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Host Specificity and Sensitivity of Established and Novel Sewage-Associated Marker Genes in Human and Nonhuman Fecal Samples

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ABSTRACT Microbial source tracking (MST) methods measure fecal contamination levels and identify possible sources using quantitative PCR (qPCR) that targets host-associated fecal microorganisms. To date, most established MST assays for human sources, especially bacterial markers, have shown some nonhuman host cross-reactions. Recently developed assays, such as the crAssphage CPQ_056, *Lachnospiraceae* Lachno3, and *Bacteroides* BacV6-21, have more limited information on host sensitivity and host specificity for human or sewage sources, particularly in countries other than the United States. In this study, we rigorously evaluated six sewage-associated MST assays (i.e., *Bacteroides* HF183, human adenovirus [HAdV], human polyomavirus [HPyV], crAssphage CPQ_056, Lachno3, and BacV6-21) to show advantages and disadvantages of their applications for MST. A total of 29 human and 3 sewage samples and 360 nonhuman fecal samples across 14 hosts collected from a subtropical region of Australia were tested for marker host specificity, host sensitivity, and concentrations. All sewage samples were positive for all six marker genes tested in this study. Bacterial markers were more prevalent than viral markers in human feces. Testing against animal hosts showed human feces (or sewage)-associated marker gene specificity was HAdV (1.00) > HPyV (0.99) > crAssphage CPQ_056 (0.98) > HF183 (0.96) > Lachno3 (0.95) > BacV6-21 (0.90), with marker concentrations in some animal fecal samples being 3 to 5 orders of magnitude lower than those in sewage. When considering host specificity, sensitivity, and concentrations in source samples, the HF183, Lachno3, and crAssphage CPQ_056 tests were the most suitable assays in this study for sewage contamination tracking in subtropical waters of Australia.

IMPORTANCE Large financial investments are required to remediate fecal contamination sources in waterways, and accurate results from field studies are crucial to build confidence in MST approaches. Host specificity and sensitivity are two main performance characteristics for consideration when choosing MST assays. Ongoing efforts for marker assay validation will improve interpretation of results and could shed light on patterns of occurrence in nontarget hosts that might explain the underlying drivers of cross-reaction of certain markers. For field applications, caution should be taken to choose appropriate MST marker genes and assays based on available host specificity and sensitivity data and background knowledge of the contaminating sources in the study area. Since many waterborne pathogens are viruses, employing both viral and bacterial markers in investigations could provide insight into contamination dynamics and ecological behavior in the environment. Therefore, combined usage of marker assays is recommended for more accurate and informative sewage contamination detection and fecal source resolution.

KEYWORDS microbial source tracking

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Microbial source tracking (MST) is a group of techniques or analytical tools that are used to determine the sources of fecal contamination in various types of water bodies (1, 2). The fundamental hypothesis of MST is that some species of microorganisms are specific to the gut of human or animals due to differences in diet and physiology. These microorganisms are excreted in the feces of that particular animal or human. Thus, their presence in water bodies indicates fecal contamination has occurred from specific host groups. Most commonly used MST tools identify or quantify a DNA sequence(s) or marker gene(s) from the host-associated microorganisms by PCR or quantitative PCR (qPCR) assays (3–6).

Numerous MST marker genes or microorganism(s) have been identified to detect sewage (7, 8), cattle (9), pig (10), seagull (11), chicken (12), dog (13), duck (14), possum (15), and other types of animal fecal contamination in water bodies. When a microorganism or a marker gene associated with a microorganism is identified and proposed for MST field studies, a series of performance characteristic evaluations need to be undertaken to determine its suitability and accuracy for successful field application (1, 16). This is because performance characteristics of a microorganism or a marker gene may vary geographically and should always be verified in a new geographical region (1).

Host specificity is arguably one of the most critical performance characteristics. This is because a highly host-specific marker gene can identify the sources of fecal contamination accurately (17). An accurate source assignment is important for water quality managers or regulators who are responsible for making regulatory decisions to improve the environmental water quality. Incorrectly assigned sources may lead to wasted capital investment or mitigation efforts that may not ultimately improve the water quality (18). Host specificity is defined as the proportion of nontarget host fecal samples that produce negative results (1). Host specificity is determined by testing the presence of the target marker gene in fecal samples collected from a range of nontarget animal species that are commonly found in the catchments. None of the bacterial markers exhibit absolute host specificity; they occasionally are detected in nontarget animal species, probably due to physiology, colonization, similar diets, and cohabitation. For example, HF183, BacHum-UCD, and *Methanobrevibacter smithii nifH* marker genes have shown limited cross-reactivity with chicken, dog, and other animals (19–21). In contrast, certain viruses, such as human adenovirus (HAdV) and human polyomavirus (HPyV), have shown absolute host specificity, although other viruses currently used for MST field studies, such as pepper mild mottle virus (PMMoV) and crAssphage, have been detected sporadically in nonhuman fecal samples (22, 23).

Sensitivity is another important performance characteristic of the MST marker genes. In the context of MST, host sensitivity is the proportion of positive samples in which the marker is detected (16, 24). A marker could be highly host sensitive (i.e., present in feces of all or most individuals), but the concentration can be low in the target host feces. Such a marker gene may not be frequently detected in contaminated water samples due to dilution and losses through sample processing steps than a marker which is highly prevalent as well as abundant in the feces of target hosts.

Urbanization and extreme weather events such as floods and wet/dry weather overflows result in an increased amount of sewage being discharged into water bodies (3, 25–27). Sewage-contaminated water bodies are potential public health hazards and can cause severe illnesses to humans due to the presence of infectious pathogens (28). Various MST marker genes have been proposed to identify the source of sewage contamination and mitigate the human health risks (1, 17). Sewage-associated MST marker genes such as HF183 or BacHum-UCD have been evaluated rigorously in terms of host specificity and sensitivity. However, none of the markers showed absolute host specificity and are occasionally detected in nonhuman fecal samples (1, 17). Therefore, researchers are searching for novel microorganisms or marker genes in the feces of animals that are highly host specific and sensitive. Little is known regarding the performance characteristics of recently developed sewage-associated marker genes,

TABLE 1 Background information on animal and human fecal samples

Host	No. of archived DNA samples used	No. of fecal samples collected in this study	Total no. of samples	Sample type	Source(s) of samples	Wet wt (mg) (or vol [ml] where indicated) used for DNA extraction	Range (ng) of DNA per μl of extract
Animals							
Birds	9	21	30	Individual	Veterinary hospital, wild	100–120	4.02–260
Cat	14	6	20	Individual	Veterinary hospital	200–220	17–83.2
Cow	10	20	30	Individual	Various farms	200–220	10.2–108
Chicken		30	30	Individual	Various households	180–200	11.1–70.2
Deer	12	18	30	Individual	Deer sanctuary	200–220	20.1–80.3
Dog	17	13	30	Individual	Dog beach	200–220	14–230
Emu	14	6	20	Individual	Farm	200–220	14–220
Goat	10		10	Individual	Farm	180–220	6–18.0
Horse	18	12	30	Individual	Horse racecourse	200–220	8.2–78.0
Kangaroo	14	16	30	Individual	Wild	100	16.9–75.2
Koala	12	3	15	Individual	Koala sanctuary	200	4–20.1
Pig		35	35	Individual	Abattoir	200–220	10–70.1
Sheep	14	16	30	Individual	Various farms	200–220	3–15
Waterfowl	9	11	20	Individual	Various lakes	120–150	11–38
Human							
Human	13	16	29	Individual	Brisbane area	200–220	80.1–180
Untreated sewage	3		3	Composite	Primary influent of a WWTP[a]	0.1 ml	10.3–23.4

[a]WWTP, wastewater treatment plant.

such as crAssphage (8), Lachno3 (belonging to family *Lachnospiraceae*) (29), and the novel BacV6-21 (belonging to *Bacteroides*) (30), which is specific to sewer pipes.

