Supporting Information for:

Ruminants contribute fecal contamination to the urban household environment in Dhaka, Bangladesh

Authors: Angela R. Harris[1], Amy J. Pickering[2], Michael Harris[3], Solaiman Doza[4], M. Sirajul Islam[4], Leanne Unicomb[4], Stephen Luby[2], Jennifer Davis[1, 2], and Alexandria B. Boehm*[1]

[1]Environmental and Water Studies, Department of Civil and Environmental Engineering, Stanford University, Stanford, CA

[2]Woods Institute for the Environment, Stanford University, Stanford, CA

[3]Emmet Interdisciplinary Program for Environment and Resources, Stanford University, Stanford, CA

[4]icddr,b; Dhaka, Bangladesh

A manuscript for

Environmental Science and Technology

Includes 14 pages, 6 tables, and 2 figures.

Methods

Calculating concentrations of molecular targets

For each assay, a master standard curve was created by combining the standard curves from individual plates[1,2]. The master standard curve was used to calculate concentrations in samples using the sample's mean cycle threshold (C_T). A sample was considered detected within the range of quantification if the sample's mean C_T value corresponded to a concentration between 10^1 copies per µL DNA extract to 10^5 copies per µL DNA extract. If the sample had a mean C_T value that corresponded to less than 10^1 copies per µL DNA extract, then the sample was reported as detected but not quantifiable. If the sample had a concentration above 10^5 copies per µL DNA extract, then the sample was decimally diluted until its concentration was within the range of quantification. If the sample had an undetermined mean C_T value, then the sample was reported as a non-detect. For the fecal samples, if 2 or more of the triplicate reactions were undetermined, the sample was reported as a non-detect. For the environmental samples, if 1 or more of the duplicate reactions was undetermined, the sample was reported as a non-detect.

Data analysis- Binary analysis of microbial source tracking assay performance

The sensitivity and specificity of the assays was also determined using molecular results in a binary, presence/absence format, which is commonly used to report these metrics for PCR assays. A sample was recorded as positive for the molecular target if the target was detected but not quantifiable, or if it was detected within the range of quantification of the assay. A sample was recorded as negative if the molecular target was not detected. The sensitivity (reported as a percentage) was calculated as the number of target animal samples that tested positive for the molecular target (true positive) divided by the total number of target animal samples processed:

$$Sensitivity = \frac{True\ Positive}{True\ Positive + False\ Negative} \quad (1)$$

The specificity (reported as a percentage) was calculated as the number of non-target animal samples correctly identified as negative (true negative) divided by the total number of non-target animal samples:

$$Specificity = \frac{True\ Negative}{True\ Negative + False\ Positive} \quad (2)$$

An assay was considered sensitive and specific if both of these indicators exceeded 80% [3].

Household sample collection and processing: Sample Volumes for Molecular Analysis

Forty floor and 32 hand rinse samples were processed at 50 mL volumes. For 4 floor and 10 hand rinse samples, 100 mL volumes were processed; however, because of high turbidity of the samples, the 50 mL volume was selected for the remaining samples. Two hand samples had high turbidity so 47 mL and 55 mL volumes were processed (50 mL and 100 mL volumes were attempted, respectively).

Fecal Composite Duplicate Processing

The cow, goat, human, and chicken duplicate fecal composites were processed for the following microbial source tracking assays: GenBac3, Avian GFD, BacHum, HF183, BacCow, BacR, and Rum2Bac. The duck duplicate composite was processed for the GenBac3, Avian GFD, and BacCow assays; there was not sufficient DNA extract for the remaining assays. The duplicate fecal composites were processed for each assay listed in duplicate qPCR reactions.

Inhibition tests

To determine if there was any PCR inhibition, a modified 'spike and dilute' method was used.[4] One fecal composite per animal source type was processed to test for inhibition for each microbial source tracking assay, for a total of 5 fecal samples tested for inhibition. Fecal samples were processed at two different 10-fold dilution levels, and each dilution was spiked with a different concentration of standard. Chicken and duck samples were processed undiluted and at a 1:10 dilution, and the undiluted sample was spiked with 10^4 copies per µL standard and the 1:10 diluted sample was spiked with 10^3 copies per µL standard. Cow, goat, and human samples were processed at 1:10 and 1:100 dilutions; these samples were not processed undiluted, because the DNA concentration of the undiluted DNA extract was greater than 100 ng DNA / µL. The 1:10 dilution was spiked with 10^4 copies per µL standard and the 1:100 dilution was spiked with 10^3 copies per µL standard. For the environmental samples, 5 floor samples and 5 hand rinse samples were

