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ORIGINAL PAPER

# Performance Evaluation of Human-Specific Viral Markers and Application of Pepper Mild Mottle Virus and CrAssphage to Environmental Water Samples as Fecal Pollution Markers in the Kathmandu Valley, Nepal

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**Abstract**
Monitoring of environmental water is crucial to protecting humans and animals from possible health risks. Although numerous human-specific viral markers have been designed to track the presence of human fecal contamination in water, they lack adequate sensitivity and specificity in different geographical regions. We evaluated the performances of six human-specific viral markers [Aichi virus 1 (AiV-1), human adenoviruses (HAdVs), BK and JC polyomaviruses (BKPyVs and JCPyVs), pepper mild mottle virus (PMMoV), and crAssphage] using 122 fecal-source samples collected from humans and five animal hosts in the Kathmandu Valley, Nepal. PMMoV and crAssphage showed high sensitivity (90–100%) with concentrations of 4.5–9.1 and 6.2–7.0 $\log_{10}$ copies/g wet feces ($n = 10$), respectively, whereas BKPyVs, JCPyVs, HAdVs, and AiV-1 showed poor performances with sensitivities of 30–40%. PMMoV and crAssphage were detected in 40–100% and 8–90%, respectively, of all types of animal fecal sources and showed no significantly different concentrations among most of the fecal sources (Kruskal–Wallis test, $P > 0.05$), suggesting their applicability as general fecal pollution markers. Furthermore, a total of 115 environmental water samples were tested for PMMoV and crAssphage to identify fecal pollution. PMMoV and crAssphage were successfully detected in 62% (71/115) and 73% (84/115) of water samples, respectively. The greater abundance and higher mean concentration of crAssphage ($4.1 \pm 0.9$ $\log_{10}$ copies/L) compared with PMMoV ($3.3 \pm 1.4$ $\log_{10}$ copies/L) indicated greater chance of detection of crAssphage in water samples, suggesting that crAssphage could be preferred to PMMoV as a marker of fecal pollution.

**Keywords** CrAssphage · Fecal contamination · Microbial source tracking · Pepper mild mottle virus

## Introduction

Identifying the source of fecal contamination of water bodies is an important first step in controlling pollution and managing health risk. Microbial source tracking (MST) is a technique to identify specific sources of fecal pollution introduced into water bodies through humans or animals. One of the library-independent MST methods, using host-specific *Bacteroidales* genetic markers, such as HF183, BacHum, and Human-Bac1, is used extensively to

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differentiate between human and animal fecal-source contamination (Betancourt and Fujioka 2006; Okabe et al. 2007; Odagiri et al. 2016; Haramoto 2018; Haramoto and Osada 2018; Malla et al. 2018a). However, application of *Bacteroidales* genetic markers has been criticized because of cross-amplification among human and animal species (e.g., cat and dog) living in close proximity and animals with similar digestive physiologies (e.g., human and swine) (Layton et al. 2006; Kildare et al. 2007; Okabe et al. 2007). In addition, *Bacteroidales* genetic markers are at times not directly correlated with human enteric viruses (Harwood et al. 2014), and waterborne viral outbreaks have also been reported even when general bacterial fecal indicators were not detected or were below the guideline values (Sinclair et al. 2009).

Although bacterial markers have been used for fecal-source identification, some studies have suggested the use of viral markers (Okoh et al. 2010; Rodriguez-Lazaro et al. 2012; Symonds and Breitbart 2015). Viral markers are gaining special attention for MST due to their great abundance in the feces and urine of hosts, their high persistence in environmental waters, and their highly host-specific nature (Ahmed and Harwood 2017). Human enteric viruses, such as human adenoviruses (HAdVs), Aichi virus 1 (AiV-1), and human polyomaviruses, have been investigated as human fecal markers because of their abundance in wastewater (Kitajima et al. 2014) and rivers contaminated with sewage (Rusinol et al. 2014) and their non-seasonal nature (Kitajima et al. 2014). Because these viruses are causes of waterborne diseases in human, their presence in water reflects a direct health risk.

