SUPPLEMENTARY INFORMATION

Elevated fecal mitochondrial DNA from symptomatic norovirus infections suggests potential health relevance of human mitochondrial DNA in fecal source tracking

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O Before norovirus challenge × Peak of norovirus shedding

Figure S1. hCYTB484 and HF183/BacR287 counts in samples taken from US norovirus challenge study comparing fecal samples taken before the norovirus challenge to fecal samples taken during the peak of norovirus shedding for individuals who became symptomatic or remained asymptomatic for norovirus. Counts of both markers are normalized to ng of dsDNA as determined by Qubit and reported as log_{10} (concentration + 1).

Table S1. Comparisons between biological replicates for hCYTB484 and HF183/BacR287 markers showing a mean percent difference of 24% for hCYTB484 and 37% for HF183/BacR287.

Sample	hCYTB484 Biological Replicate 1 [copies / ng of dsDNA]	hCYTB484 Biological Replicate 2 [copies / ng of dsDNA]	hCYTB484HF183/BacR2Percent87 BiologicalDifferenceReplicate 1[%][copies / ng of dsDNA]		HF183/BacR2 87 Biological Replicate 2 [copies / ng of dsDNA]	HF183/BacR2 87 Percent Difference [%]
1	4.6E+02	2.4E+02	48	DBNQ	DBNQ	Not applicable
2	2.5E+02	2.7E+02	-9	DBNQ DBNQ		Not applicable
3	6.8E+02	5.7E+02	17	17 DBNQ		Not applicable
4	7.6E+02	5.7E+02	24	4.0E+01 2.4E+01		40
5	6.8E+02	6.1E+02	10	2.2E+03 8.9E+		60
6	5.9E+02	8.4E+02	-43	DBNQ DBNQ		Not applicable
7	1.8E+03	1.2E+03	32	2.2E+03 2.6E		-17
8	2.6E+03	1.5E+03	42	No Amplification	No Amplification	Not applicable
9	6.0E+03	1.9E+03	67	DBNQ	5.3E-01	Not applicable
10	3.8E+03	2.1E+03	46	1.8E+00 5.9E+00		-230
11	2.0E+03	2.1E+03	-6	Amplification No below aLoD Amplific		Not applicable
12	3.9E+03	2.2E+03	42	42 Amplification below aLoD		Not applicable
13	6.3E+03	3.7E+03	41	41 Amplification Ampl below aLoD belo		Not applicable
14	5.5E+03	4.7E+03	14	Amplification below aLoD	No Amplification	Not applicable
15	1.4E+04	1.0E+04	28	No Amplification	No Amplification	Not applicable
Mean			24 (range: -9 to 67)			-37 (range: - 230 to 60)

Table S2. Minimum Information for Publication of Quantitative Digital PCR Experiments (dPCR MIQE) for the ddPCR materials and methods used in this study.

ITEM TO CHECK	COMMENT			
1. SPECIMEN				
Detailed description of specimen type and numbers	We extracted DNA from archived samples collected in previous studies. Detailed descriptions of specimens can be found elsewhere ¹ ³ . Number of samples used in this study can be found in Table 1 of the main text.			
Sampling procedure (including time to storage)	Detailed descriptions of sampling procedures can be found elsewhere ^{1–3} .			
Sample aliquotation, storage conditions and duration	All samples were archived at -80°C before DNA extraction for this study.			
2. NUCLEIC ACID EXTRACTION				
Description of extraction method including amount of sample processed	We used the MO BIO PowerSoil® kit (Carlsbad, CA, USA) DNA extraction kit for the US samples and the Qiagen QIAamp® 96 PowerFecal QIAcube® HT Kit automated on the Qiagen QIAcube® HT platform (Hilden, Germany) for the Mozambiquan and Bangladeshi samples.			
Volume of solvent used to elute/resuspend extract	We eluted each sample using 100 µL of elution buffer: Buffer EB for PowerSoil kit and Qiagen Solution C6 for PowerFecal.			
Number of extraction replicates	We extracted $\sim 7\%$ (n = 15) of the samples in duplicate. See Table S1 for comparisons of extraction replicates.			
Extraction blanks included?	At least one extraction blank was done with each batch of extractions. No extraction blanks were above the analytical limit of detection.			
3. NUCLEIC ACID ASSESSMENT AND STORAGE				
Method to evaluate quality of nucleic acids	We did not assess the quality of nucleic acids.			
Method to evaluate quantity of nucleic acids (including molecular weight and calculations when using mass)	We used a Qubit 3 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA) with Qubit dsDNA HS Assay Kits to quantify the yield of dsDNA (ng of dsDNA) in each sample.			
Storage conditions: temperature, concentration, duration, buffer, aliquots	We stored eluted DNA in Buffer ATE (for the PowerFecal extracts) and Buffer C6 (for the PowerSoil extracts) in both diluted and undiluted aliquots for up to 2 years before analyzing on ddPCR at - 80°C.			
Clear description of dilution steps used to prepare working DNA solution	To make any dilutions, we UV-treated microcentrifuge tubes for 20 minutes and made 1 in 10 dilutions using 5 μ L of extract and 45 μ L of the respective elution buffer (Buffer EB or Solution C6).			

