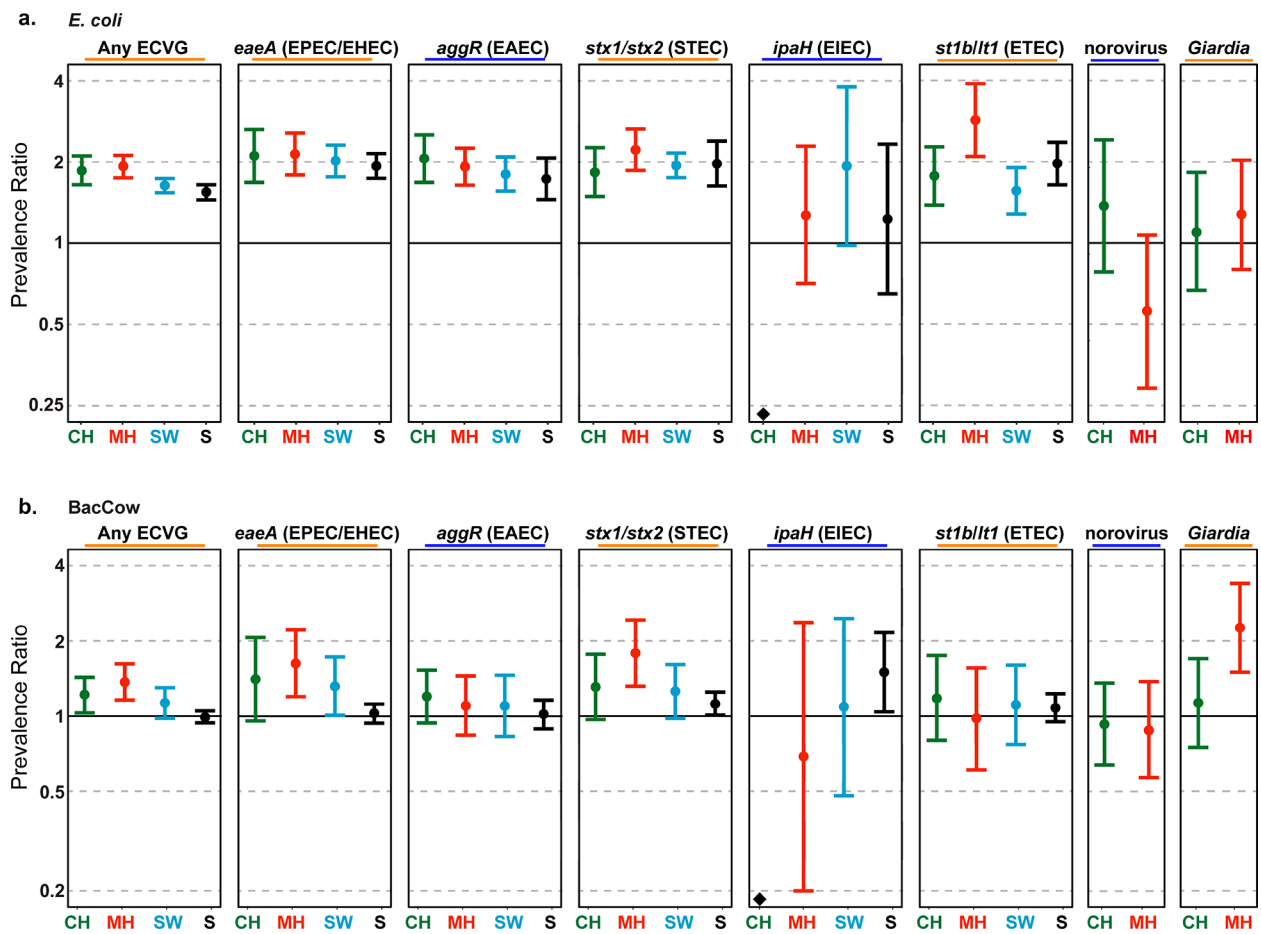
## RESULTS AND DISCUSSION

**Presence of Pathogen Genes and Indicators.** Culturable *E. coli* were detected in 95% of soil, 88% of stored water, 75% of mother hands, and 73% of child hands (Table 2). Culturable *E. coli* were the most commonly detected indicator in soil and stored water followed by BacCow and HumM2. On hands, BacCow was detected most frequently (98% of child

hands and 96% of mother hands) followed by *E. coli* (see above) and HumM2 (22% of child hands and 18% of mother hands).