HAdVs and JC polyomaviruses (JCPyVs) are highly prevalent all over the world in different environmental water matrices (Biofill-Mas et al. 2000; McQuaig et al. 2009). Although high sensitivity and specificity of HAdVs have been reported (Hundesa et al. 2006; Ahmed et al. 2010a), the detection of HAdVs in fecal-source samples of cattle, swine, poultry, and wild avian using a microarray technique has been reported (Li et al. 2015). Previous studies have reported high sensitivity of JCPyVs and BK polyomaviruses (BKPyVs) in human fecal-contaminated environmental samples (McQuaig et al. 2009) and high specificity when evaluated using fecal samples of potential hosts, such as dogs, chickens, cow, sheep, ducks, humans, and birds (McQuaig et al. 2009; Ahmed et al. 2010b; Staley et al. 2012). AiV-1 was abundant in wastewater samples and in different environmental water matrices including river water and groundwater samples (Kitajima et al. 2011; Kitajima and Gerba 2015; Haramoto and Kitajima 2017).

Pepper mild mottle virus (PMMoV), a plant pathogen that originates from processed pepper products and is excreted in human feces at high concentrations, has been proposed as an indicator of human fecal pollution (Rosario et al. 2009; Hamza et al. 2011; Haramoto et al. 2013; Betancourt et al. 2014; Kuroda et al. 2014; Kitajima et al. 2018; Symonds et al. 2018). The advantage of using PMMoV as an indicator of fecal pollution is that this virus is dietary in origin, independent of active human infection and seasons, and is more abundant than the pathogenic viruses (Rosario et al. 2009; Hata et al. 2018; Kitajima et al. 2018). However, the significant variation in the occurrence of PMMoV among geographic regions (Haramoto et al. 2013) and differences in dietary preferences in different parts of the world highlight the need for validation studies in a specific geographic region prior to its use as a human fecal marker (Rosario et al. 2009).

CrAssphage, a novel bacteriophage, was reported to be present in the majority of published human fecal metagenomes in a recent metagenomics study (Dutilh et al. 2014). CrAssphage has been proposed as a human fecal indicator (Garcia-Aljaro et al. 2017; Stachler and Bibby 2014; Stachler et al. 2017, 2018) and has been applied to the tracking of fecal-source pollution (Ahmed et al. 2018a, b; Stachler et al. 2018) because its abundance was higher than that of known human-associated viruses, including noroviruses and adenoviruses (Stachler and Bibby 2014) and other phages in the human gut (Dutilh et al. 2014). However, because of geographic dependence in the abundance of crAssphage, previous studies have recommended further testing, using fecal samples from a wide variety of animal species and sewage collected across a broad geographical range prior to the use of crAssphage as a human-specific fecal marker (Stachler and Bibby 2014; Stachler et al. 2017; Ahmed et al. 2018a). It has also been recommended for use in combination with other markers (Ahmed et al. 2018a).

Kathmandu, the capital city of Nepal, has a population of 2.51 million (CBS 2012) and is facing a severe water scarcity (KUKL 2017). In the valley, surface water, the main source of municipal water (KUKL 2017), and the groundwater sources, the alternative water sources of households (Shrestha et al. 2017), have been reported contaminated with pathogens (Haramoto et al. 2011; Haramoto and Kitajima 2017; Ghaju Shrestha et al. 2017; Tandukar et al. 2018). The primary objective of this study was to investigate the performances of human-specific viral markers, PMMoV, and crAssphage, in order to examine their suitability for tracking human fecal pollution using fecal-source samples from human and non-human host groups collected in the valley. In addition, the selected markers, PMMoV and crAssphage, were applied to test for fecal pollution in different environmental water samples collected in the study area.

## Materials and Methods

### Collection and Processing of Fecal-Source Samples

Altogether, 122 composite fecal-source samples, including 54 samples that had been analyzed for the validation of

host-specific *Bacteroidales* assays in our previous study (Malla et al. 2018a), were collected in sterilized containers by using sterilized disposable spoons from different species from various locations in the Kathmandu Valley in December 2015, December 2016, and August 2017, including human sewage ($n = 10$), feces of ruminants ($n = 12$), pigs ($n = 10$), dogs ($n = 30$), chickens ($n = 30$), and ducks ($n = 10$), and manures of ruminants ($n = 10$) and chickens ($n = 10$). Each composite fecal-source sample contained approximately 5 g of fecal-source sample from five different individuals of the same target host. Animal fecal-source samples were collected from the ground within the farms, except for dog samples, in which samples were collected from a dog training school, veterinary clinics, and dog shelters. Human sewage samples were collected from the collection tanks of mobile public toilet vans and each composite sample contained approximately 5 g of human feces each from five different spots within a tank. Samples were stored in an icebox and transported to the laboratory within 6 h of collection. Samples were stored at − 25 °C after performing initial processing.