4. NUCLEIC ACID MODIFICATION				
Template modification (digestion, sonication, pre-amplification, bisulphite etc.)	We did not perform template modification in this study.			
Details of repurification following modification if performed	We did not perform any repurification following DNA extraction.			
5. REVERSE TRANSCRIPTION				
cDNA priming method and concentration				
One or two step protocol (include reaction details for two step)				
Amount of RNA added per reaction				
Detailed reaction components and conditions	We did not perform reverse transcription in this study.			
Estimated copies measured with and without addition of RT*				
Manufacturer of reagents used with catalogue and lot numbers				
Storage of cDNA: temperature, concentration, duration, buffer and aliquots				
6. dPCR OLIGONUCLEOTIDES DESIGN AND TARGET INFORMATION				
Sequence accession number or official gene symbol	The two assays we used in this study were: (1) hCYTB484 targeting a human-specific region of the cytochrome <i>b</i> gene within the mitchondrial genome and (2) HF183/BacR287 targeting human- associated members of the <i>Bacteroides</i> genus.			
Method (software) used for design and <i>in silico</i> verification	We used NCBI BLAST to design and verify in silico ⁴ .			
Location of amplicon	The hCYTB484 amplicon begins at the 484 bp position of the cytochrome b gene of the revised Cambridge Reference Sequence (reference human mtDNA genome). The HF183/BacR287 amplicon begins at the 180 bp position partial 16S rRNA gene sequence of Bacteroides dorei (accession number AB242143).			
Amplicon length	The hCYTB484 assay has a length of 121 bp. The HF183/BacR287 assay has a length of 126 bp.			

	[5' 3']			
	hCVTB604P. CCTCCAACAATACCACCTC			
Primer and probe sequences (or	hCVTB520TM· ACCCTCACACGATTCTTTACCTTTCACT			
amplicon context sequences (of	HE182. ATCATGACTTCACATCTCCC			
amplicon context sequence).	DeeD297: ATCATCACTTCACATCTCCC			
	DacK20/: ATCATCACTTCACATOTCCO			
	Dack254: ATCATGAGTTCACATGTCCG			
	Back234IAC: AICAIGAGIICACAIGICCG			
	The hCY IB484 assay had a Zen quencher (Integrated DNA			
	Technologies, Coraville, IA, USA): 5'-/56-FAM/ACC CTC ACA			
Location and identity of any	/ZEN/CGA TIC TIT ACC TIT CAC T/3IABkFQ/-3. The			
modifications	HF183/BacR28/ assay had two MGB probes: BacP234MGB: FAM-			
	CTAATGGAACGCATCCC-MGBNFQ and BacP234IAC: HEX-			
	AACACGCCGTTGCTACA-MGBNFQ			
	All primers and probes used in this study were manufactured by IDT			
Manufacturer of oligonucleotides	(Coraville, IA, USA) except for the MGB probes used in			
internet of ongoine controls	HF183/BacR287. MGB probes were manufactured by Applied			
	Biosystems (Waltham, MA, USA).			
7. dPCR PROTOCOL				
Manufacturer of dDCD instrument and	We used the QX200 droplet digital PCR platform manufactured by			
instrument model	Bio-Rad (Hercules, CA, US).			
Instrument model				
	We used BioRad's ddPCR Supermix for Probes (No dUTP) (cat. No.			
Buller/kit manufacturer with catalogue	1863024). We did not record buffer lot numbers for this study.			
and lot number				
	For hCTYB484: primers at 900 nM and probe at 250 nM. For			
Primer and probe concentration	HF183/BacR287: primers at 1000 nM and probe at 250 nM.			
Dra magning volume and some site	The pre-reaction total volume (before droplet generation) was 22 μ L			
Pre-reaction volume and composition	and composed of 4.99 µL of molecular grade H ₂ O, 11 µL of BioRad			
(incl. amount of template and if	ddPCR Supermix for Probes (no dUTP), 0.055 µL of probe, 1.98 µL			
restriction enzyme added)	of primers, and 2 μ L of template. We did not add restriction enzymes.			
	We did not do any treatments of the template.			
Template treatment (initial heating or				
chemical denaturation)				
	The concentration of divalent cations was 3.8 mM, and the			
Polymerase identity and concentration,	concentration of dNTPs was 0.8 mM.			
Mg++ and dNTP concentrations***				
	We used 10 min at 95 °C, followed by 40 cycles of 30 s at 95 °C and			
	60 s at the assay-specific annealing temperature (annealing			
Complete thermocycling parameters	temperature of 58 °C for HF183/BacR287 and 59 °C for			
	hCYTB484), followed by a 10-min hold at 98 °C. All ramp rates set			
	at 2 °C/s.			

