Validation of *Bacteroidales* quantitative PCR assays targeting human and animal fecal contamination in the public and domestic domains in India

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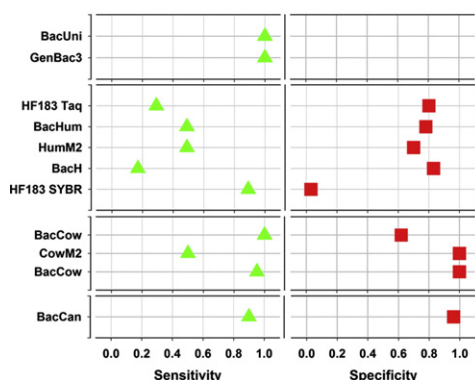[d] Departments of Epidemiology and Pediatrics, Center for Global Health and Development, College of Public Health, University of Nebraska Medical Center, Omaha, NE 68198, USA
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HIGHLIGHTS

- First report of *Bacteroidales* qPCR assay validation in India
- Ten qPCR assays evaluated with fecal samples from human and animal sources in India
- BacHum best of five human-associated assays tested on Indian fecal samples
- BacUni, BacHum, HumM2, BacCan and BacCow recommended for future MST use in India

GRAPHICAL ABSTRACT

ABSTRACT

We compared host-associated *Bacteroidales* qPCR assays developed in the continental United States and Europe for the purpose of measuring the effect of improved sanitation on human fecal exposure in rural Indian communities where both human and animal fecal loading are high. Ten candidate *Bacteroidales* qPCR assays were tested against fecal samples (human, sewage, cow, buffalo, goat, sheep, dog and chicken) from a test set of 30 individual human, 5 sewage, and 60 pooled animal samples collected in coastal Odisha, India. The two universal/general *Bacteroidales* assays tested (BacUni, GenBac3) performed equally well, achieving 100% sensitivity on the test set. Across the five human-associated assays tested (HF183 Taqman, BacHum, HumM2, BacH, HF183 SYBR), we found low sensitivity (17 to 49%) except for HF183 SYBR (89%), and moderate to high cross-reactivity with dog (20 to 80%) and chicken fecal samples (60 to 100%). BacHum had the highest accuracy (67%), amplified all sewage samples within the range of quantification (ROQ), and did not cross-react with any fecal samples from cows, the most populous livestock animal in India. Of the ruminant- and cattle-associated assays tested (BacCow, CowM2), BacCow was more sensitive in detecting the full range of common Indian livestock animal fecal sources, while CowM2 only detected cow sources with 50% sensitivity. Neither assay cross-reacted with human sources.

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BacCan, the dog-associated assay tested, showed no cross-reactivity with human sources, and high sensitivity (90%) for dog fecal samples. Overall, our results indicate BacUni, BacHum, HumM2, BacCan and BacCow would be the most suitable MST assays to distinguish and quantify relative amounts of human-associated and livestock/domestic animal-associated contributions to fecal contamination in Odisha, India.

1. Introduction

Microbial contamination from human fecal matter is a leading cause of preventable infectious disease, disability, and death in communities lacking sanitation and good hygiene (Wagner and Lanoix, 1958). In India, where 626 million mostly rural people practice open defecation (WHO/UNICEF, 2012) and 535,000 children under age five die each year from diarrhea (Boschi-Pinto et al., 2008), the situation is of particular concern. Adequate human sanitation is clearly necessary to prevent environmental contamination and reduce exposure to diarrhea and other excreta-related human pathogens, for example, soil-transmitted helminths. Yet questions remain about the effectiveness of simple on-site household sanitation facilities, such as pit latrines, in reducing contamination and diarrhea diseases on their own, as well as the level of community coverage necessary to achieve impacts (Clasen et al., 2012).

As part of a large study in Coastal Odisha, India on the effectiveness of providing household latrines to reduce diarrhea and helminth infections in rural communities (Clasen et al., 2012), changes in human fecal contamination and exposure in study communities are being examined. To assess exposures, fecal–oral pathways need to be considered in both the public domain (for example, local surface and groundwater sources located in public places and used for drinking, washing, and bathing by a number of people in the community) and domestic domain (for example, on hands and in stored drinking water in the home usually under the control of a household) of disease transmission, for which contamination sources and risks may differ (Cairncross et al., 1996). Furthermore, as is true across rural India, households in study communities own livestock with which they share public and private spaces and animal sanitation is poor. Cattle are among the most commonly owned species, unsurprising given their important role as a source of nutrition, fuel, transport and wealth for many rural Indian households (Tipathy, 2001). Traditional fecal indicator bacteria (FIB) such as total coliforms, thermotolerant coliforms, *Escherichia coli*, and members of the genus *Enterococcus* (the enterococci), are widely used to assess fecal contamination in the environment, but originate from both humans and animals (Leclerc et al., 2001). Thus, in order to assess changes in human fecal exposure attributable to improved human sanitation and understand its health impacts in communities with poor animal sanitation, new tools are needed that can distinguish human from non-human animal fecal contamination in study settings.