processed for inhibition. These samples were tested undiluted and at a 1:10 dilution, and spiked with 10^4 copies per µL standard and 10^3 copies per µL standard, respectively. The samples were processed in duplicate reactions. Samples decimally diluted would have a 3.32 difference in mean C_T values, if the qPCR assay were 100% efficient. For the inhibition tests, a margin of error of 1.3 C_T was allowed, which is within the range of previous studies assessing inhibition with internal amplification controls[4–6]. Therefore, if the difference in mean C_T values between the two dilutions tested was greater than 2.0, the more concentrated sample tested was considered uninhibited.

Results

Blanks, duplicates, and inhibition tests

All laboratory and field blanks processed using the IDEXX assays were negative. All qPCR no-template controls were negative for target detection. Twelve DNA extraction blanks were processed. The extraction blank created when extracting DNA of the cow and goat fecal samples had the GenBac3 and BacR target detected below the range of quantification (10 copies per uL of DNA extract). Because these targets were detected in high concentrations in the cow and goat samples (Figure 1 and 2), the contamination was negligible. Eleven field blanks and 12 lab blanks were processed for molecular analysis in the field lab. All of these blanks had the GenBac3 target detected below the range of quantification. All fecal composites and environmental samples (except for 2 floor samples) had the GenBac3 target detected within the range of quantification; therefore, the cross-contamination was negligible. Concentrations of *E. coli* and enterococci detected in the duplicate fecal composites were within the same order of magnitude as the original sample. Concentrations of the microbial source tracking molecular targets in the duplicates were also within the same order of magnitude as the original sample, except the cow duplicate processed for the HF183 assay. For the original cow sample, the HF183 target was detected but not quantifiable, and for the duplicate cow sample, the HF183 target was not detected.

Based on the inhibition tests, dilutions of fecal DNA extracts with no observed inhibition were chosen to run for each microbial source tracking assay (Table S4). When the

molecular target was detected above the range of quantification, further dilution was necessary. Environmental samples were processed undiluted, as only 1 of 5 tested floor samples and 0 of 5 tested hand rinse samples showed signs of inhibition.

Concentrations of fecal indicator bacteria in fecal samples

Log_{10}-mean concentrations of the EC, ENT, and GenBac3 in fecal sources were compared using one-way ANOVA Tukey's post-hoc test (Table 1). The avian fecal composites have lower GenBac3 concentrations compared to the ruminant and human fecal composites ($p<0.05$ for all) by an average of 2.1 log_{10} units. EC concentrations in feces from different host species are similar, except chicken fecal composites have higher concentrations than cow fecal composites ($p<0.05$) by 1.7 log_{10} units, on average. Cow fecal composites have lower concentrations of ENT than other host composites ($p<0.05$) by 2.9 log_{10} units, and goat fecal composites have lower concentrations of ENT than chicken fecal composites ($p<0.05$) by 2.0 log_{10} units.

Detection of microbial source tracking targets in fecal samples- binary analysis

The sensitivity and specificity of each microbial source tracking assay was also computed using Equations 1 and 2, which considers the target 'detected but not quantifiable' as a positive result (Table S6). Assays with a sensitivity of 80% or greater include BacHum, Avian GFD, Rum2Bac, BacR, and BacCow. Only Rum2Bac and BacR had both a sensitivity and a specificity of 80% or greater. Because the BacR target was detected in higher concentrations in fecal material from ruminants than the Rum2bac target, we chose to use the BacR assay on the household environmental samples collected. As the avian GFD assay did not satisfy either the binary or quantitative specificity metric, it was not used on the environmental samples. Similarly, given the low sensitivity and specificity of the human assays, these were not used for the environmental samples.

Table S1 Reported by animal source type, the number of donors feces was collected from, the number of individual specimens included in each composite, and the number of composites processed.

FECAL SAMPLES	No. of donors	No. of donors per composite	No. of composites
Chicken	20	4	5
Cow	20	4	5
Duck	15	3	5
Goat	20	4	5
Human	15	3	5

Table S2 Number and type of household samples collected for microbiological analysis.

DESCRIPTION	N
Households with child hand rinse sample only	15
Households with floor sponge sample only	15
Households with both hand rinse and floor sponge samples	29
Total number of households visited	59

Table S3 Methods for microbial source tracking assays used in this study. Includes target fecal host, mastermix, BSA concentration, standard material, and reference for the method protocol. ABI is Applied Biosystems (Carlsbad, CA).