The objective of this study was to compare the host specificity, sensitivity, and concentration of six sewage-associated marker genes across a large number of human and nonhuman fecal samples in a subtropical region of Australia, including three recently developed sewage markers. In addition, we have provided insights and general guidance on performance characteristics based on our professional judgements and understandings. This study will provide valuable information to water quality managers and regulators for choosing appropriate sewage-associated marker genes for identifying sewage contamination tracking in catchment waters.

RESULTS

Host specificity. We assessed 29 individual fecal and 3 untreated sewage samples and 360 nonhuman fecal samples for six different sewage-associated bacterial and viral marker genes (Table 1). HAdV was not detected in any of the animal fecal samples tested in this study. Similarly, HPyV was not found in any animal fecal samples except one bird fecal sample. The crAssphage CPQ_056 marker gene was detected in five cat fecal samples. Among the bacterial marker genes tested, HF183 was detected less frequently than BacV6-21 and Lachno3 marker genes. The HF183 marker gene was detected in five chicken, four emu, and three koala fecal samples, while the BacV6-21 marker gene was detected in 13 bird, 1 cow, 12 chicken, 4 dog, 4 emu, and 1 sheep fecal sample. Lachno3 was also detected in 2 bird, 1 cat, 3 deer, and 11 horse fecal samples. Overall, bacterial markers were more prevalent than viruses in animal fecal samples (Table 2). The host specificity values of the markers were >0.90. HAdV showed absolute host specificity (1.00) against the fecal samples tested in the study, followed by HPyV (0.99) > crAssphage CPQ_056 (0.98) > HF183 (0.96) > Lachno3 (0.95) > BacV6-21 (0.90).

We also determined the concentrations of sewage-associated marker genes in nonhuman fecal samples. The concentrations are presented per gram of wet feces as well as per nanogram of DNA (Fig. 1). HPyV could not be quantified in the bird fecal sample that produced a PCR-positive signal. crAssphage was quantifiable in all five cat fecal samples, and the concentrations ranged between 6.02 and 8.59 $\log_{10}$ gene copies (GC)/g of feces. Of the four chicken fecal samples positive for HF183, three samples were quantifiable, with concentrations ranging between 4.36 and 4.65 $\log_{10}$ GC/g of feces. The BacV6-21 marker gene was quantifiable in 5 of 13 bird fecal samples, and the

TABLE 2 Host specificity of sewage-associated marker genes in nonhuman fecal samples

Nonhuman fecal samples/ performance characteristic	No. of samples collected	No. (%) of samples positive for marker gene:					
		HF183	BacV6-21	Lachno3	HAdV	HPyV	CPQ_056
Birds	30	0 (0)	13 (43.3)	2 (6.66)	0 (0)	1 (3.33)	0 (0)
Cat	20	1 (5)	0 (0)	1 (7.14)	0 (0)	0 (0)	5 (25)
Cow	30	0 (0)	1 (3.33)	0 (0)	0 (0)	0 (0)	0 (0)
Chicken	30	5 (16.6)	12 (40.0)	0 (0)	0 (0)	0 (0)	0 (0)
Deer	30	0 (0)	0 (0)	3 (10.0)	0 (0)	0 (0)	0 (0)
Dog	30	0 (0)	4 (13.3)	0 (0)	0 (0)	0 (0)	0 (0)
Emu	20	4 (20)	4 (20)	0 (0)	0 (0)	0 (0)	0 (0)
Goat	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Horse	30	0 (0)	0 (0)	11 (36.7)	0 (0)	0 (0)	0 (0)
Kangaroo	30	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Koala	15	2 (13.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Pig	35	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Sheep	30	0 (0)	1 (3.33)	0 (0)	0 (0)	0 (0)	0 (0)
Waterfowl	20	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
True negative		347	325	342	360	358	355
False positive		12	35	17	0	1	5
Host specificity		0.96	0.90	0.95	1.00	0.99	0.98

concentrations ranged between 4.48 and 6.18 $\log_{10}$ GC/g of feces. The concentrations of BacV6-21 in 8 of 12 chicken fecal samples ranged from 2.25 to 9.16 $\log_{10}$ GC/g of feces. Lachno3 could not be quantified in two bird fecal samples that produced positive PCR results. The concentrations of Lachno3 in 3 deer and 11 horse fecal samples ranged from 5.04 to 6.90 and 4.68 to 6.29 $\log_{10}$ GC/g, respectively. In comparing the concentrations of marker genes expressed in grams of feces to the equivalent nanograms of DNA, 1 g of nonhuman fecal sample has 3 to 5 $\log_{10}$ GC more than 1 ng of DNA (see Table S1 in the supplemental material).

Host sensitivity. Among the 29 individual human fecal samples tested, HF183 was the most prevalent (72.4%), followed by Lachno3 (68.9%) and crAssphage CPQ_056 (37.9%) marker genes, corresponding to host sensitivity values of 0.72, 0.69, and 0.38, respectively (Table 3). BacV6-21 is associated with sewer pipes, while HPyV is associated with human urine. Therefore, these markers should not be present in the individual human fecal samples. Nonetheless, individual fecal samples were tested for the presence of these markers but could not be detected. HAdV, although an enteric pathogen, could not be detected in any of the healthy human fecal samples tested in this study. All three untreated sewage samples were positive for all six sewage-associated marker genes tested in this study, yielding a host sensitivity value of 1 for sewage samples.

The concentration of crAssphage in PCR-positive human feces ranged from 6.30 to 8.92 $\log_{10}$ GC/g with a mean concentration of 7.35 $\log_{10}$ GC/g of human feces (Fig. 2 and Table S2). Similarly, the concentrations of the HF183 marker ranged from 6.69 to 9.37 with a mean of 7.89, and those of Lachno3 ranged from 4.59 to 9.37 with a mean of 7.33 $\log_{10}$ GC/g of feces. The concentrations of HF183, BacV6-21, Lachno3, and crAssphage CPQ_056 were within the same order of magnitude in untreated sewage samples and ranged from 9.22 (Lachno3) to 9.46 (BacV6-21) $\log_{10}$ GC/liter of sewage. The concentrations of sewage-associated marker genes per gram of human feces and per liter of untreated sewage samples were 3.5 to 6.5 orders of magnitude greater than those per nanogram of DNA (Tables S2 and S3).