Microbial source tracking (MST) is an emerging approach to discriminate and quantify human and other animal fecal contamination sources in the environment. Among a variety of proposed MST techniques, host-associated *Bacteroidales* genetic markers are increasingly used as a complement or alternative to standard FIB in the developed world. Members of the order *Bacteroidales* are strictly anaerobic and highly abundant bacteria in human and other animal intestines and feces (Paster et al., 1994). Furthermore, *Bacteroidales* populations adapt to their hosts differently, allowing identification of host sources of fecal contamination using host-associated genetic markers (Bernhard and Field, 2000a,b). Recent studies have shown the relevance of MST using *Bacteroidales* genetic markers to identify fecal sources and assist in targeting public health interventions in less developed countries, including Kenya (Jenkins et al., 2009), Tanzania (Pickering et al., 2011, 2012; Mattioli et al., 2012) and Bangladesh (Ahmed et al., 2010; Ferguson et al., 2012; Knappett et al., 2011). To our knowledge, however, no MST *Bacteroidales* assays have been applied to address fecal pollution problems in India. Recently, performance of several human and non-human targeting MST assays was

thoroughly assessed in a large-scale multi-laboratory MST method comparison study (Boehm et al., 2013). Yet applicability of the results to other regions and countries is potentially limited as all fecal samples were collected in California (Boehm et al., 2013). Because geographical differences significantly affect sensitivity and specificity of host-associated *Bacteroidales* assays (Gawler et al., 2007; Balleste et al., 2010; Ahmed et al., 2009; Jenkins et al., 2009; Tambalo et al., 2012b; Reischer et al., 2013), performance assessment is necessary prior to application of these assays in a new region of interest such as India.

The primary objectives of this research were, therefore, (1) to evaluate the performance of candidate assays for application in India, in terms of sensitivity and specificity to distinguish human and major animal fecal contamination, by testing the assays against fecal samples of known origin collected in Coastal Odisha, and (2) to identify the best performing host-associated MST assays, based on testing results, for large-scale application to evaluate sanitation impacts in Odisha, India. The secondary objective was to explore potential variability in MST assay performance on feces from healthy humans versus patients with diarrhea.

2. Materials and methods

2.1. Study area overview

This study was conducted in rural and urban areas of Puri and Khorda districts in Coastal Odisha, along the Bay of Bengal, including Bhubaneswar, Odisha's state capital (Fig. 1). Study area populations have access to improved drinking water sources from public deep and private shallow tube wells or to municipal water systems. However, access to improved sanitation is poor, resulting in large segments practicing open defecation, while open ponds continue to be used in rural areas for bathing, washing, and anal cleansing after open defecation. Over 80% of rural Odisha households own livestock, with populations highest for cows, followed by goats, sheep, and buffalos (Government of Odisha, 2013). Pigs are rare, accounting for less than 2.5% of the total livestock population, while poultry are relatively common. In addition to livestock animals, free roaming domestic dogs can be observed in large numbers in rural and urban communities in Odisha.

2.2. Identification of candidate Bacteroidales qPCR assays

A candidate set of ten host-associated assays, comprising two universal, five human, two cows, and one dog, was targeted for identification from the literature, as the first step toward selection of a validated optimal subset for application in the Odisha sanitation study.

Only one previous study testing the human-associated *Bacteroidales* qPCR assay HF183 SYBR has been reported in South Asia, in Dhaka, Bangladesh, reporting 87% sensitivity and 93% specificity (Ahmed et al., 2010). Thus, HF183 SYBR was identified as one of the five candidate human-associated assays. We further searched the peer-reviewed literature to identify assays performing well in developed and/or less developed countries. One problem, however, was that assay performance varied considerably from one study to the next because of different performance evaluation criteria, use of a small number of test samples, and potential influence of inter-laboratory variability, making it difficult to assess and compare performance and geographical stability across studies. Recently, a comprehensive large scale MST assay evaluation was completed in California (Boehm et al., 2013). The study assessed

Fig. 1. Overview map of the study area in Odisha, India, where fecal samples were collected for comparative testing of different *Bacteroidales* assays.

the performance of 41 assays against 64 blind fecal samples in 27 laboratories. Drawing on this comprehensive evaluation, we identified the remaining candidate human- (HF183 Taqman, BacHum, HumM2 and BacH), ruminant- (BacCow), cattle-(CowM2) and dog- (BacCan) associated assays. Note that BacCow was reclassified as a ruminant-associated assay (Raith et al., 2013). For the two candidate universal/general *Bacteroidales* assays, we compared BacUni and GenBac3. BacUni has been reported to perform well not only in the US but also in a developing country, Kenya, where people have a very different diet from the US (Kildare et al., 2007; Jenkins et al., 2009; Silkie and Nelson, 2009). GenBac3 markers were reported to be abundant in human sources and 20 representative animal sources collected across the US, using a large number of test samples ($n = 223$) (Kelty et al., 2012).

2.3. Fecal sample collection

Fresh animal, human and raw sewage samples, were collected in sterile 20-ml tubes (Clinicol, Himedia) from villages across Puri and Khordha districts, and locations around Bhubaneswar over the dry season period from April to May 2012. Separately, human diarrhea samples were collected from hospital wards from April to July 2012, in order to explore potential variability in assay performance on feces from healthy versus diseased human guts. All samples were transported on ice to the Asian Institute of Public Health (AIPH) lab facility in Bhubaneswar and stored there at −70 °C for up to three months prior to DNA extraction.

2.3.1. Animal sample collection

Specimens from 346 individual animals, comprising cow ($n = 50$), buffalo ($n = 50$), sheep ($n = 50$), goat ($n = 50$), chicken ($n = 96$), and dog ($n = 50$), were collected and pooled to create 60 composite animal samples. Chicken samples came from several chicken farms in Puri district and Bhubaneswar, dog samples came from different locations in Bhubaneswar, while all other animal samples came from several well-dispersed villages in Puri and Khorda districts. Pooled cow, buffalo, sheep, goat and dog samples consisted of material from five animals. Pooled chicken samples consisted of material from six to ten birds. Approximately 1 g (wet weight) of feces from each individual animal was collected, combined and well-mixed using a sterile spoon in the field or the laboratory to form a pooled animal sample.