8. ASSAY VALIDATION	
Details of optimisation performed	For each assay, we conducted a series of experiments to optimize the annealing temperature. First, we ran a temperature gradient spanning approximately 8°C; then, we ran a finer scale temperature gradient (spanning approximately 2°C) by identifying the highest temperatures in which the separation between negative and positive bands reached a limit in the previous gradient. We selected an annealing temperature from the finer scale gradient that gave us the most separation while remaining a relatively high temperature to avoid non-specific amplification. We also experimented with 94°C, 95°C, and 96°C denaturation cycles, finding that 95°C provided the best separation between positive and negative partition signals for the assays used in this study.
Analytical specificity (vs. related sequences) and limit of blank (LOB)	Analytical specificity: 97% for hCYTB484 and 80% for HF183/BacR287 ⁴ . We did not use a limit of blank but instead treated amplification under the analytical limit of detection as "amplification below the analytical limit of detection." We considered amplification below the analytical limit of detection as not detected.
Analytical sensitivity/LoD and how this was evaluated	Analytical sensitivity: 100% for hCYTB484 and 51% for HF183/BacR287. This was evaluated by testing 22 cow, 34 pig, 8 chicken, 22 goat, and 222 human feces samples ⁴ .
Testing for inhibitors (from biological matrix/extraction)	We tested for inhibition using the internal amplification control as described in Green et al. 2014 ⁵ .
9. DATA ANALYSIS	
Description of dPCR experimental design	Detailed description of the dPCR methods can be found in Zhu et al. 2020 ⁴ .
Comprehensive details negative and positive of controls (whether applied for QC or for estimation of error)	We ran at least two wells of UV-treated (for at least 15 minutes) molecular grade water as our negative controls for each plate. For positive controls, we used
Partition classification method (thresholding)	Based on previous experience with partitions with intermediate fluorescence, we adopted a moderate approach to partition thresholding. Our method starts with the histogram of the partition fluorescence of each entire plate run. We select the amplitude value for the peak of both the negative and positive bands. We determine the negative and positive bands by comparing with the no template controls and positive controls run on each plate. We then find the midpoint value between the peaks of the positive and negative partition bands and set the threshold at the midpoint value. We argue that this approach has value in fecal source tracking (FST) due to possibilities of detecting degraded target in environmental samples as well as close but not exact sequence matches.