The mean concentrations of the HAdV (6.50 $\log_{10}$ GC/liter) and HPyV (6.71 $\log_{10}$ GC/liter) markers were 2 to 3 orders of magnitude lower than those of all bacterial and crAssphage CPQ_056 marker genes. One-way analysis of variance (ANOVA) with Tukey's *post hoc* test indicated that the concentrations of HAdV and HPyV in sewage samples were significantly ($P < 0.05$) lower than those of HF183, BacV6-21, Lachno3, and CPQ_056 marker genes regardless of the units of measurement used (Tables S4 and S5). The concentration of HAdV did not differ significantly ($P > 0.05$) from that of HPyV in sewage samples for both units of measurement. The differences in concentrations of

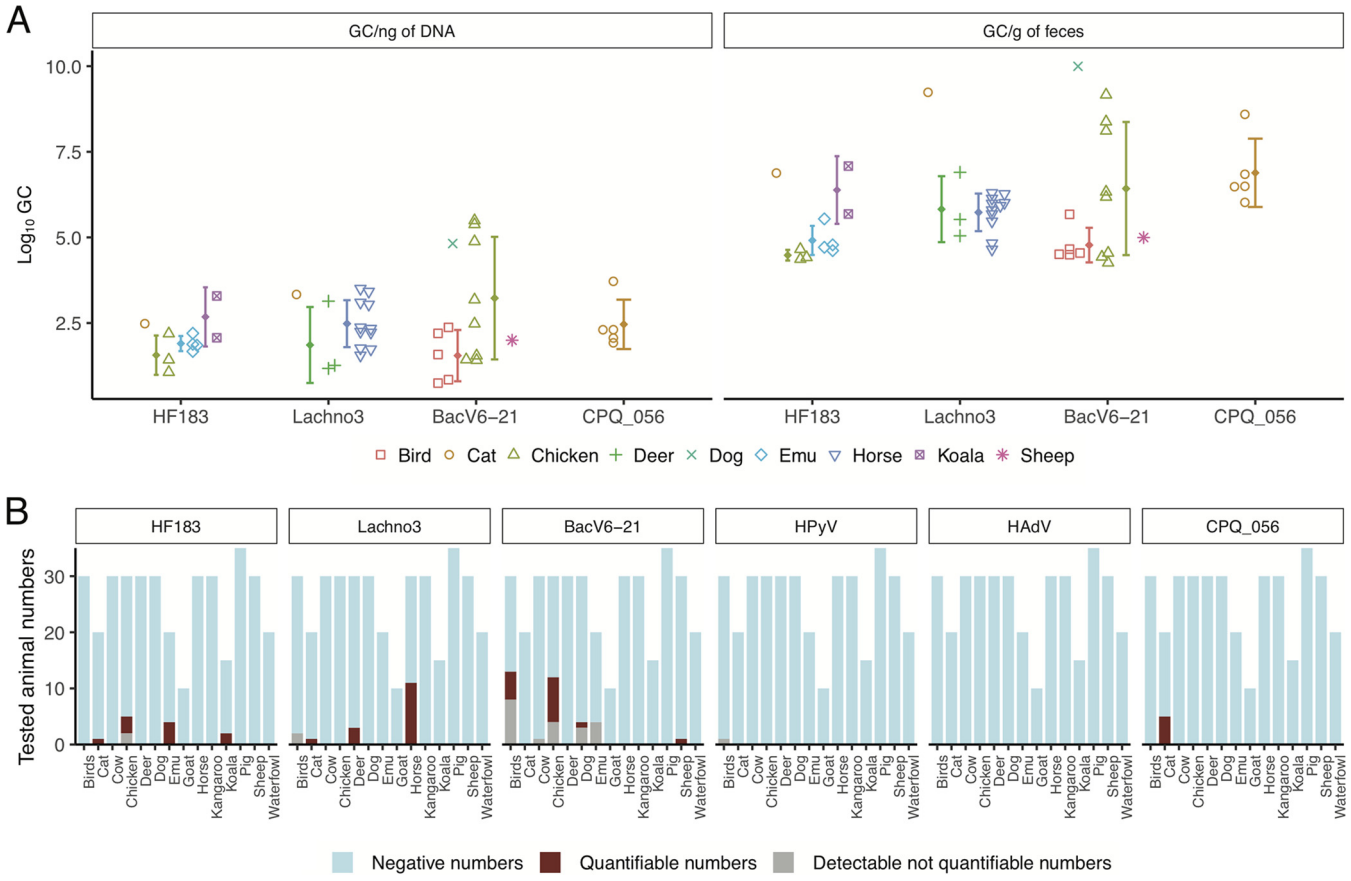

FIG 1 Concentrations of sewage-associated marker genes in nonhuman fecal samples. (A) Gene copies (GC) of HF183, Lachno3, BacV6-21, and crAssphage CPQ_056 markers in animal hosts. Results are calculated in GC/ng of DNA and GC/g of feces. Each animal host is represented in unique color and shape. Error bars represent marker GC means ± standard deviations. (B) Overall nonhuman sample test results for six markers where the bar height represents sample numbers. Quantifiable sample numbers are shown in maroon bars, negative sample numbers are shown in light blue bars, and detectable but not quantifiable (positive) numbers are shown in gray bars.

HF183, Lachno3, and CPQ_056 marker genes per gram of feces were not statistically significant ($P > 0.05$) from each other. Notably, the concentrations of CPQ_056 and Lachno3 per nanogram of human feces were significantly ($P < 0.05$) greater than that of HF183 per nanogram of sewage. CPQ_056 concentration was significantly ($P < 0.05$) different from those of HF183 and Lachno3 when nanogram was used as a unit of measurement.

Accuracy. We also determined the accuracy of the marker genes by combining host specificity and sensitivity values for each marker gene. When we combined the host specificity (for all nonhuman fecal samples) and host sensitivity (untreated sewage

TABLE 3 Host sensitivity of sewage-associated marker genes in human feces and untreated wastewater samples

Human fecal samples/ performance characteristic	No. of samples collected	No. (%) of samples positive for marker gene:					
		HF183	BacV6-21	Lachno3	HAdV	HPyV	CPQ_056
Human feces	29	21 (72.4)		20 (68.9)	0 (0)		11 (37.9)
True positive		21		20	0		11
False negative		8		9	19		18
Host sensitivity		0.72		0.69	0		0.38
Untreated wastewater	3	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
True positive		3	3	3	3	3	3
False negative		0	0	0	0	0	0
Host sensitivity		1.00	1.00	1.00	1.00	1.00	1.00