2.3.2. Human and wastewater sample collection

Thirty anonymous healthy individuals varying in age and sex from Puri and Khordha districts, and Bhubaneswar donated fresh fecal samples. Another 20 samples were collected from anonymous patients with diarrhea admitted to three local hospitals (Capital Hospital in Bhubaneswar, Puri District Headquarter Hospital, and Puri Government Area Hospital). Details of healthy and diarrhea human samples are provided in Supplementary Material (Table S1 and S2). Sewage samples ($n = 5$) were collected from municipal sewer lines at 5 different locations in Bhubaneswar.

2.4. Performance of candidate Bacteroidales qPCR assays

DNA extraction, DNA concentration measurements and all qPCR work including standard curve establishment and quantification of target markers were conducted in microbiology laboratories at AIPH and Kalinga Institute of Industrial Technology (KIIT) in Bhubaneswar, India.

2.4.1. DNA extraction

DNA was extracted from 180–220 mg of stored fecal/wastewater samples using QIAmp Stool DNA Kit (Qiagen) according to the manufacturer's directions. DNA extraction controls consisting of DNAase free water were included in each extraction batch. DNA concentrations were measured with the Epoch Micro-Volume Spectrophotometer System (BioTek). In every batch, one method blank with DNA-free water was run for quality assurance.

2.4.2. qPCR amplification

For all probe-based qPCR assays, each 25 µl of qPCR mixture contained 12.5 µl of TaqMan Environmental Master Mix 2.0 (Applied Biosystems) and 2.5 µl solution of primer and probe mixture (Table S3 in Supplementary Material). For HF183 SYBR, each 25 µl of qPCR mixture contained 10 µL 10× qPCR Buffer of SYBR Green 1 (Eurogentec), 0.25 µM of each primer, 200 µM of each deoxynucleoside triphosphate, 2.0 mM MgCl$_2$, 2.5 U of HotGold Star DNA polymerase, and 0.75 µl of Sybr Green I 1/10. For all qPCR reactions, 10 µl of diluted extracted genomic DNA from each fecal and wastewater sample was added to the reagents. To reduce the effects of qPCR inhibition, two dilutions (1:10 and 1:100) of DNA extracts were assayed. All samples were amplified with a Mastercycler ep Realplex (Eppendorf). For all probe-based assays except BacH, thermocycler conditions were as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. For BacH, thermal conditions were as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C, 15 s at 61 °C and 45 s at 72 °C. Despite the higher number of cycles for BacH, the same limit of detection ($C_t = 39$) was applied so as to be able to compare all human-associated assays equally. For HF183 SYBR thermal conditions were as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 60 s at 53 °C and 60 s at 60 °C. The dissociation stage was set at 15 s at 95 °C, 20 s at 60 °C and 15 s at 95 °C. Positive samples for HF183 SYBR were the wells showing amplification as

well as having a dissociation temperature between 76 and 79 °C. The greater dissociation temperature range was deemed necessary because different abundances of target markers in fecal samples can change the melting temperature more than 1 °C (Ririe et al., 1997). In each plate, at least four reagent blanks with DNA free water substituted for DNA template were included.

2.4.3. Standard curves and interpretation of qPCR results

Eight 10-fold serial dilutions (10^1 to 10^8 gene copies per reaction) of DNA plasmid standard containing target sequences were run to generate the standard curve for each assay. Regression analysis was performed to eliminate outliers by removing C_t values with a residual value larger than $+3$ or smaller than -3 (Schriewer et al., 2013). A limit of detection (LOD) was defined as the concentration calculated at $C_t = 39$ for all assays (Schriewer et al., 2013). A C_t value of 39 was chosen as a cutoff to be able to distinguish this amplification from artifacts or accidental bumps in the baseline. The lower limit of quantification (LLOQ) was determined for each assay using the lowest concentration on the standard curve where at least 5 of 6 replicates of eight serial dilutions were amplified (Schriewer et al., 2013). Each sample replicate was considered as follows: not detected (ND) when no amplification was detected by LOD; detected but not quantifiable (DNQ) when LLOQ < C_t < LOD; or detected within the range of quantification (ROQ) when C_t < LLOQ (Schriewer et al., 2013). Standard curve values and LLOQ are provided in Supplementary Material (Table S4).

2.5. Selection of assays for application in the Orissa (Odisha) sanitation trial

Assays were evaluated based on presence/absence sensitivity and specificity metrics with DNQs as positives. To evaluate and compare assay performance, accuracy was calculated, and the assay within each class with the highest accuracy considered the best class performer. Two further criteria were considered important for the performance of an optimal human-associated assay namely, 100% detection of sewage samples amplified within ROQ, and zero (or lowest) cross-reactivity with cow fecal samples, cows being the most common livestock in the study communities. We calculate the joint sensitivity and specificity of each paired combination of the five human-associated assays tested, to evaluate the utility of using two human-associated assays to increase detection of human fecal contamination.

2.5.1. Sensitivity, specificity and accuracy calculations

Sensitivity, specificity and accuracy of each MST assay were calculated using results for all samples, but not including results for human diarrhea samples. Fecal material from patients with diarrhea may not be representative of the microflora found in the guts of the dominant population of health individuals (Gorkiewicz et al., 2013; Goldberg et al., 2014; Monira et al., 2013). Even in low-resource settings such as the study area, diarrhea rates are very low (Clasen et al., 2012), indicating diarrheal fecal mass is a very small portion of total daily generated human fecal load. Healthy human and sewage samples were considered human sources for calculation of human-associated assay sensitivity. As originally reported, cattle and dog samples were classified as target sources of CowM2 and BacCan assay, respectively (Kildare et al., 2007; Shanks et al., 2008). Sensitivity and specificity for BacCow was calculated by treating the assay in two ways: 1) as a ruminant-associated assay, and 2) as a livestock/domestic animal-associated assay. In the latter case, all tested animal samples were considered target sources, and only healthy human and sewage samples were regarded as non-targets.