Of the 360 child hand and 720 mother hand samples, 32% of child and 22% of mother hand samples were positive for at least one *E. coli* virulence gene (ECVG), the most common being *stx1/2* (15% of child and 8% of mother hand samples) (Table 2). *Stx1/2* were also the most abundant genes in stored water (16%), whereas *eaeA* was the most frequently detected ECVG in soil (38%). More than half (60%) of soil samples contained at least one ECVG. Across all sample types, few (≈1%) were positive for *ipaH*. Norovirus and *Giardia* genes were found in <5% of mother hand and child hand samples. As mentioned previously, we detected few positive samples for the *Cryptosporidium* spp. gene in our initial testing of 100 samples and thus did not assay the remainder of the samples. The *Cryptosporidium* spp. gene was inhibited in soil (see SI), which could have impacted our ability to detect the gene in this sample type.

The concentrations of *E. coli* detected on hands, in stored water, and in soil were similar to those in previously published WASH Benefits studies.<sup>32,45</sup> However, the concentration of *E. coli* in soil was significantly higher than soil in Tanzania, a non-WASH Benefits study in Bangladesh, and in Zimbabwe.<sup>34,46,47</sup> Variation in *E. coli* concentration could be due to differences between the study contexts and sampling season, and differences between enumeration methods could also play a role (all WASH Benefits studies used the same IDEXX Colilert-18 method, whereas other studies used membrane filtration and plating). The prevalence of BacCow in this study was slightly higher than was found on hands and in stored water in rural India.<sup>48</sup> In a different study in urban Kenya,



**Figure 2.** Prevalence ratios and 95% confidence intervals indicating the average increase in prevalence of norovirus GII, *Giardia lamblia*, and *E. coli* virulence genes for a 1 log<sub>10</sub> increase in (a) MPN *E. coli*/sample matrix and (b) gene copies of BacCow/sample matrix. ◆ indicates too few samples were positive to model. Pathogens and virulence genes associated with human or human and animal sources are underlined in blue and orange, respectively. CH: child hands, MH: mother hands, SW: stored water, S: soil. ECVG: *E. coli* virulence gene, EHEC: Enterohemorrhagic *E. coli*, EPEC: Enteropathogenic *E. coli*, EAEC: Enteroaggregative *E. coli*, STEC: Shiga toxin-producing *E. coli*, ETEC: Enterotoxigenic *E. coli*, EIEC: Enteroinvasive *E. coli*.

protozoa were much more prevalent (*Cryptosporidium*: 67% and *Giardia*: 18%) in soil in public areas than protozoa in our study.<sup>49</sup> The occurrence of pathogenic *E. coli* genes in soil was similar in Bangladesh and Kenya, except for the *stx1/2* genes. 25% of soil samples were positive for *stx1/2* in this study and less than 2% of soil were STEC positive in Kenya. Overall, it is noteworthy that both studies provide evidence of widespread presence of pathogenic *E. coli* in environmental reservoirs.

Our detection methods for indicators and pathogen genes have some limitations. First, the sampling unit was different for hand rinses, stored water, and soil samples, limiting the ability to compare the relative percentage of pathogens in different environmental reservoirs. The binary data on pathogen presence and concentrations of BacCow in stored water and soil are influenced by different sampling volumes of stored water and varying moisture content in soil. Looking at the ECVGs detected, some, such as *eaeA*, are common to both EPEC and EHEC, whereas *ipaH* is common to both EIEC and *Shigella*.<sup>20,50</sup> Although *ipaH* is present in both EIEC and *Shigella*, IDEXX Colilert-18 has been shown to be selective for *E. coli*.<sup>51,52</sup> The prevalence ratios measuring the association between both *stx1/2* and *eaeA* genes and both *E. coli* and BacCow exhibited similar trends (Figure 2a and b), which could be due to the co-occurrence of *stx1/2* and *eaeA* genes in

EHEC, or due to co-occurrence of different bacteria in the same samples with these genes (e.g., EPEC and STEC). Co-occurring *eaeA* and *stx1/2* genes were found in 2–5% of hand and water samples and 14% of soil samples (SI Table S7). It should also be noted that there is a potential for false positives in detection of *E. coli* genes using PCR and gel electrophoresis, as we did not use confirmation by gene probe or sequencing. Lastly, the presence of PCR targeted genes for norovirus and *Giardia* does not indicate the presence of infectious organisms. *E. coli* virulence genes likely originated from viable *E. coli*, due to the IDEXX culturing step, similar to enrichment PCR. For *Giardia* and norovirus, our methods capture the presence of genetic material, and our results could, therefore, overestimate the potential for infection with *Giardia* and norovirus.