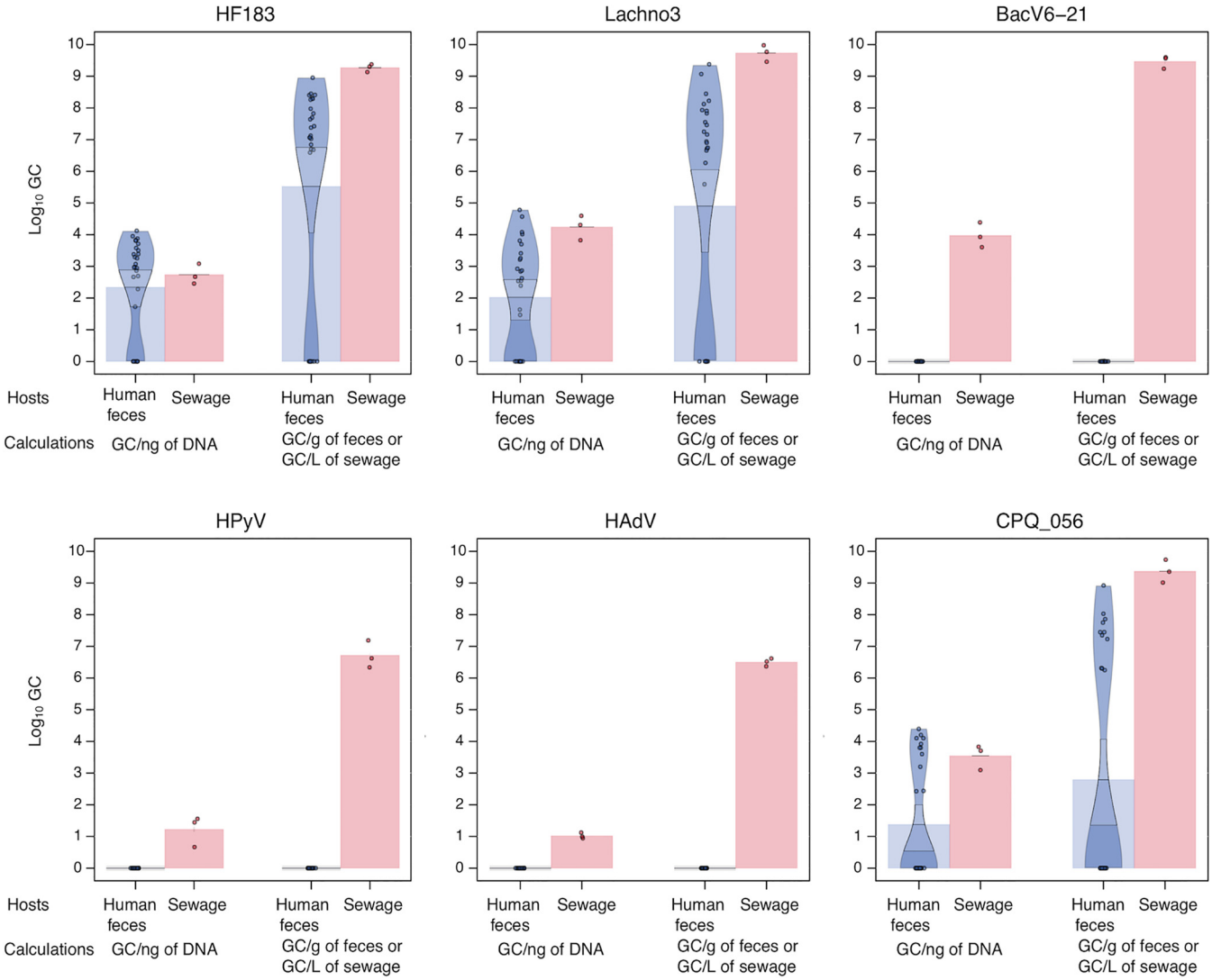

FIG 2 Concentrations of sewage-associated markers in sewage and human feces. The *y* axis represents $\log_{10}$ gene copy (GC) numbers, and the *x* axis shows groups of human feces and sewage. Each marker is shown as results of GC/ng DNA and GC/g of feces or per liter of sewage to better visualize between unequal group sizes. Horizontal line represents mean value, and band represents 95% highest density intervals.

samples only) values, the marker gene accuracy values were HAdV (1.00) > HPyV (0.99), crAssphage CPQ_056 (0.98) > HF183 (0.96) > Lachno3 (0.95) > BacV6-21 (0.90). When we combined the host specificity of nonhuman fecal samples with the sensitivity of individual human fecal samples, the marker gene accuracy values were HF183 and crAssphage (0.94) > Lachno3 (0.93) > HAdV (0.92).

DISCUSSION

In this study, we rigorously evaluated the host specificity and sensitivity of six sewage-associated marker genes, including viruses, by testing a large number of human and animal fecal samples collected from a subtropical region of Australia. Among the marker genes analyzed, some are well characterized (i.e., HF183, HAdV, and HPyV), and the others (BacV6-21, Lachno3, and crAssphage CPQ_056) are relatively new. Little is known on the performance characteristics of BacV6-21, Lachno3, and crAssphage CPQ_056 marker genes. Our study has determined the performance characteristics of a wide range of bacterial and viral sewage-associated marker genes in a head-to-head comparison study in subtropical Australia.

The host specificity values of the marker genes were >0.90 (maximum value of 1.00),

with HAdV showing absolute host specificity. At present, there is no benchmark criteria for host specificity value. However, a host specificity value of >0.80 is considered useful (31) and >0.90 is excellent (17, 32). Most of the marker genes show limited cross-reactivity with nonhuman fecal DNA samples. This issue can be resolved by testing two or more marker genes simultaneously (1, 22, 29). For example, based on the data obtained in this study, if we pair or synergistically use HF183 with the crAssphage CPQ_056 marker gene, then the potential false-positive results due to the presence of HF183 in chicken, emu, and koala can be resolved when assigning the true sources of fecal contamination in water samples. In cases of discrepant results, additional assays can be easily performed on the extracted DNA samples with little cost, and this will increase the confidence level that the sources of contamination have been correctly identified.

Most qPCR assays are based on the analysis of a single marker gene at a time to identify the sources of fecal contamination. We recently developed a duplex qPCR assay for the simultaneous quantification of HF183 and crAssphage CPQ_056 in sewage and environmental water samples (33). Such an assay has the potential to minimize the risk of false-positive results in environmental water samples due to cross-reactivity. In addition, duplex or multiplex qPCR assays will facilitate rapid analysis of water samples for the presence of sewage contamination with reduced cost and increased certainty.

In this study, sewage-associated marker genes were detected in several nonhuman fecal samples. However, the concentrations were 3 to 5 orders of magnitude lower than those of untreated sewage in some samples, and other samples were not quantifiable. Nonquantifiable samples or samples with low levels ($<10^3$ to 10^4 GC/g) of sewage-associated marker genes found in nonhuman fecal samples should not be seen as a pitfall. This is because in the event of contamination by a confounding source in urban areas, such low levels will be difficult to detect due to loss of marker genes through sample concentration and DNA extraction processes and also dilution factor, which will likely mask their detection (34). Further, individual animals were also sporadically positive, further reducing the likelihood that a detectable signal would interfere with interpretation of results. The use of two sewage-associated markers alleviates this concern. In particular, pairing a bacterial marker with a viral marker such as crAssphage CPQ_056 is useful for environmental applications where the fate and transport of viral pathogens may be different than that of the bacterial markers.

The mechanisms for the presence of sewage-associated marker genes in nonhuman fecal samples is unclear and requires further investigation. It is possible that the assay is amplifying a related sequence in high abundance, and slight modification, such as increasing the annealing temperature, may resolve the issue. Microorganisms that contain the marker genes may transiently colonize in the gut and become a stable member. Factors such as diets and cohabitation can influence the sharing of host-associated microorganisms among animals (35). Another explanation is that some genome regions of sewage-associated marker genes may have homology to microorganisms in animals (36). Cross-contamination of fecal samples within a farm environment is another factor that cannot be completely ruled out.