Sensitivity was calculated as the number of target host samples identified correctly as positives, divided by the total number of target host samples tested:

$$\text{Sensitivity} = \frac{\text{TP}}{(\text{TP} + \text{FN})} \tag{1}$$

where TP and FN are true positives and false negatives, respectively, of target host samples tested.

Specificity was calculated as the number of non-target host samples identified correctly as negatives, divided by the total number of non-target host samples tested:

$$\text{Specificity} = \frac{\text{TN}}{(\text{TN} + \text{FP})} \tag{2}$$

where TN and FP are true negatives and false positives, respectively, of non-target host samples.

Accuracy was calculated as the number of both target and non-target host samples identified correctly, divided by the total number of samples tested:

$$\text{Accuracy} = \frac{(\text{TP} + \text{TN})}{(\text{TP} + \text{FP} + \text{TN} + \text{FN})} \tag{3}$$

2.5.2. Joint sensitivity and specificity of paired human-associated assays

Samples were considered positive when either one or two assays showed amplification, and negative otherwise; joint sensitivity and specificity for each pair of human-associated assays was calculated using Eqs. (4) and (5).

$$\text{Joint sensitivity} = \frac{\text{TP}_1 \cup \text{TP}_2}{(\text{TP}_1 \cup \text{TP}_2) + (\text{FN}_1 \cap \text{FN}_2)} \tag{4}$$

where $\text{TP}_1 \cup \text{TP}_2$ denotes true positives of target host samples tested, confirmed by either one of two or both assays, and $\text{FN}_1 \cap \text{FN}_2$ represents false negatives of target host samples tested, confirmed by both assays.

$$\text{Joint specificity} = \frac{\text{TN}_1 \cap \text{TN}_2}{(\text{TN}_1 \cap \text{TN}_2) + (\text{FP}_1 \cup \text{FP}_2)} \tag{5}$$

where $\text{TN}_1 \cap \text{TN}_2$ denotes true negatives of target host samples tested, confirmed by both assays, and $\text{FP}_1 \cup \text{FP}_2$ represents false positives of target host samples tested, confirmed by either one of two or both assays.

More details on joint sensitivity and specificity calculations are available in Supplementary Material.

2.6. Statistical analysis

To compare abundance of universal *Bacteroidales* markers in healthy human and diarrhea samples, and abundance of BacUni and GenBac3 markers in all fecal/wastewater samples, a Mann–Whitney *U*-test was performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, U.S.A.). A p-value < 0.05 was considered statistically significant.

2.7. Licensing of assays

A research license was obtained from the U.S. Environmental Protection Agency (US EPA) to use the patent-pending GenBac3, HumM2, and CowM2 assays for the duration of our study.

3. Results

Assay sensitivity and specificity performance is reported in Tables 1, 2 and 3, and quantitative aspects are shown in Fig. 2 for the ten candidate MST qPCR assays. Human-associated marker concentrations in each fecal source type are reported in Table 4. Detailed information on abundance of universal and animal-associated markers as well as joint sensitivity and specificity of pairs of human-associated assays is available in Supplementary Material.

Table 1
Performance of *Bacteroidales* assays using Indian fecal and wastewater samples.

Source	No. of samples	Percentage of positive[a] stool or wastewater samples with listed qPCR assay targeting									
		Warm-blooded animal hosts		Humans						Ruminants[b]	Cattle	Dogs
		BacUni	GenBac3	HF 183 Taqman	BacHum	HumM2	BacH	HF183 SYBR	BacCow	CowM2	BacCan
Human	30 [c]	100	100	16.7	40.0	40.0	13.3	86.7	0	0	0
Sewage	5	100	100	100	100	100	40.0	100	0	0	0
Cow	10	100	100	0	0	10.0	0	100	100	50.0	10.0
Buffalo	10	100	100	0	10.0	0.00	0	100	100	0	0
Goat	10	100	100	0	10.0	60.0	0	100	100	0	20.0
Sheep	10	100	100	0	0	30.0	0	100	100	0	0
Dog	10	100	100	40.0	40.0	20.0	30.0	80.0	90.0	0	90.0
Chicken	10	100	100	80.0	70.0	60.0	70.0	100	80.0	0	0
Human (diarrhea)	20	95.0	100	40.0	40.0	10.0	30.0	0	N.M.[d]	N.M.	N.M.

[a] Calculated with DNQ (detected, not quantifiable) samples as positive.
[b] BacCow was reclassified as a ruminant-associated assay (Raith et al., 2013).
[c] 20 human fecal samples were tested for BacCow, ComM2 and BacCan.
[d] Not measured.

3.1. Bacteroidales qPCR assay diagnostic sensitivity and specificity

3.1.1. Universal assay performance

The two candidate universal *Bacteroidales* assays, BacUni and GenBac3, each amplified all fecal and sewage samples tested, providing 100% diagnostic sensitivity on the test set of 95 samples (excluding human diarrhea) (Table 1). GenBac3 amplified all human diarrhea samples tested, whereas all but one were amplified by BacUni. Results obtained from both 1:10 and 1:100 dilutions of DNA extracts did not change the number of positives and concentrations of BacUni markers, suggesting that the effect of qPCR inhibition was negligible in this test set.