The collected composite fecal-source sample was processed to obtain a 1% fecal suspension in phosphate-buffered saline as described previously (Malla et al. 2018a).

## Collection of Water Samples

In addition to the 97 water samples collected for the identification of fecal contamination sources using host-specific *Bacteroidales* assays (Malla et al. 2018b), an additional 18 water samples were collected from rivers ($n = 8$) and springs ($n = 10$), to identify fecal pollution, covering different parts of the Kathmandu Valley in the dry (February and March; $n = 52$) and wet seasons (August and September; $n = 63$) of 2016. The water samples, 1100 mL for all water source types except for river for which 100 mL was collected, were collected in sterilized plastic bottles, stored in an icebox, and transported to the laboratory within 6 h of sample collection.

## Enumeration of *E. coli* in Fecal-Source and Water Samples

*E. coli* in fecal-source and water samples was measured using the Colilert method (IDEXX Laboratories, Westbrook, MA, USA) following the manufacturer's protocol, as described previously (Ghaju Shrestha et al. 2017; Malla et al. 2018b). Of the total of 122 composite fecal-source samples, 54 samples had been analyzed previously (Malla et al. 2018a), whereas 97 of the 115 water samples had also been analyzed in a previous study (Malla et al. 2018b).

## Processing of Viruses in Fecal-Source and Water Samples

Fecal-source samples were concentrated by the centrifugation of a 1.5 mL of 1% fecal suspension at 7500×*g* for 10 min at 4 °C, and the resulting supernatant (1 mL, equivalent to 10 mg of wet fecal-source sample) was used as a virus concentrate. An electronegative membrane-vortex method (Haramoto et al. 2012) with some modifications was used for the concentration of viruses in the water samples as described previously (Shrestha et al. 2018; Tandukar et al. 2018). Briefly, for the concentration step, 10 mL and 500 µL of 2.5 M $MgCl_2$ were added to 1 L of groundwater samples and 50 mL of river water samples, respectively, which were then filtered through a mixed cellulose-ester membrane (pore size, 0.8 µm; diameter, 90 mm; Merck Millipore, Billerica, MA, USA). After filtration, vigorous vortexing of the membrane was performed with 10 mL of elution buffer containing 0.2 g/L $Na_4P_2O_7{\cdot}10H_2O$, 0.3 g/L $C_{10}H_{13}N_2O_8Na_3{\cdot}3H_2O$, and 0.1 mL/L Tween-80 in a 50-mL plastic tube. This procedure was repeated using 5 mL of the elution buffer. Subsequently, centrifugation was performed at 2000×*g* for 10 min at 4 °C, and the resulting supernatant was filtered using a disposable membrane filter unit (pore size, 0.45 µm; diameter, 28 mm; Advantec, Tokyo, Japan). A Centriprep YM-50 ultrafiltration device (Merck Millipore) was used to further concentrate the filtrate, following the manufacturer's protocol. The final volume of a virus concentrate obtained was noted and stored in a freezer at − 25 °C for future use.

## Extraction of Viral Nucleic Acid and Reverse Transcription

An aliquot (200 µL) of the viral concentrate was used for the extraction of viral DNA (crAssphage, HAdVs, BKPyVs, and JCPyVs) using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) to obtain a 200-µL DNA extract. Similarly, for the extraction of viral RNA (AiV-1 and PMMoV), the viral concentrate (140 µL) was used to obtain a 60-µL RNA extract, using QIAamp Viral RNA Mini Kit (QIAGEN), following the manufacturer's protocol. Virus extractions were performed using a QIAcube automated platform (QIAGEN). A High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used for the reverse transcription of 30-µL viral RNA to obtain 60-µL cDNA, following the manufacturer's protocol.