**Indicator *E. coli* and Pathogen Genes.** The concentration of indicator *E. coli* across all samples types was significantly associated with the prevalence of *eaeA*, *aggR*, *stx1/2*, and *st1b/l1t1* (Figure 2a and SI Table S8). The detection of ECVGs increased 86% (prevalence ratio [PR]: 1.86 (95% CI: 1.65–2.11),  $p < 0.001$ ) on child hands, 93% (PR: 1.93 (1.75–2.12),  $p < 0.001$ ) on mother hands, 64% (PR: 1.64 (1.54–1.74),  $p < 0.001$ ) in stored water, and 55% (PR: 1.55 (1.45–1.65),  $p < 0.001$ ) in soil with every log<sub>10</sub> increase in *E. coli* concentration. A log<sub>10</sub> increase in the concentration of *E.*

*coli* was associated with a doubling of the prevalence of *eaeA* in all sample types: child hands (PR: 2.11 (1.68–2.64),  $p < 0.001$ ); mother hands (PR: 2.14 (1.79–2.56),  $p < 0.001$ ); stored water (PR: 2.02 (1.76–2.31),  $p < 0.001$ ); soil (PR: 1.93 (1.74–2.15),  $p < 0.001$ ). A similar magnitude of association was observed between *aggR*, *stx1/2*, and *st1b/lt1* and indicator *E. coli* concentration (Figure 2a). However, no statistically significant association was observed between the prevalence of *ipaH* and *E. coli* concentration in any sample type. Similarly, there were no statistically significant associations between the prevalence of norovirus GII or *Giardiagenes* and the concentration of indicator *E. coli*.

Our results confirm that higher concentrations of *E. coli*, as detected by Colilert-18, are correlated with a higher prevalence of pathogenic *E. coli* genes. Our findings are consistent with previous studies that found higher concentrations of indicator *E. coli* were correlated with more ECVGs on mother hands, and in stored and source water in Tanzania.<sup>37,53</sup> Unlike the Tanzania study, we did not find a significant association between concentration of *E. coli* and *ipaH* in stored water. However, the study in Tanzania reported a higher prevalence of *ipaH* (32%) in stored water compared to 1% in our study. There are several reasons why concentrations of indicator *E. coli* and pathogenic *E. coli* might not be correlated. Studies have identified “naturalized” *E. coli* in tropical environments; if these naturalized strains are nonpathogenic and are a significant contributor to the *E. coli* measured in a sample, then we would not expect a relationship between indicator and pathogenic *E. coli*.<sup>54–57</sup> Also, studies have elucidated physiological differences between strains of *E. coli* sourced from different environments, including pathogenic strains, that impact survival and transport in the environment.<sup>58–60</sup> Thus, it is meaningful that *E. coli* concentrations provided quantitative information on the presence of pathogenic *E. coli* in different household reservoirs, when cultured using IDEXX Colilert-18. It should be noted that we did not measure genes from other bacterial pathogens to explicitly assess the ability of indicator *E. coli* to predict the presence of non-*E. coli* bacterial pathogens; each bacterial pathogen may vary in its transport and survival in the environment.

The bacterial indicator *E. coli* was not a good indicator of norovirus or the protozoan *Giardia*. This could be due to differences in organism structure that dictate fate and survival in the environment.<sup>61</sup> Bacteria, viruses, and protozoa respond differently to environmental conditions such as desiccation or sunlight exposure, and only bacteria have the capacity to grow in the environment and become naturalized.<sup>61,62</sup> With regards to source tracking, the norovirus gene and *E. coli* were not correlated. This may be because norovirus only infects humans, whereas indicator *E. coli* are present in both humans and animal feces.<sup>26,32</sup> However, *Giardia* can infect both humans and animals, making this lack of association notable.<sup>25</sup>