3.1.2. Human-associated assay performance

Performance of the five candidate human-associated assays varied considerably. The largest number of healthy human fecal samples were measured positive by HF183 SYBR (26/30) followed by BacHum (12/30) and HumM2 (12/30) (Table 1). All sewage samples were detected by four of the human-associated assays, but not by BacH (2/5). HF183 SYBR provided the highest sensitivity (89%) across the set of 30 healthy human and 5 sewage samples, followed by BacHum and HumM2 (49%) (Table 2).

All candidate human-associated assays showed high cross-reactivity with chicken fecal samples (60 to 100%) and some cross-reactivity with dog fecal samples (20 to 80%). No cross-reactivity of the HF183 Taqman and BacH assays with any of the four ruminant livestock animal samples was found, while HF183 SYBR showed 100% cross-reactivity on all samples of the four ruminant livestock animals. BacHum showed no cross-reactivity with cow or sheep, and low levels of cross-reactivity with

buffalo (1/10) and goat (1/10). HumM2 measured positive in cow (1/10), goat (6/10) and sheep (3/10), but not buffalo. The highest specificity was obtained with BacH, at 83%, followed by HF183 Taqman (80%) (Table 2).

Based on accuracy, which accounts for the number of both true positives and true negatives identified correctly, five human-associated assays were evaluated. BacHum showed the highest accuracy (0.67) among five tested human-associated assays, followed by HumM2 (0.62). Despite the highest sensitivity of HF183 SYBR, high levels of cross-reactivity with animal fecal samples provided the lowest accuracy of this assay (0.35).

3.1.3. Ruminant-, cattle- and dog-associated assay performance

The ruminant- and cattle-associated assays preformed very differently on the animal fecal samples (Table 1). BacCow markers were detected in 100% of ruminant samples, but were also found in most dog (9/10) and chicken (8/10) samples. On the other hand, the CowM2 assay was less sensitive but highly specific to cow (5/10), not reacting with any of the other animal sources. Importantly, the BacCow and CowM2 genetic markers were absent in all healthy human and sewage samples. Overall diagnostic sensitivity and specificity for detecting cow-associated fecal material was 100% and 62%, respectively, for BacCow, and 50% and 100%, respectively, for CowM2 (Table 3). The finding that BacCow markers were detected in all types of animal feces we tested, but not in any human source samples tested, indicates the BacCow assay can be used as a livestock/domestic animal assay whose target hosts include cow, buffalo, goat, sheep, dog and chicken in this setting. Considered as a livestock/domestic animal associated assay, the sensitivity and specificity of BacCow were 95% and 100%, respectively.

Table 2
Specificity and sensitivity of universal and human-associated *Bacteroidales* assays using Indian fecal[a] and wastewater samples.

Metric	DNQ[b] outcome scored as	Assay targets						
		Warm-blooded animal hosts		Humans				
		BacUni	GenBac3	HF183 Taqman	BacHum	HumM2	BacH	HF183 SYBR
Specificity		–	–	0.80	0.78	0.70	0.83	0.03
Sensitivity	Positive	1.00	1.00	0.29	0.49	0.49	0.17	0.89
Accuracy		–	–	0.61	0.67	0.62	0.59	0.35
Specificity		–	–	0.80	0.80	0.92	0.83	0.63
Sensitivity	ND[c]	1.00	1.00	0.29	0.29	0.26	0.17	0.37
Accuracy		–	–	0.61	0.61	0.67	0.59	0.54

[a] Diarrhea samples are excluded for the calculation.
[b] Detected but not quantifiable.
[c] Not detected.

Table 3
Specificity and sensitivity of ruminant-, cattle-, livestock/domestic animal- and dog-associated *Bacteroidales* assays using Indian fecal and wastewater samples.

Metric	DNQ[a] outcome scored as	Assay targets			
		Ruminants	Livestock/domestic animals[b]	Cattle	Dogs
		BacCow	BacCow	CowM2	BacCan
Specificity		0.62	1.00	1.00	0.96
Sensitivity	Positive	1.00	0.95	0.50	0.90
Accuracy		0.80	0.96	0.94	0.95
Specificity		0.62	1.00	1.00	0.99
Sensitivity	ND[c]	1.00	0.95	0.50	0.90
Accuracy		0.80	0.96	0.94	0.98

[a] Detected but not quantifiable.
[b] BacCow was treated as "Livestock/domestic animal-associated assay", and its targets included all tested animal samples (cow, buffalo, goat, sheep, dog and chicken); non-target targets included human and sewage samples.
[c] Not detected.

The dog-associated assay, BacCan, amplified 90% of dog fecal samples, while showing limited cross-reactivity to cow (1/10) and goat (2/10) samples. No healthy human and sewage samples were amplified by BacCan, resulting in 90% diagnostic sensitivity and 96% specificity on the test set for detecting dog-associated fecal contamination.

3.1.4. Effect of DNQ classification on sensitivity and specificity

The sensitivity and specificity were re-analyzed treating DNQ as the absence rather than presence of amplification to examine the effect on assay performance metrics (Tables 2 and 3). Sensitivity and specificity of BacUni, GenBac3, HF183 Taqman, BacCow, CowM2, and BacCan did not change. Meanwhile, sensitivity was significantly decreased, in some cases by more than half, for the remaining four human-associated assays, BacHum, HumM2, BacH and HF183 SYBR, while specificity improved for two of them (HumM2 and HF183 SYBR).