Assay	Target fecal host	Master Mix	BSA concentration (mg/mL)	Standard	Reference
				Bacteroidales thetaiotaomicron	
GenBac3	General	ABI Universal	0.2	gDNA	Siefring et al.[7]
BacR	Ruminant	ABI Universal	0.2	circular plasmid[a]	Reischer et al.[8]
Rum2bac	Ruminant	ABI Universal	0.4	circular plasmid[a]	Mieszkin et al.[9]
Baccow	Ruminant	ABI Environmental	0.05	circular plasmid[b]	Kildare et al.[10]
HF183 Taqman	Human	ABI Universal	0.2	*Bacteroidales dorei* gDNA	Haughland et al.[11]
HumM2	Human	ABI Universal	0.2	circular plasmid[a]	Shanks et al.[12]
BacHum	Human	ABI Universal	0.05	circular plasmid[b]	Kildare et al.[10]
Avian GFD	Avian	ABI SYBR Green	0.04	circular plasmid[a]	Green et al.[13]

[a]Synthetic plasmid from Integrated DNA Technologies (IDT, Coralville, Iowa, US).
[b]Plasmid extracted from *E. coli* transformed with the target plasmid vector using Qiagen Miniprep kit (Valencia, CA, USA).

Table S4 Dilutions of DNA extract by fecal source type processed for each microbial source tracking assay. The DNA extract was either processed undiluted or decimally diluted 1:10 or 1:100.

Assay	Chicken	Duck	Cow	Goat	Human
GenBac3	undiluted	undiluted	1:100 dilution	1:100 dilution	1:100 dilution
BacR	undiluted	undiluted	1:100 dilution	1:100 dilution	1:10 dilution
Rum2bac	undiluted	undiluted	1:100 dilution	1:100 dilution	1:10 dilution
Baccow	undiluted	undiluted	1:100 dilution	1:100 dilution	1:10 dilution
HF183 taqman	undiluted	undiluted	1:10 dilution	1:10 dilution	1:10 dilution
HumM2	undiluted	undiluted	1:10 dilution	1:10 dilution	1:10 dilution
BacHum	undiluted	undiluted	1:10 dilution	1:10 dilution	1:10 dilution
Avian GFD	undiluted	undiluted	1:10 dilution	1:10 dilution	1:10 dilution

Table S5 Sensitivity and specificity of the microbial source tracking assays as concluded from the binary analysis, and whether assay is deemed sensitive or specific based on the binary and quantitative metrics.

Assay	Binary analysis		Binary metric		Quantitative Metric	
	Sensitivity	Specificity	Sensitive	Specific	Sensitive	Specific
HumM2	40%	55%	no	no	no	no
HF183 Taqman	60%	40%	no	no	no	no
BacHum	80%	40%	yes	no	no	no
Rum2Bac	100%	100%	yes	yes	yes	yes
BacR	100%	100%	yes	yes	yes	yes
BacCow	100%	33%	yes	no	yes	yes
Avian GFD	100%	33%	yes	no	no	no

Table S6 Study household (n=59) characteristics.

Household Characteristic	N	Percent
Drinking water source located inside compound	50	85%
Always treat drinking water by boiling	29	49%
Primary toilet located inside compound	53	90%
Primary toilet shared with other households	52	88%
Animals kept within household's compound	15	25%
Floor in child’s sleep area made of concrete	53	90%
Handwashing station with soap and water observed	33	56%