The positive predictive value for the presence of ECVGs increased from 45% (95% CI: 41–49%) using >1 MPN/100 mL as the cutoff for *E. coli* concentration to 65% (58–72%) using >100 MPN/100 mL as the cutoff (SI Table S9). While the likelihood of detecting pathogen genes increased at higher cutoff concentrations of indicator *E. coli*, the number of samples below the cutoff that were positive for pathogen genes also increased (decreased sensitivity, more false negatives). Overall, these findings are consistent with the results of the generalized linear model and suggest that higher concentrations of indicator *E. coli* are associated with an increase in

the presence of pathogenic *E. coli* genes. We demonstrate that indicator *E. coli* is an appropriate indicator of exposure to pathogenic *E. coli* in rural Bangladesh, when cultured using IDEXX Colilert-18. These results are also consistent with previous work that found increased concentrations of indicator *E. coli* in drinking water were associated with increased prevalence of child diarrhea.<sup>63,64</sup> Interestingly, in another WASH Benefits study, the concentration of indicator *E. coli* on child hands, and not in stored water, was associated with diarrheal incidence.<sup>65</sup> It is important to note that the association between indicator *E. coli* in soil and child diarrhea remains unclear, and it is difficult to extend conclusions about relationships found for drinking water and hands to soil, because soil does not undergo treatment (in the case of treated drinking water) or washing (in the case of hands), and contains its own complex microbial community which may include naturalized *E. coli*.

**Human Fecal Marker and Pathogen Genes.** There were no statistically significant relationships between the presence of the human fecal marker, HumM2, and any pathogen genetic marker on child hands, mother hands, or in stored water (SI Figure S2 and Table S10).

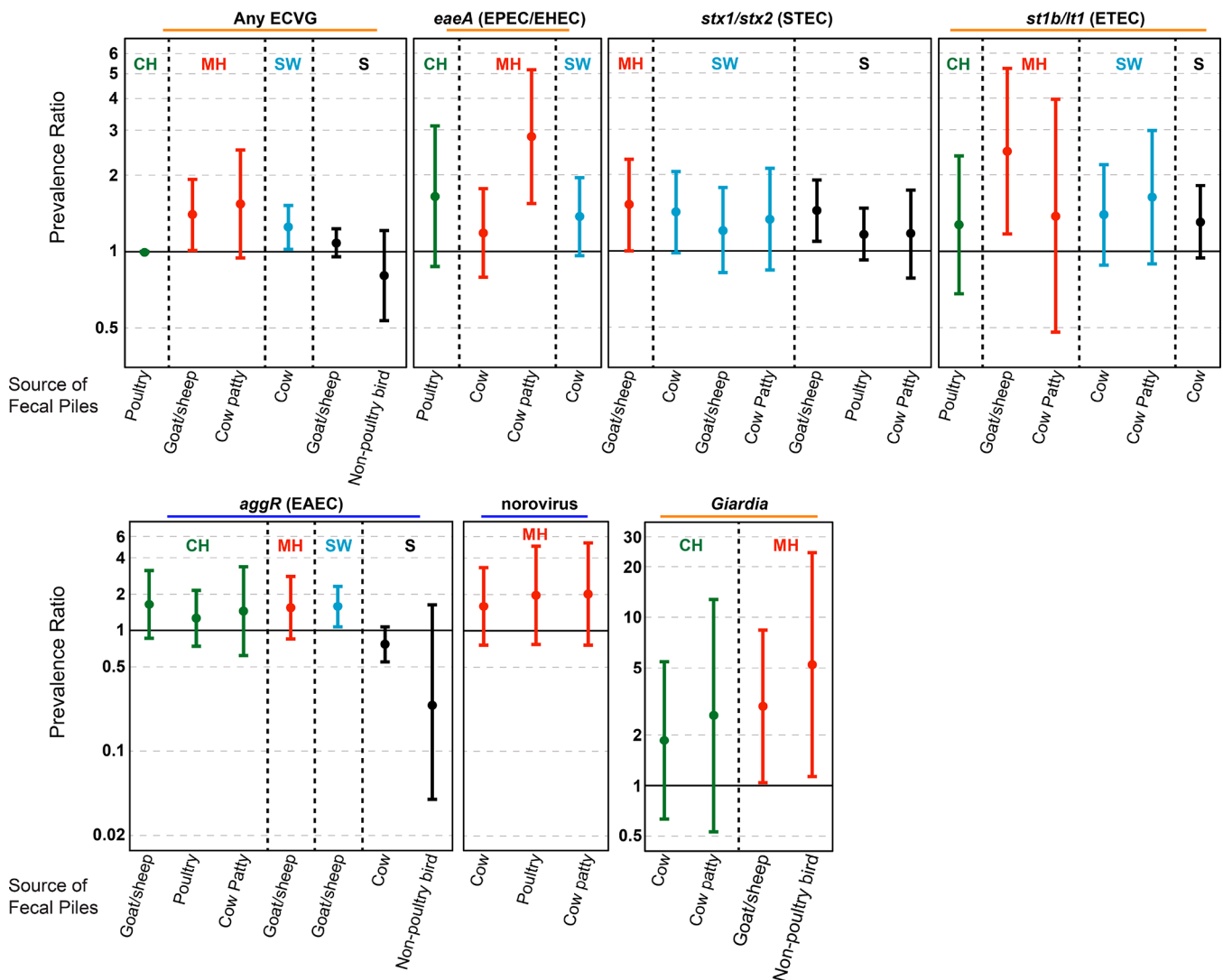
The human marker was not a good predictor of enteric pathogen genes in hand rinses as indicated by the PPV (SI Table S11). On mother and child hands there was only a 28% (95% CI: 18–39%) and 24% (17–32%) chance of observing any ECVG when HumM2 was present. The likelihood of detecting ECVGs when the human fecal marker was present was higher in stored water, 50% (26–75%) and soil, 61% (53–70%). Negative predictive values for many of the ECVGs were highest for mother hands. High NPVs indicate there was a low probability of ECVGs in the absence of HumM2. Therefore, HumM2 could be considered a somewhat conservative indicator of enteric pathogens on mother and child hands in the rural Bangladeshi environment.