3.2. Bacteroidales qPCR assay target marker abundance

The mean log_{10} concentration of target markers per nanogram of total DNA was calculated for positive samples within the range of quantification (ROQ) (Fig. 2). Quantifiable levels of BacUni and GenBac3
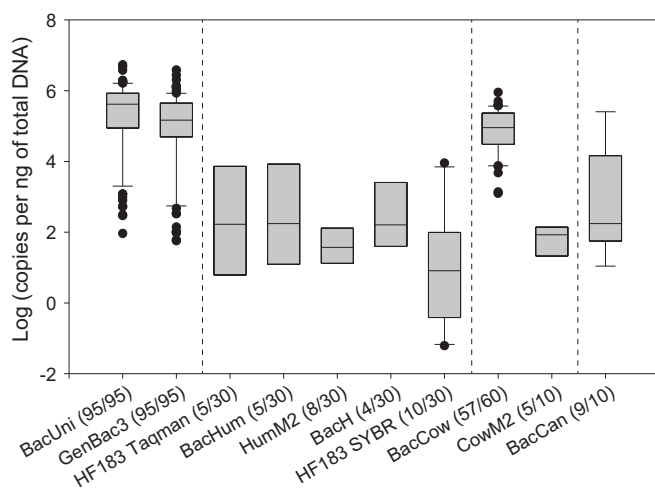
Fig. 2. Concentration of each marker in target fecal DNA extracts when present and quantifiable (i.e., within the range of quantification (ROQ)). The top and bottom of each box denotes the 75th and 25th percentiles, respectively. The top and bottom bars represent 10th and 90th percentiles, respectively. Closed circles represent outliers. Numbers in parentheses refer to the number of target fecal samples within ROQ/number of target fecal samples tested. Only results from fecal samples obtained from healthy humans were included for human-associated assays. BacCow is treated as livestock/domestic animal assay whose targets include cow, buffalo, goat, sheep, dog and chicken.

markers were present in all samples tested except for three and one human diarrhea samples, respectively. The abundance of BacUni was significantly greater than that of GenBac3 across the samples ($p < 0.05$). Mean abundance of HF183 Taqman, BacHum and BacH markers was similar in human stool and in sewage samples. Interestingly, the concentration of these markers varied by five orders of magnitude across individual human samples (from -0.14 to $4.49\ log_{10}$ copies per nanogram of total DNA). Compared to these three assays, the mean concentration of HumM2 markers was notably lower. HF183 Taqman and BacHum were the only assays amplifying all five sewage samples within ROQ, a key sensitivity criterion in selecting a human-associated assay for this study. HF183 SYBR had the lowest mean marker concentration in human stool samples despite its high presence/absence sensitivity. Among non-target samples, chicken fecal samples were found to contain very high levels of human-associated markers, especially those of HF183 Taqman, BacHum and HF183 SYBR. Mean log_{10} copies of HF183 SYBR markers per nanogram of total DNA were two orders of magnitude greater in chicken samples than in human samples (Table 4).

The mean abundance in ruminant samples of BacCow markers was more than 3 orders of magnitude greater than the mean abundance of CowM2 markers were in cattle samples (5.05 versus 1.77 log_{10} copies per nanogram of total DNA) (Fig. 2 and Table S6 in Supplementary Material). Considering BacCow as a livestock/domestic animal-associated assay, the mean log_{10} concentration of BacCow markers in target samples decreases slightly to 4.85. Abundance of BacCan varied across tested dog samples, ranging from 1.04 to 5.40, with a mean of 2.87 log_{10} copies per nanogram of total DNA (Fig. 2 and Table S6 in Supplementary Material).

3.3. Joint sensitivity and specificity of paired human-associated assays

We compared joint sensitivity and specificity for each possible paired combination to seek for possibilities to obtain higher sensitivity than that of BacHum alone, whose individual assay accuracy was the highest (Table S7 in Supplementary Material). All assays paired with HF183 SYBR significantly increased joint sensitivity up to 0.94, but also drastically decreased joint specificity as low as 0.03 due to high cross-reactivity of HF183 SYBR with animal fecal samples, indicating that these pairs would not be applicable. BacHum–HumM2 had the highest sensitivity (0.66), followed by HF183 Taqman–HumM2 (0.57). Joint specificity of BacHum–HumM2, however, was reduced by 16 percentage points due to HumM2's much lower specificity. These results indicate that, compared to BacHum alone, the pair BacHum–HumM2 provides a meaningful increase in the chance of detecting individual human-associated fecal contamination signals, but this comes at a cost of an increasing chance of also detecting false-positive signals.

4. Discussion

4.1. Human-associated assay performance and selection

Compared to diagnostic sensitivities reported by original assay developers (81% to 100%), all five human-associated assays we tested, except HF183 SYBR, demonstrated lower sensitivity in this study, ranging from 17 to 49% (Table 2). Geographical differences may explain this finding (Wuertz et al., 2011). A recent study also showed that sensitivity and specificity of BacH and BacHum were lower than originally reported after testing against human and animal fecal samples from 16 countries (Reischer et al., 2013). Similarly, the sensitivity of BacHum on fecal samples collected in Kenya was as low as 18% (Jenkins et al., 2009). These studies underscore the need for further testing in different geographic regions, especially for the HF183 Taqman and HumM2 assays, which to date have been applied only in North America and Australia. HF183 SYBR has been tested in several regions including developing countries, with sensitivity ranging from 65 to 90%, suggesting the sensitivity

Table 4
Abundance of human-associated *Bacteroidales* markers in fecal and wastewater samples collected in India.