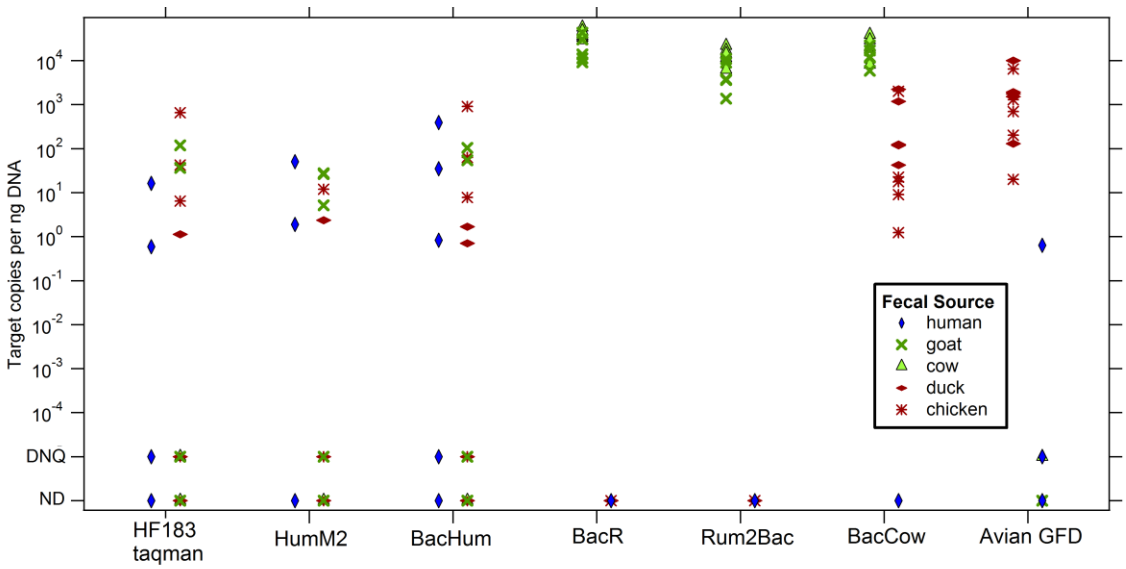

Figure S1 Concentrations of microbial source tracking assay target copies per ng DNA in fecal material from humans, goats, cows, ducks, and chickens. The target source of the microbial source tracking assay is aligned to the left of the assay tick, and non-target sources are aligned to the right of the assay tick. Samples in which the microbial source tracking target was detected but not quantified (DNQ), or in which the target was not detected (ND), are plotted at the bottom of the figure.

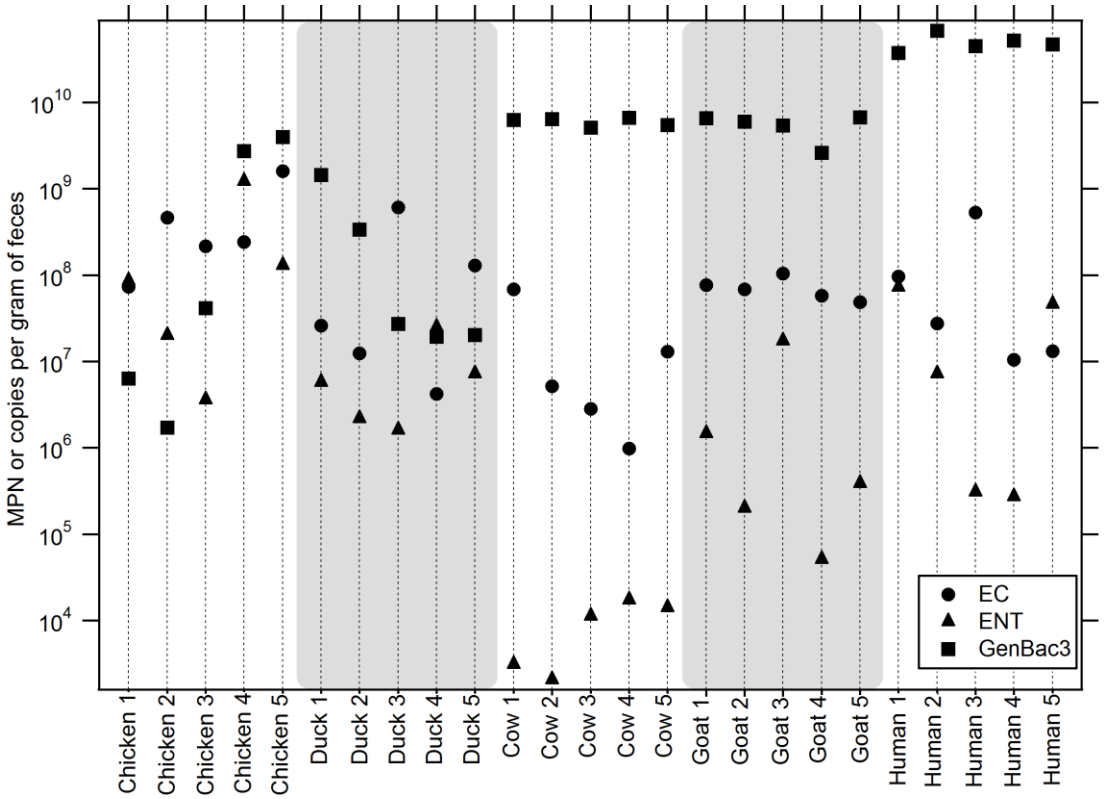

Figure S2 Concentrations of general fecal indicators per gram of wet-weight feces by fecal composite. EC and ENT concentrations are measured in MPN per g wet weight by defined-substrate assay. GenBac3 concentrations are measured by qPCR in units of 16S rRNA gene copies per g wet weight.