	Ch1 Pos:4019 Neg:29940 Channel 1 Histogram				
Examples of positive and negative experimental results (including fluorescence plots in supplemental material)	20000 D12 G12 G12 G12 G12 G12 G12 G12 G12 G12 G				
Description of	We performed technical replicates for 25% of the samples.				
technical replication					
Repeatability (intra- experiment variation)	Coefficient of variation data can be found in Zhu et al. 2020 ⁴ .				
Reproducibility (inter- experiment/user/lab etc. variation)	No evaluation of user or lab variability: only one user analyzed the samples in only one lab.				
Number of partitions measured (average and standard deviation)	Mean = 14,113 partitions and standard deviation of 1,665 partitions				
Partition volume	We did not measure partition volume; instead, we used the assumed volume of 0.85 nL set in Bio-Rad QuantaSoft Version 1.7.4.0917.				
Copies per partition (λ or equivalent) (average and standard deviation)	Mean $\lambda = 0.37$. Standard deviation of $\lambda = 0.81$.				
dPCR analysis program (source, version)	Bio-Rad QuantaSoft (Version 1.7.4.0917)				
Description of normalisation method	We normalized to DNA yield by (1) calculating marker copies per μ L of extract, (2) quantifying DNA yield per μ L of extract via Qubit 3.0 Fluorometer with Qubit High Sensitivity DNA kits (ThermoFisher Scientific, Waltham, MA, USA), and (3) dividing marker copies per μ L of extract by ng of dsDNA per μ L of extract to obtain marker copies per ng of dsDNA.				
Statistical methods used for analysis	We applied the Wilcoxon signed rank paired test to the pre- and post-challenge US samples. For the Mozambique and Bangladesh sample sets (cross-sectional data), w used the Kruskal-Wallis test, followed with the Dunn test with Benjamini-Hochberg adjustment. We calculated effect sizes for log10 transformed concentrations through				

	difference in means approach using Cohen's d, the difference between the two means
	divided by the pooled standard deviation. To compare the relative influences of potential
	confounders, we fitted a generalized linear model (GLM) using a Gaussian identity
	function to the Mozambique and Bangladesh sample sets using reported diarrhea and
	norovirus GI/GII detected/not detected (as determined by the GPP) as the independent
	variables and log10 values of hCYTB484 normalized to ng of dsDNA as the dependent
	variable while adjusting for number of pathogens detected (as determined by the GPP),
	sex, age (continuous, number of months), and study population (Mozambique or
	Bangladesh).
	ddPCR results are available in supplementary info as an Excel file.
Data transparency	

Generalized Linear Model. We fit a generalized linear model (GLM) to the log₁₀ transformed copies of hCYTB484 normalized to ng of dsDNA using the glm() function in R version 4.0.1. We made the decision to log₁₀ transform hCYTB484 concentrations based off of Box-Cox transformation tests. We fit the GLM to only data from the Mozambiquan and Bangladeshi samples due to the differences in environment (US as a high-income country) and study population (children versus adults) when compared with the US study. Regression coefficient information is shown in Table S3. To assess the fit of the GLM, we plotted the residuals of the GLM using a normal quantile-quantile plot (Figure S2) to assess if the residuals were normally distributed as well as plotted the residuals versus predicted values to assess if the residuals were of constant variance (Figure S3).



Figure S2. A normal quantile-quantile plot of the residuals of the GLM showing linearity, demonstrating that the residuals are normally distributed. Normally distributed residuals of the GLM support the appropriateness of the GLM fit to the data.



Figure S3. A residual by predicted values plot showing that the residuals have approximately constant variance (as indicated by the roughly constant horizontal band pattern as opposed to any curvature pattern), supporting the appropriateness of the GLM fit to the data.

Table S3. Coefficients of the generalized linear model (GLM) fitted using only the Bangladesh and Mozambique samples. We fitted the GLM using reported diarrhea and norovirus GI/GII detected/not detected as the independent variables and log10(hCYTB484 copies / ng of dsDNA) as the dependent variable while adjusting for sex, age, and study population.

	Intercept	Diarrhea	Norovirus (Detected / Not detected)	Number of GI Infections (GPP)	Sex (Male / Female)	Age (months)	Study Population (Bangladesh / Mozambique)
Estimate	3.4	0.38	0.15	0.0084	-0.082	-0.0052	-0.26
Std. Error	0.13	0.19	0.18	0.050	0.093	0.0033	0.11
95% Confidence Interval Range	3.14 – 3.66	0-0.76	-0.21 - 0.51	-0.092 – 0.011	-0.27 – 0.10	-0.012 - 0.0014	-0.480.04
<i>p</i> -value	<2e-16	0.045	0.38	0.87	0.38	0.12	0.018
Standardized Regression Coefficient	N/A	0.16	0.12	0.023	-0.064	-0.12	0.19
95% Confidence Interval Range for Standardized Regression Coefficients	N/A	0-0.32	-0.16 - 0.39	-0.25 – 0.03	-0.21	-0.28 – 0.03	-0.360.03

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