Source		Mean Log$_{10}$ gene copies per ng of total DNA of positive samples[a] ($\pm$ S.D.)									
		HF183 Taqman		BacHum		HumM2		BacH		HF183 SYBR	
	n	Mean	No. samples within ROQ[b]	Mean	No. samples within ROQ[b]	Mean	No. samples within ROQ[b]	Mean	No. samples within ROQ[b]	Mean	No. samples within ROQ[b]
Human	30	2.31 (1.71)	5	2.46 (1.61)	5	1.57 (0.67)	8	2.40 (0.97)	4	0.96 (1.64)	10
Sewage	5	2.29 (0.72)	5	2.20 (0.75)	5	1.95 (−)	1	2.03 (0.35)	2	1.67 (0.47)	3
Cow	10	N.D.	0	N.D.	0	N.D.	0	N.D.	0	1.63 (−)	1
Buffalo	10	N.D.	0	N.D.	0	N.D.	0	N.D.	0	1.41 (0.49)	8
Goat	10	N.D.	0	1.66 (−)	1	2.17 (−)	1	N.D.	0	2.23 (1.00)	2
Sheep	10	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
Dog	10	1.49 (1.12)	4	1.56 (1.08)	4	0.81 (−)	1	1.09 (0.87)	4	1.04 (1.00)	4
Chicken	10	3.52 (1.16)	8	3.62 (1.09)	7	0.88 (0.36)	3	2.49 (1.22)	7	3.09 (1.11)	7
Human (diarrhea)	20	1.70 (1.27)	6	2.27 (1.06)	5	1.99 (0.97)	2	2.24 (1.03)	2	N.D.	0
Target[c]		2.30 (1.23)	10	2.33 (1.62)	10	1.61 (0.64)	9	2.28 (0.79)	6	1.12 (1.46)	13
Non-target[d]		2.84 (1.48)	12	2.77 (1.44)	13	1.12 (0.64)	5	2.07 (1.27)	11	1.96 (1.15)	22

[a] Calculated with DNQ samples as negative.
[b] Range of quantification.
[c] Targets include (healthy) human and sewage samples.
[d] Non-targets include cow, buffalo, goat, sheep, dog and chicken samples.

observed in this study, 89%, is in line with past reports (Kildare et al., 2007; Seurinck et al., 2005; Ahmed et al., 2010; Jenkins et al., 2009). Our results add to the evidence that HF183 SYBR markers are likely to be distributed in human feces regardless of geographic differences.

To our knowledge, this is the first report investigating sensitivity of *Bacteroidales* human-associated assays against human diarrhea samples. Interestingly, HF183 Taqman showed higher sensitivity (40%) on the limited number of tested diarrhea samples ($n = 20$) than on healthy human samples (n = 30; 17%). Poor amplification of diarrhea samples was evident for HumM2 (10%), while HF183 SYBR did not amplify any diarrhea samples despite having the highest sensitivity on healthy human fecal samples. BacHum and BacH amplified 40% and 30% of diarrhea samples, respectively, showing similar levels of detection compared to those of samples from healthy humans.

In contrast to the relatively low sensitivity observed for most of the human-associated assays we tested, all assays demonstrated relatively high specificity (70 to 83%) except for HF183 SYBR (3%). All five human-associated assays, however, showed high levels of cross-reactivity with chicken and low levels of cross-reactivity with dog fecal samples. HF183 Taqman, HumM2 and HF183 SYBR assays have displayed cross-reactivity with chicken fecal samples in other countries (Ahmed et al., 2012; Shanks et al., 2009, 2010a). Moreover, several studies have shown that the HF183 forward primer can pick up target markers in chicken fecal samples in combination with the reverse primer Bac708R (Gawler et al., 2007; Balleste et al., 2010; Gourmelon et al., 2007; Ahmed et al., 2012). These reports indicate HF183 amplifiable markers are ubiquitous in chicken fecal samples, which we have further confirmed here in this study. The cross-reactivity of BacHum and BacH assays with chicken fecal samples observed in this study has also recently been shown when fecal samples from six continents were tested (Reischer et al., 2013). Excluding HumM2, four of the human-associated assays have previously been reported to amplify dog fecal samples (Kildare et al., 2007; Ahmed et al., 2009, 2010; Layton et al., 2013; Silkie and Nelson, 2009; Shanks et al., 2010a). Observed cross-reactivity with dog samples for all five assays tested in this study, including HumM2, confirms the absence of assays capable of complete discrimination between human and dog fecal sources worldwide.

BacHum had the highest accuracy among the five human-associated assays tested in this study, amplified all sewage samples within ROQ, and did not cross-react with any cow fecal samples (representing material from 50 individual cows). HumM2, with the second highest accuracy, failed both criteria. Thus, BacHum provides the best human-associated assay for application in this setting. While human fecal contamination detected in the public domain can be considered a composite from many individuals, contamination in the domestic domain can occur

from a single individual. Thus, higher human source sensitivity than provided by BacHum (49%) may be required to detect human fecal contamination in samples collected in the domestic domain. To aid in detecting human contamination in domestic domain samples from study areas (e.g. on mothers' and children's hands, and in household stored drinking water), HumM2 may be applied following a failure to detect a human-associated signal with BacHum to enhance detection of human contamination from 49% with BacHum alone, to 66% using BacHum and HumM2 together.

To account for observed cross-reactivity of BacHum with dog fecal samples in this setting (4/10), the dog-associated assay BacCan, which achieved 90% sensitivity against dog fecal samples and showed no cross-reactivity with human fecal sources, should be applied to samples positive for BacHum to confirm these samples are true human signals. High sensitivity of BacCan has also been reported in the US and Canada (Tambalo et al., 2012b; Silkie and Nelson, 2009; Schriewer et al., 2013).