The prevalences of the HF183 (72.4%) and Lachno3 (68.9%) markers were higher than that of crAssphage CPQ_056 (37.9%) in individual human fecal samples. A recent study determined the occurrence of crAssphage in 255 human fecal samples from patients with diarrhea in China. Among the 255 samples, 49 (19.6%) were PCR positive for the crAssphage marker (37). Similarly, Cinek and colleagues (38) also reported highly variable concentrations of crAssphage in 20 (7.24%) of 274 fecal samples collected from children from Azerbaijan, Finland, Jordan, Nigeria, and Sudan. The reasons for complete absence or an uneven distribution of crAssphage and other sewage-associated markers in human feces are not clearly understood and require further investigation. Factors such as diet, host age, and animal health may influence the host sensitivity and stability of a marker gene (35) within a host. BacV6-21 and HPyV were not detected in human feces, as these are associated with sewer pipes and human urine (30, 39). Quantitative data for the concentrations of sewage-associated marker genes in human and nonhu-

man fecal samples provide important information with regard to the ability to detect their presence in environmental waters. Because the concentrations of sewage-associated marker genes are relatively consistent in sewage (3, 26, 40), concentrations in environmental waters can be compared with the concentrations in untreated sewage to obtain information on the amount of sewage present immediately after a contamination event.

In this study, we have provided quantitative data of sewage-associated markers in both human and nonhuman fecal samples. An important factor that may influence the estimation of sewage-associated markers or target pathogens in human and nonhuman hosts is the unit of measurement (41). A number of strategies, such as (i) fecal wet mass, (ii) fecal dry mass, (iii) GC of genetic markers, and (iv) a mass of total DNA, have been tested (41–43). The majority of the studies in the research literature provided quantitative data per gram of wet weight feces or liter of wastewater (43–46). The advantage of this approach is that the total loading of fecal contamination in catchment waters can be calculated, and such data can be used in reverse quantitative microbial risk assessment (QMRA) analysis to determine the potential health risks. However, it will require correction of concentration data due to loss of DNA through extraction processes (47). Using the mass of DNA has some advantages, such as avoiding the need to measure and correct for DNA extraction efficiencies and error introduced by sample variability (solid or liquid phases). The standardization process involves dilution of DNA, which may also relieve PCR inhibition. However, little information is available on what mass of DNA should be used in qPCR amplifications. One study determined the concentrations of marker genes in primary influent and fecal samples from 20 animal species across the United States by standardized DNA concentration to 1 ng per qPCR reaction (41). However, several samples at the test concentration fell below the assay lower limit of quantification (ALLOQ). Care should be taken, because the lack of detection data due to small test quantity of DNA may affect host sensitivity and host specificity values.

The following is a list of recommendations and guidance which can be considered by regulators and scientists implementing MST tools for water quality monitoring.

- Assessments for the host specificity, sensitivity, and concentrations of marker genes should be an ongoing practice by testing a panel of human and nonhuman fecal samples. This is important given the high degree of variability of microbes in the gut due to cohabitation, diet, physiology, feeding habits, and geographical stability. In addition, *in silico* host specificity assessment should be performed using BLAST searches against the growing NCBI database of microbiome sequences NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) but should not be a replacement for testing fecal samples.
- Quantitative PCR assay lower limit of detection (ALLOD), ALLOQ, method lower limit of detection (MLLOD), method lower limit of quantification (MLLOQ), DNA extraction efficiency (percent), and inhibition test results should be reported.
- Marker gene selection should consider host specificity and sensitivity and their concentration in the host feces. Background information can be obtained by literature review on the performance characteristics of these marker genes followed by a validation study prior to field testing.
- Markers should be validated when used in a new geography (on the scale of states for the case of Australia) or in catchments/watersheds with different land uses or sources than previously investigated. The numbers of samples tested and the regime for testing should consider limits of detection and coverage of animal populations (separate farms, etc.). Human and nonhuman fecal samples should be carefully selected by performing a thorough sanitary survey in the study area and based on local area knowledge. For example, if the study area is highly urbanized with limited or no agricultural input of fecal contamination, then cattle or other agricultural animals can be excluded from the performance characteristics study. Geographic information system (GIS) analysis can serve as an important tool to

identify land use patterns and intensive farming areas in close proximity to waterways. How many nontarget fecal samples should be included in the host specificity testing is not currently known. The more the better, but inclusion criteria such as the most abundant animals in the catchments, size of the fecal pat, and their close proximity to waterways need to be considered. The U.S. EPA MST guide document recommends that 10 or more samples per host should be used for host specificity and should not be collected from the same farm or location within a catchment (31).

- Individual fecal samples should be tested for host specificity analysis over pooled or composite fecal samples. If a false-positive detection is obtained for composite fecal samples, it is not possible to find out how many individual fecal samples contributed to that result. It is also possible that the signal from one positive fecal sample could be missed because the signal is diluted by the other fecal samples in the pool or composite. However, when resources are limited, pooled or composite fecal samples can be used in the host specificity assay. In this way, multiple fecal samples can be tested simultaneously with reduced cost. If a pooled sample is positive, then the individual fecal samples that were included in the pool should be tested separately to identify the number of true positives in the pool.
- Composite fecal samples are ideal for the host sensitivity assay. This is especially important for sewage contamination or agricultural wastewater tracking in developing countries. Sewage and septic wastewater are more likely to reach water bodies due to rapid urbanization. Since open defecation is not practiced in developing nations, human feces are unlikely to contaminate water bodies. The number of samples that are required to determine host sensitivity has not been determined. However, multiple-sample collection at different time periods may be required to capture the temporal variability of marker genes.
- It is unlikely that sewage-associated marker genes will exhibit absolute host specificity globally. Conditional probability associated with combinations of marker genes can be determined using Bayesian statistics. This will allow the calculation of probabilities of correctly identifying sewage pollution in the catchments.
- Consistent terminology should be used. The term "host specificity" is more appropriate for viruses than bacteria. However, not all viruses are host specific. For bacteria, the term "host associated" is more accurate. Therefore, to avoid confusion, the term "host associated" should be used for both bacteria and viruses. Researchers need to reach a consensus on key terminologies that are currently used in MST studies.
- Host specificity and sensitivity data must be presented in the quantitative form, not as binary (presence/absence) data. Quantitative results should be presented per gram or liter as well as per nanogram of DNA to compare results among studies.
- Two markers per host should be used in field investigations to improve the confidence in results. Any marker with specificity of <0.8 and sensitivity of <0.3 should be used with caution and must be paired with additional markers to avoid false-positive and false-negative results. Currently, there is no universal criterion for a specificity measure, but it is generally agreed that assays with <0.8 host specificity may not be useful in most cases.