There was no association between HumM2 and the norovirus gene. Norovirus infection is specific to human hosts; therefore, we would expect the human marker and norovirus to co-occur. On child hands we observed a prevalence ratio of 1.26 (0.36–4.41,  $p = 0.72$ ) and PPV of 5% (0–10%). However, the number of samples positive for norovirus (4%) was low, leading to an imprecise estimate of prevalence ratio and low PPV. We would need an even larger sample size to adequately test the relationship between HumM2 and norovirus, given the low prevalence. There have been mixed results in previous literature about the association between human-associated microbial source-tracking markers and human-associated viruses. In coastal waters, both a positive association and no association have been reported.<sup>66,67</sup> In our study, it is also possible that we did not observe an association between norovirus and HumM2 due to the low host-specificity of the HumM2 assay (75% specific to human feces) in rural Bangladesh.<sup>36</sup>

**Animal Feces and Pathogen Genes.** There was a statistically significant increase in the prevalence of the *Giardia lamblia* gene (PR: 2.26 (1.50–3.40),  $p < 0.001$ ), any ECVG (PR: 1.37 (1.16–1.62),  $p < 0.001$ ), *eaeA* (PR: 1.63 (1.20–2.22),  $p < 0.001$ ), and *stx1/2* (PR: 1.79 (1.32–2.43),  $p < 0.001$ ) with every  $\log_{10}$  increase in the concentration of the animal fecal marker (BacCow) on mother hands (Figure 2b and SI Table S12).

We observed increases in the prevalence of many pathogen genes with an increase in the number of fecal piles from





**Figure 3.** Adjusted prevalence ratios and 95% confidence intervals indicating the prevalence of norovirus GII, *Giardia lamblia*, and *E. coli* virulence genes in households with fecal piles or cow patties above and below a threshold value. For cow, goat/sheep, and nonpoultry birds, the threshold value was zero. For chicken/nonchicken poultry the threshold value was 5 (>5 versus ≤5). Feces types with a *p*-value < 0.2 in bivariate models between outcomes and exposures were included in the adjusted models (models adjusted for the presence of other feces types in households if these were associated with the outcome pathogen in a bivariate model at *p*-value < 0.2). Pathogens and virulence genes associated with human or human and animal sources are underlined in blue and orange, respectively. Prevalence ratios for EIEC were omitted due to their large confidence intervals. CH: child hands, MH: mother hands, SW: stored water, S: soil. ECVG: *E. coli* virulence gene, EHEC: enterohemorrhagic *E. coli*, EPEC: enteropathogenic *E. coli*, EAEC: enteroaggregative *E. coli*, STEC: Shiga toxin-producing *E. coli*, ETEC: enterotoxigenic *E. coli*, EIEC: enteroinvasive *E. coli*.