4.2. Ruminant- and cattle-associated assay performance and selection

Interestingly, the BacCow assay amplified all ruminants and most of dog and chicken fecal samples, but no human source samples in the test set. The abundance of BacCow markers in these animal samples was consistently high. Thus, BacCow may be effectively used as a livestock/domestic animal-associated assay in India, rather than a cow-associated assay as originally reported (Kildare et al., 2007), providing 95% sensitivity and 100% specificity when target hosts are expanded to include all primary livestock (cow, buffalo, sheep, goat and chicken) and domestic animals (dog). In California, BacCow has been reported to cross-react with deer, chicken, dog, goose, gull, horse and pig in an inter-laboratory comparison study (Boehm et al., 2013) and reclassified as ruminant-associated (Raith et al., 2013). Another study, looking at MST assay performance globally using fecal samples from 16 countries, also showed cross-reactivity of BacCow with several types of animals such as sheep, goat, dog and chicken (Reischer et al., 2013). These findings together suggest that BacCow has the potential to detect a broader range of animal feces regardless of geographic region; however, thorough validation of the BacCow assay is necessary in new study regions to define target hosts. Unlike BacCow, the CowM2 assay failed to detect five cow fecal samples, resulting in lower sensitivity (50%) than reported for this assay in the US and Canada (Tambalo et al., 2012a; Shanks et al., 2008, 2010b; Raith et al., 2013). Abundance of CowM2 markers in cow feces was three orders of magnitude lower than that of BacCow, likely due to differences between the 16S rRNA and non-ribosomal genes targeted by these two assays (Raith et al., 2013). Specificity of

CowM2, however, was 100%, and matches previous studies in Canada and the US (Tambalo et al., 2012a; Shanks et al., 2008, 2010b), indicating that this assay is highly cattle specific regardless of geographic region.

4.3. Universal assay performance and selection

Both universal assays, BacUni and GenBac3, showed 100% sensitivity in the Odisha area, matching findings reported for the US and Kenya (Kildare et al., 2007; Jenkins et al., 2009; Kelty et al., 2012). Together, this evidence suggests universal *Bacteroidales* markers are likely to be abundant in warm-blooded animal feces across continents. BacUni and GenBac3 both amplified all or nearly all diarrhea samples with significantly lower copy numbers per nanogram of total DNA compared to that detected in healthy human samples ($p < 0.05$) (Table S5 in Supplementary Material). The observed reduction in overall abundance (mean $\log_{10}$ gene copies per ng DNA) of universal *Bacteroidales* markers in diarrheal patients in our study may be related to the stress level in these individuals. The phylum *Bacteroidetes* has been shown to decrease in infectious as well as osmotic diarrheal states (Gorkiewicz et al., 2013). An inverse relationship between *Clostridium difficile* diarrhea and *Bacteroidetes* (Goldberg et al., 2014) and a severity-linked reduction of *Bacteroidetes* in cholera infection have been described (Monira et al., 2013). Treatment with macrolide antibiotics is also known to selectively disrupt *Bacteroidales*, *Fusobacteria*, and *Moraxella* in the gut in dogs (Suchodolski et al., 2009). While detailed clinical parameters were not collected, all hospital patients in our study had clinically significant diarrhea and were expected to be treated with antibiotics (a common practice even in the absence of culture proven diarrhea). Thus, purging and antibiotics could be incriminated as the causes of significant reduction of *Bacteroidales* in our diarrheal patients, and highlight the need for vigilance while dealing with diarrheal (a common condition in coastal regions of Odisha state) stools compared to those from healthy individuals. However, it remains unclear why only some of the genetic markers had reduced concentrations in patients with diarrhea compared to healthy individuals.

The validation findings demonstrate that either universal *Bacteroidales* assay can perform equally well in the study area. We recommend BacUni because of significantly greater abundance of the marker in test samples, extensive experience using BacUni, and its unrestricted use and free availability, compared to GenBac3, which requires a license from the US EPA.

5. Conclusions

We evaluated the performance of 10 *Bacteroidales* qPCR assays based on presence/absence metrics using human and non-human fecal samples from Odisha, India. The following conclusions are made:

- Both universal/general *Bacteroidales* assays, BacUni and GenBac3, performed equally well, achieving 100% sensitivity against fecal samples collected in Odisha, India.
- There was lower sensitivity of the human-associated assays tested than has been reported for other geographic regions, with the exception of HF183 SYBR, suggesting that human-associated markers in human feces collected from this study area were not as prevalent as in the US or Europe. High and moderate levels of cross-reactivity with chicken and dog fecal samples, respectively, were confirmed for all five human-associated assays tested.
- BacHum performed best among the five tested human-associated assays in this setting, based on highest accuracy, amplification of all tested sewage samples within ROQ, and zero cross-reactivity with cow fecal samples.
- Using BacHum and HumM2 together enhances detection of individual human fecal contamination, indicating a potential benefit from applying HumM2 in addition to BacHum, especially for samples collected from the domestic domain of disease transmission where higher individual

sensitivity may be required than for samples from the public domain.
- Neither ruminant-associated BacCow nor cattle-associated CowM2 amplified any human sources, while BacCow showed high sensitivity not only to ruminants, but also to other animals, including chicken and domestic dog fecal contamination sources present in our study area.
- BacCan, the dog-associated assay, performed well, showing no cross-reactivity with human sources, and high sensitivity to detect dog fecal contamination. Applying the BacCan assay to environmental samples positive for a human-associated assay can help verify that human-positive signals are not derived from dog contamination.
- Taken together, the assays BacUni, BacHum, HumM2, BacCan and BacCow are recommended for future use in microbial source tracking studies in these regions of India, until a better performing human-associated assay for application in India can be developed.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2014.09.040.

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