References

(1) Bell, A.; Layton, A. C.; McKay, L.; Williams, D.; Gentry, R.; Sayler, G. S. Factors influencing the persistence of fecal Bacteroides in stream water. *Journal of Environmental Quality.* **2009**, *38* (3), 1224–1232.

(2) Ebentier, D. L.; Hanley, K. T.; Cao, Y.; Badgley, B. D.; Boehm, A. B.; Ervin, J. S.; Goodwin, K. D.; Gourmelon, M.; Griffith, J. F.; Holden, P. a; et al. Evaluation of the repeatability and reproducibility of a suite of qPCR-based microbial source tracking methods. *Water Research.* **2013**, *47* (18), 6839–6848.

(3) Boehm, A. B.; Van De Werfhorst, L. C.; Griffith, J. F.; Holden, P. a; Jay, J. a; Shanks, O. C.; Wang, D.; Weisberg, S. B. Performance of forty-one microbial source tracking methods: a twenty-seven lab evaluation study. *Water Research.* **2013**, *47* (18), 6812–6828.

(4) Cao, Y.; Griffith, J. F.; Dorevitch, S.; Weisberg, S. B. Effectiveness of qPCR permutations, internal controls and dilution as means for minimizing the impact of inhibition while measuring Enterococcus in environmental waters. *Journal of Applied Microbiology.* **2012**, *113* (1), 66–75.

(5) Haugland, R. a; Siefring, S. C.; Wymer, L. J.; Brenner, K. P.; Dufour, A. P. Comparison of Enterococcus measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. *Water Research.* **2005**, *39* (4), 559–568.

(6) Griffith, J. F.; Weisberg, S. B. Challenges in implementing new technology for beach water quality monitoring : Lessons from a California demonstration project. *Marine Technology Society Journal.* **2011**, *45*, 65–73.

(7) Siefring, S.; Varma, M.; Atikovic, E.; Wymer, L.; Haugland, R. A. Improved real-time PCR assays for the detection of fecal indicator bacteria in surface waters with different instrument and reagent systems. *Journal of Water and Health.* **2008**, *6* Mieszkin, S.; Yala, F.; Joubrel, R.; Gourmelon, M. Phylogenetic analysis of Bacteroidales 16S rRNA gene sequences from human and animal effluents and assessment of ruminant faecal pollution by real-time PCR. *Journal of Applied Microbiology.* **2010**, *108* (3), 974–984. (2), 225–237.

(8) Reischer, G. H.; Kasper, D. C.; Steinborn, R.; Mach, R. L.; Farnleitner, A. H. Quantitative PCR method for sensitive detection of ruminant fecal pollution in freshwater and evaluation of this method in alpine karstic regions. *Applied and Environmental Microbiology.* **2006**, *72* (8), 5610–5614.

(9) Mieszkin, S.; Yala, F.; Joubrel, R.; Gourmelon, M. Phylogenetic analysis of Bacteroidales 16S rRNA gene sequences from human and animal effluents and assessment of ruminant faecal pollution by real-time PCR. *Journal of Applied Microbiology.* **2010**, *108* (3), 974–984.

(10) Kildare, B. J.; Leutenegger, C. M.; McSwain, B. S.; Bambic, D. G.; Rajal, V. B.; Wuertz, S. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: A Bayesian approach. *Water Research.* **2007**, *41* (16), 3701–3715.

(11) Haugland, R. A.; Varma, M.; Sivaganesan, M.; Kelty, C.; Peed, L.; Shanks, O. C. Evaluation of genetic markers from the 16S rRNA gene V2 region for use in quantitative detection of selected Bacteroidales species and human fecal waste by qPCR. *Systematic and Applied Microbiology.* **2010**, *33* (6), 348–357.

(12) Shanks, O. C.; Kelty, C. A.; Sivaganesan, M.; Varma, M.; Haugland, R. A. Quantitative PCR for genetic markers of human fecal pollution. *Applied and Environmental Microbiology.* **2009**, *75* (17), 5507–5513.

(13) Green, H. C.; Dick, L. K.; Gilpin, B.; Samadpour, M.; Field, K. G. Genetic markers for rapid PCR-based identification of gull, Canada goose, duck, and chicken fecal contamination in water. *Applied and Environmental Microbiology.* **2012**, *78* (2), 503–510.