In conclusion, two important advantages of the sewage-associated marker genes over traditional fecal indicator bacteria (FIB) and pathogen monitoring are that (i) these marker genes are highly associated with sewage and (ii) their concentrations are much greater in sewage than that of enteric pathogens, thus facilitating new opportunities for water quality monitoring. Among the marker genes tested, HF183, Lachno3, and crAssphage CPQ_056 appeared to be the most suitable for sewage contamination

tracking in environmental waters and should be used synergistically. BacV6-21 shows lower specificity and could be a useful confirmatory tool. Low concentrations of enteric viruses such as HAdV and HPyV may hinder their application for sensitive detection of low levels of sewage contamination in water and, if used, should be paired with an additional marker gene, such as HF183, Lachno3, or crAssphage.

MATERIALS AND METHODS

Animal and human fecal sample collection. To determine the host sensitivity of the sewage-associated marker genes, 32 human (29 individual feces and 3 untreated sewage samples) and 360 nonhuman fecal samples were collected. These samples were comprised of our archived DNA samples ($n = 169$) extracted from fecal samples in a previous study (3) and new fecal samples ($n = 223$) collected in this study (Table 1). Three composite untreated sewage samples representing human hosts were collected from the influent of three sewage treatment plants (STPs) from Brisbane, Perth, and Tasmania. In this study, fresh human fecal samples were collected from healthy individuals. We collected cat fecal samples from the veterinary hospital located at University of Queensland, Gatton Campus, and six different households. Cattle fecal samples were collected from five farms located on the outskirts of Brisbane, whereas chicken fecal samples were collected from the backyard of six different households and a chicken processing farm in Brisbane. Deer fecal samples were sourced from a deer sanctuary in Mount Samson, Brisbane, on two separate occasions. We collected dog fecal samples from three dog parks and a veterinary hospital. Emu fecal samples were collected from an emu farm on two separate occasions. Deer sanctuaries, dog parks, and emu farms had no animal species other than deer, dog, and emu. Goat fecal samples were collected from the veterinary hospital located at University of Queensland, Gatton Campus. Horse fecal samples were collected from two different horse racecourses. Kangaroo fecal samples were collected from a sanctuary and the wild. Koala fecal samples were collected from a Koala sanctuary. Pig fecal samples were collected from two abattoirs. Sheep fecal samples were collected from the veterinary hospital located at University of Queensland, Gatton Campus, and three farms located in Toowoomba region. Waterfowl fecal samples were collected from the banks of ponds and three lakes in Brisbane.

DNA extraction. DNA was extracted from an aliquot of 100 μl of untreated sewage sample using the DNeasy PowerSoil kit (Qiagen, Carlsbad, CA), with minor modifications. A QIAamp DNA stool minikit or DNeasy PowerSoil kit was used to extract DNA from individual fecal samples. DNA concentrations were measured with a spectrophotometer (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE, USA).

PCR inhibition. An experiment was conducted to determine the presence of PCR inhibitors in untreated sewage and fecal DNA samples using a Sketa22 qPCR assay (48). Untreated sewage, human, and animal fecal samples with a 2-quantification cycle (C_q) delay were considered to have potential PCR inhibitors (3). Samples with PCR inhibitors were subjected to a 10-fold dilution with Tris-EDTA (TE) buffer. PCR-uninhibited and 10-fold-diluted (inhibition relieved) samples were used for qPCR analysis of sewage-associated marker genes.

qPCR assays. Previously published qPCR assays were used for the analysis of the sewage-associated marker genes (7, 8, 29, 30, 49, 50). The primers and probes for each assay are shown in Table S6 in the supplemental material along with qPCR cycling parameters. gBlocks gene fragments were used to prepare qPCR standards, ranging from 10^6 to 1 GC/μl of DNA (Integrated DNA Technology, Coralville, IA). All qPCR amplifications were performed in 20-μl reaction mixtures using SsoAdvanced universal probes supermix (Bio-Rad Laboratories, Richmond, CA). qPCR mixtures contained 10 μl of Supermix, 1,000 nM forward primer, 1,000 nM reverse primer, 100 nM probe (for HF183, BacV6-21, Lachno3, and CPQ_056), 200 nM forward primer, 200 nM reverse primer and 100 nM probe (for HAdV and HPyV), and 2 μl of template DNA. The qPCR assays were performed using a Bio-Rad CFX96 thermal cycler. All qPCR reactions were performed in triplicate. For each qPCR run, a series of standards (2×10^6 to 2 GC per reaction) and no-template controls ($n = 3$) were included.

qPCR assay performance characteristics, lower limit of detection, and quantification. The qPCR standards were analyzed to determine the amplification efficiencies (E) and the correlation coefficient (R^2) for each assay. The qPCR assay's lower limit of detection (ALLOD) and quantification (ALLOQ) values were determined from C_q values of the standards. The qPCR ALLOD was defined as the number of copies that could be detected in 2 out of 3 qPCR assays, while the ALLOQ was the number of copies that could be quantified in 2 out of 3 qPCR assays. Four separate standard curves were generated for each qPCR assay. The optimal reaction conditions were determined based on the following criteria: a slope factor of -3.09 to -3.59, corresponding to a PCR efficiency of 90% to 110%, and a correlation coefficient of >0.98. The qPCR efficiencies were within the optimal range as recommended by MIQE guidelines (51). The qPCR ALLOQ and ALLOD were determined to be 20 and 2 GC/reaction, respectively, for all assays.

Quality control. A reagent blank was included for each batch of DNA samples to ensure no carryover contamination occurred from DNA extraction reagents. No carryover contamination was observed in extracted DNA samples. To minimize qPCR contamination, DNA extraction and qPCR setup were performed in separate laboratories.

Data analysis. The host specificity and sensitivity values of the studied marker genes were determined as follows. Host sensitivity was determined by TP/(FN + TP) and host specificity by TN/(FP + TN), where TP is true positive (human fecal and untreated sewage samples were positive for sewage-associated marker genes), FN is false negative (human and untreated sewage samples were negative for the tested marker genes), TN is true negative (nonhuman fecal samples were negative for sewage-associated marker genes), and FP is false positive (nonhuman fecal samples were positive for the

sewage-associated marker genes) (16, 17) (Table S7). The accuracy of each assay was determined as (TP + TN)/(TP + FP + FN + TN). Samples were considered quantifiable when the crAssphage marker gene levels were above the qPCR ALLOQ. Samples that were below the ALLOQ and above the ALLOD levels and generated PCR amplification were considered positive but not quantifiable. Samples below the ALLOD were considered negative. For statistical analysis, sewage-associated marker gene concentrations in sewage and fecal samples were $\log_{10}$ transformed. The nonparametric Kruskal-Wallis one-way analysis of variance (ANOVA) with Tukey's honestly significance difference (HSD; $P \leq 0.05$) *post hoc* test at the 95% confidence level was performed to determine the significant differences among the concentrations of sewage-associated marker genes in sewage and fecal samples.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00641-19.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