animals found in the courtyard of study households (Figure 3 and SI Table S13). However, only the association between the prevalence of *eaeA* on mother’s hands in households with observed cow patties compared to those with no cow patties was significant in the adjusted model and after correcting for multiple comparisons (PR: 2.82 (1.54–5.17), *p* < 0.001). Because *eaeA* is known to occur in *E. coli* from animal hosts, this association is expected. However, it should also be noted that the association between BacCow and *ipaH* in soil (PR:1.5 (1.04–2.16), *p* = 0.03) and between norovirus and increased number of cow feces, chicken feces, and cow patties on mother hands were trending toward significance, and that norovirus and EIEC are human associated, suggesting there may be other factors that impact these relationships.

On mothers’ hands, both higher concentration of animal fecal marker (BacCow) and greater number of feces in

household courtyards were positive predictors of zoonotic pathogen genes. We found the most significant association between BacCow and pathogenic genes *eaeA*, *stx1/2*, and the *Giardia* gene, which indicate the presence of EPEC or EHEC (*eaeA*), STEC (*stx1/2*), and *Giardia lamblia*. EPEC, STEC, and *Giardia lamblia* have been found in animal reservoirs and can be transmitted in the environment through animal feces.<sup>8</sup> In the fecal pile analysis, *eaeA* was most associated with cow patties on mother hands. These findings are consistent with a previous study in Bangladesh that found the presence of animals and animal feces were associated with increased concentrations of indicator *E. coli* in household reservoirs, but the most significant associations were evident in soil, in contrast to mother hands in this study.<sup>32</sup> In urban Kenya, the presence of domestic animals (specifically chicken, cattle, goats, and sheep) was associated with increased pathogen

diversity in public domains but not with increased concentrations of the indicator bacteria, *enterococci*.<sup>49</sup> In our study, the animal molecular marker and the fecal pile survey findings suggest specific sources for these pathogens in the environment. Particularly, cow patties are candidates for the source of EPEC/EHEC, which can ultimately make their way to hands when mothers handle animal feces. While 69% of households reported using tools to clean up feces, 34% reported using their hands, cloth, or scraps for animal feces removal (SI Table S14). Additionally, cow patties are used for cooking fuel which results in caregivers frequently switching between food handling and handling dung patties as they cook.<sup>68</sup> Based on these household practices, it is possible mothers are both picking up animal feces and handling their children, which can provide an opportunity for transmission of zoonotic pathogens to children.

In areas with high fecal contamination, such as rural Bangladesh, it is important to not only measure indicator organisms, but also assess the performance of these indicators to monitor for pathogens. In rural Bangladeshi households, the concentration of culturable *E. coli* was a useful indicator for the presence of pathogenic *E. coli*, despite the potential presence of naturalized *E. coli* which can compound the relationship between indicator *E. coli* and pathogens in environmental reservoirs (SI Figure S3). Conversely, culturable *E. coli* were not an effective indicator for the presence of norovirus or *Giardia*, two nonbacterial pathogens. The human marker (HumM2) was a poor indicator for the presence of pathogen genes, including those known to originate from humans, but these relationships could be impacted by the low host-specificity of HumM2 in the rural Bangladeshi setting. The animal fecal marker (BacCow) was indicative of the presence of animal-associated pathogen genes on mother hands, but not in other household reservoirs. Of the potential sources of zoonotic pathogens, assessed using household surveys, cow patties could be a source of the EPEC/EHEC associated gene (*eaeA*) on mother hands. It is plausible that significant associations between animal fecal contamination and pathogen genes were found on mother hands and not in other reservoirs due to the domestic duties of caretakers such as cleaning household floors. We provide evidence that animals could be important contributors to enteric pathogens in the rural household environment, and therefore, greater animal fecal containment may be needed to reduce the transmission of zoonotic pathogens.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.est.8b07192](https://doi.org/10.1021/acs.est.8b07192).

Details on molecular methods, generalized linear model results, conditional probability results, and a summary figure (PDF)

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## Notes

The authors declare no competing financial interest.

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