REFERENCES

1. Harwood VJ, Staley C, Badgley BD, Borges K, Korajkic A. 2014. Microbial source tracking markers for detection of fecal contamination in environmental waters: relationships between pathogens and human health outcomes. FEMS Microbiol Rev 38:1–40. https://doi.org/10.1111/1574-6976.12031.
2. Unno T, Staley C, Brown CM, Dukki H, Sadowsky MJ, Hur H-G. 2018. Fecal pollution: new trends and challenges in microbial source tracking using next-generation sequencing. Environ Microbiol 20:3132–3140. https://doi.org/10.1111/1462-2920.14281.
3. Ahmed W, Payyappat S, Cassidy M, Besley C, Power K. 2018. Novel crAssphage marker genes ascertain sewage pollution in a recreational lake receiving urban stormwater runoff. Water Res 145:769–778. https://doi.org/10.1016/j.watres.2018.08.049.
4. Haramoto E, Osada R. 2018. Assessment and application of host-specific *Bacteroidales* genetic markers for microbial source tracking if river water in Japan. PLoS One 13:e0207727. https://doi.org/10.1371/journal.pone.0207727.
5. Jarde E, Jeanneau L, Harrault L, Quenot E, Solecki O, Petitjean P, Lozach S, Cheve J, Gourmelon M. 2018. Application of a microbial source tracking based on bacterial and chemical markers in headwater and coastal catchments. Sci Total Environ 610-611:55–63. https://doi.org/10.1016/j.scitotenv.2017.07.235.
6. Kongprajug A, Chyerochana N, Somnark P, Leelapanang Kampaengthong P, Mongkolsuk S, Sirikanchana K. 2018. Human and animal microbial source tracking in a tropical river with multiple land use activities. Int J Hyg Environ Health 2019:30672-2. https://doi.org/10.1016/j.ijheh.2019.01.005.
7. Green HC, Haugland RA, Varma M, Millen HT, Borchardt MA, Field KG, Walters WA, Knight R, Sivaganesan M, Kelty CA, Shanks OC. 2014. Improved HF183 quantitative real-time PCR assay for characterization of human fecal pollution in ambient surface water samples. Appl Environ Microbiol 80:3086–3094. https://doi.org/10.1128/AEM.04137-13.
8. Stachler E, Kelty C, Sivaganesan M, Li X, Bibby K, Shanks OC. 2017. Quantitative CrAssphage PCR assays for human fecal pollution measurement. Environ Sci Technol 51:9146–9154. https://doi.org/10.1021/acs.est.7b02703.
9. Kildare BJ, Leutenegger CM, McSwain BS, Bambic DG, Rajal VB, Wuertz S. 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal *Bacteroidales*: a Bayesian approach. Water Res 41:3701–3715. https://doi.org/10.1016/j.watres.2007.06.037.
10. Mieszkin S, Furet JP, Corthier G, Gourmelon M. 2009. Estimation of pig fecal contamination in a river catchment by real-time PCR using two pig-specific *Bacteroidales* 16S rRNA genetic markers. Appl Environ Microbiol 75:3045–3054. https://doi.org/10.1128/AEM.02343-08.
11. Lu JR, Santo Domingo JW, Lamendella R, Edge T, Hill S. 2008. Phylogenetic diversity and molecular detection of bacteria in gull feces. Appl Environ Microbiol 74:3969–3976. https://doi.org/10.1128/AEM.00019-08.
12. Weidhaas JL, Macbeth TW, Olsen RL, Sadowsky MJ, Norat D, Harwood VJ. 2010. Identification of a *Brevibacterium* marker gene specific to poultry litter and development of a quantitative PCR assay. J Appl Microbiol 109:334–347. https://doi.org/10.1111/j.1365-2672.2010.04666.x.
13. Green HC, White KM, Kelty CA, Shanks OC. 2014. Development of rapid canine fecal source identification PCR-based assays. Environ Sci Technol 48:11453–11461. https://doi.org/10.1021/es502637b.
14. Devane ML, Robson B, Nourozi F, Scholes P, Gilpin BJ. 2007. A PCR marker for detection in surface waters of fecal pollution derived from ducks. Water Res 41:3553–3560. https://doi.org/10.1016/j.watres.2007.06.043.
15. Devane M, Robson B, Nourozi F, Wood D, Gilpin BJ. 2013. Distinguishing human and possum faeces using PCR markers. J Water Health 11:397–409. https://doi.org/10.2166/wh.2013.122.
16. Stoeckel DM, Harwood VJ. 2007. Performance, design, and analysis in microbial source tracking studies. Appl Environ Microbiol 73:2405–2415. https://doi.org/10.1128/AEM.02473-06.
17. Ahmed W, Hughes B, Harwood VJ. 2016. Current status of marker genes of *Bacteroides* and related taxa for identifying sewage pollution in environmental waters. Water 8:231. https://doi.org/10.3390/w8060231.
18. Kinzelman J, Ahmed W. 2014. Microbial source tracking: field study planning and implementation, p 3.4.5-1–3.4.5-11. *In* Yates M, Nakatsu C, Miller R, Pillai S (ed), Manual of environmental microbiology, 4th ed. ASM Press, Washington, DC.
19. Harwood VJ, Brownell M, Wang S, Lepo J, Ellender RD, Ajidahun A, Hellein KN, Kennedy E, Ye X, Flood C. 2009. Validation and field testing of library-independent microbial source tracking methods in the Gulf of Mexico. Water Res 43:4812–4819. https://doi.org/10.1016/j.watres.2009.06.029.
20. Silkie SS, Nelson KL. 2009. Concentrations of host-specific and genetic fecal markers measured by quantitative PCR in raw sewage and fresh animal feces. Water Res 43:4860–4871. https://doi.org/10.1016/j.watres.2009.08.017.
21. Odagiri M, Schriewer A, Hanley K, Wuertz S, Misra PR, Panigrahi P, Jenkins MW. 2015. Validation of *Bacteroidales* quantitative PCR assays targeting human and animal fecal contamination in the public and domestic domains in India. Sci Total Environ 502:462–470. https://doi.org/10.1016/j.scitotenv.2014.09.040.
22. Ahmed W, Lobos A, Senkbeil J, Peraud J, Gallard J, Harwood VJ. 2018. Evaluation of the novel crAssphage marker for sewage pollution tracking in storm drain outfalls in Tampa, Florida. Water Res 131:142–150. https://doi.org/10.1016/j.watres.2017.12.011.
23. Gyawali P, Croucher D, Devane M, Ahmed W, Hewitt J. 2019. Evaluation of pepper mild mottle virus as an indicator of human fecal pollution in shellfish and growing waters. Water Res 154:370–376. https://doi.org/10.1016/j.watres.2019.02.003.
24. Harwood V, Shanks O, Koraijkic A, Verbyla M, Ahmed W, Iriate M. 2017. General and host-associated bacterial indicators of faecal pollution, p 1–38. *In* Rose JB, Jiménez-Cisneros B (ed), Global water pathogen project. Michigan State University, East Lansing, MI.
25. Schneeberger CL, O'Driscoll M, Humphery C, Henry K, Deal N, Seiber K, Hill VR, Zarate-Bermudez M. 2015. Fate and transport of enteric microbes from septic systems in a coastal watershed. J Environ Health 77:22–30.
26. Olds HT, Corsi SR, Dila DK, Halmo KM, Bootsma MJ, McLellan SL. 2018. High levels of sewage contamination released from urban areas after storm events: A quantitative survey with sewage specific bacterial indicators. PLoS Med 15:e1002641.
27. Zhu H, Yuan F, Yuan Z, Liu R, Xie F, Huang L, Liu X, Jiang X, Wang J, Xu Y, Shen Z, Liu D, Zhang R, Lu Y. 2018. Monitoring of Poyang lake water for sewage contamination using human enteric viruses as an indicator. Virol J 15:3. https://doi.org/10.1186/s12985-017-0916-0.

28. Campos CJA, Kershaw S, Morgan OC, Lees DN. 2017. Risk factors for norovirus contamination of shellfish water catchments in England and Wales. Int J Food Microbiol 241:318–324. https://doi.org/10.1016/j.ijfoodmicro.2016.10.028.
29. Feng S, Bootsma M, McLellan SL. 2018. Novel human-associated Lachnospiraceae genetic markers improve detection of fecal pollution sources in urban waters. Appl Environ Microbiol 84:e00309-18. https://doi.org/10.1128/AEM.00309-18.
30. Feng S, McLellan SL. 2019. Highly specific sewage-derived Bacteroides qPCR assays target sewage polluted waters. Appl Environ Microbiol 85:e02696-18. https://doi.org/10.1128/AEM.02696-18.
31. U.S. Environmental Protection Agency. 2005. Microbial Source Tracking Guide Document EPA/600/R-05/064. U.S. Environmental Protection Agency, Washington, DC.
32. Boehm AB, Van de Werfhorst LC, Griffith JF, Holden P, Jay J, Shanks OC, Wang D, Weisberg SB. 2013. Performance of forty-one microbial source-tracking methods: a twenty seven lab evaluation study. Water Res 47:6812–6828. https://doi.org/10.1016/j.watres.2012.12.046.
33. Ahmed W, Payyappat S, Cassidy M, Besley C. 2019. A duplex PCR assay for the simultaneous quantification of Bacteroides HF183 and crAssphage CPQ_056 marker genes in untreated sewage and stormwater. Environ Int 126:252–259. https://doi.org/10.1016/j.envint.2019.01.035.
34. Zhang Q, Ishii S. 2018. Improved simultaneous quantification of multiple waterborne pathogens and fecal indicator bacteria with the use of a sample process control. Water Res 137:193–200. https://doi.org/10.1016/j.watres.2018.03.023.
35. Shanks OC, Atikovic E, Blackwood AD, Lu J, Noble RT, Domingo JS, Seifring S, Sivaganesan M, Haugland RA. 2008. Quantitative PCR for detection and enumeration of genetic markers of bovine fecal pollution. Appl Environ Microbiol 74:745–752. https://doi.org/10.1128/AEM.01843-07.
36. Stachler E, Akyon B, De Carvalho NA, Ference C, Bibby K. 2018. crAssphage qPCR markers with culturable and molecular indicators of human fecal pollution in an impacted urban watershed. Environ Sci Technol 52:7505–7512. https://doi.org/10.1021/acs.est.8b00638.
37. Liang Y, Jin X, Huang Y, Chen S. 2018. Development and application of a real-time polymerase chain reaction for detection of a novel gut bacteriophage (crAssphage). J Med Virol 90:464–468. https://doi.org/10.1002/jmv.24974.
38. Cinek O, Mazankova K, Kramna L, Odeh R, Alassaf A, Ibekwe MU, Ahmadov G, Mekki H, Abdullah MA, Elmahi BME, Hyoty H, Rainetova P. 2018. Quantitative crAssphage real-time PCR assay derived from data of multiple geographically distant populations. J Med Virol 90:767–771. https://doi.org/10.1002/jmv.25012.
39. Ahmed W, Wan C, Goonetilleke A, Gardner T. 2010. Evaluating sewage-associated JCV and BKV polyomaviruses for sourcing human fecal pollution in a coastal river in Southeast Queensland, Australia. J. Environ Qual 39:1743–1750. https://doi.org/10.2134/jeq2010.0062.
40. Sercu B, Van de Werfhorst LC, Murray JLS, Holden PA. 2011. Sewage exfiltration as a source of storm drain contamination during dry weather in urban watersheds. Environ Sci Technol 45:7151–7157. https://doi.org/10.1021/es200981k.
41. Kelty CA, Varma M, Sivaganesan M, Haugland R, Shanks OC. 2012. Distribution of genetic marker concentrations for fecal indicator bacteria in sewage and animal feces. Appl Environ Microbiol 78:4225–4232. https://doi.org/10.1128/AEM.07819-11.
42. Wright ME, Solo-Gabriele HM, Elmir S, Fleming LE. 2009. Microbial load from animal feces at a recreational beach. Mar Pollut Bull 58:1649–1656. https://doi.org/10.1016/j.marpolbul.2009.07.003.
43. Ahmed W, Sidhu JPS, Smith K, Beale DJ, Gyawali P, Toze S. 2016. Distributions of fecal markers in wastewater from different climatic zones for human fecal pollution tracking in Australian surface waters. Appl Environ Microbiol 82:1316–1323. https://doi.org/10.1128/AEM.03765-15.
44. Seurinck S, Defoirdt T, Verstraete W, Siciliano SD. 2005. Detection and quantification of human-specific HF183 Bacteroides 16S rRNA genetic marker with real-time PCR for assessment of human fecal pollution in freshwater. Environ Microbiol 7:249–259. https://doi.org/10.1111/j.1462-2920.2004.00702.x.
45. Reischer GH, Kasper DC, Steinborn R, Farnleitner AH, Mach RL. 2007. A quantitative real-time PCR assay for the highly sensitive and specific detection of human faecal influence in spring water from a large alpine catchment area. Lett Appl Microbiol 44:351–356. https://doi.org/10.1111/j.1472-765X.2006.02094.x.
46. Tambalo DD, Fremaux B, Boa T, Yost CK. 2012. Persistence of host-associated Bacteroidales gene markers and their quantitative detection of in an urban and agricultural mixed prairie watershed. Water Res 46:2891–2904. https://doi.org/10.1016/j.watres.2012.02.048.
47. Wang D, Green HC, Shanks OC, Boehm AB. 2014. New performance metrics for quantitative polymerase chain reaction-based microbial source tracking methods. Environ Sci Technol Lett 1:20–25. https://doi.org/10.1021/ez400022t.
48. Haugland RA, Siefring SC, Wymer LJ, Brenner KP, Dufour AP. 2005. Comparison of *Enterococcus* measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Res 39:559–568. https://doi.org/10.1016/j.watres.2004.11.011.
49. Heim A, Ebnet C, Harste G, Pring-Akerblom P. 2003. Rapid and quantitative detection of human adenovirus DNA by real-time PCR. J Med Virol 70:228–229. https://doi.org/10.1002/jmv.10382.
50. McQuaig S, Scott T, Lukasik J, Paul J, Harwood V. 2009. Quantification of human polyomaviruses JC virus and BK virus by TaqMan quantitative PCR and comparison to other water quality indicators in water and fecal samples. Appl Environ Microbiol 75:3379–3388. https://doi.org/10.1128/AEM.02302-08.
51. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622. https://doi.org/10.1373/clinchem.2008.112797.