## Quantification of Viral Genomes by qPCR

Table 1 shows the sequences of primers and probes of the human-specific viral markers used in this study. Each 25-µL qPCR reaction mixture contained 12.5 µL of

Probe qPCR Mix (Takara Bio, Kusatsu, Japan), 1.0 µL each of 10-pmol/µL forward and reverse primers, 1.0 µL of 5-pmol/µL TaqMan (MGB) probe, 7.0 µL of PCR-grade water, and 2.5 µL of template DNA (crAssphage, HAdVs, BKPyVs, and JCPyVs) or cDNA (AiV-1 and PMMoV). Quantification was performed using a Thermal Cycler Dice Real-Time System TP800 (Takara Bio). The thermal cycle conditions were as follows: 95 °C for 30 s, followed by 45 cycles at 95 °C for 5 s and at 60 °C for 30 s (for AiV-1, BKPyVs, and JCPyVs), at 58 °C for 30 s (for HAdVs), at 60 °C for 60 s (for PMMoV), or at 56 °C for 30 s (for crAssphage). Six tenfold serial dilutions of artificially synthesized plasmid DNA containing the amplification region sequence were used to prepare a standard curve, and the genome copy number of each virus was determined on the basis of this curve. In each qPCR run, a negative control was included to check for any contamination. In all the qPCR runs, fecal-source or water samples, standard samples, and negative controls were run in duplicates. The samples were considered negative if the threshold cycle (Ct) value was greater than 40 and considered positive if the respective virus was detected ($Ct \leq 40$) in at least one of the two wells. Limit of detection (LOD) of 5.8, 5.7, 6.7, 5.7, 3.9, and 5.7 $\log_{10}$ copies/g wet feces were used for AiV-1, HAdVs, BKPyVs, JCPyVs, PMMoV, and crAssphage, respectively, while checking their performances as human-specific markers. On the other hand, LOD of 1.6 and 2.6 $\log_{10}$ copies/L for PMMoV and crAssphage, respectively, were used for source tracking.

As recommended previously (Haramoto et al. 2018), the BKPyV-plasmid DNA was used as a qPCR control DNA to estimate the possible inhibition in the fecal-source and water samples during qPCR because of the low positive ratios of BKPyVs in these samples. In brief, 1 µL BKPyV-plasmid DNA (3.64 log copies/µL) was added to 48% (114/237) of the randomly selected fecal-source and water samples and to a non-inhibitory control (NIC) sample (PCR-grade water), which were quantified following the same procedure described earlier for BKPyVs. The efficiency was calculated as follows:

$$\text{Efficiency}(\%) = (\text{Concentration of BKPyV-plasmid DNA in a sample} / \text{Concentration of BKPyV-plasmid DNA in a NIC}) \times 100.$$

**Table 1** Primer and probe sequences of human-specific markers

| Marker | Virus type | Primer/probe | Sequence (5′–3′) | Product length (bp) | References |
|---|---|---|---|---|---|
| AiV-1 | RNA | Forward primer | GTCTCCACHGACACYAAYTGGAC | 108–111 | Kitajima et al. (2013) |
| | | Reverse primer | GTTGTACATRGCAGCCCAGG | | |
| | | TaqMan MGB probe | FAM-TTYTCCTTYGTGCGTGC-NFQ-MGB | | |
| HAdVs | DNA | Forward primer | GCCACGGTGGGGTTTCTAAACTT | 132 | Heim et al. (2003) |
| | | Reverse primer | GCCCCAGTGGTCTTACATGCACATC | | |
| | | TaqMan probe | FAM-TGCACCAGACCCGGGCTCAGGTAC TCCGA-TAMRA | | |
| BKPyVs | DNA | Forward primer | GGCTGAAGTATCTGAGACTTGGG | 78 | Pal et al. (2006) |
| | | Reverse primer | GAAACTGAAGACTCTGGACATGGA | | |
| | | TaqMan probe | FAM-CAAGCACTGAATCCCAATCACAAT GCTC-TAMRA | | |
| JCPyVs | DNA | Forward primer | GGAAAGTCTTTAGGGTCTTCTACCTTT | 89 | Pal et al. (2006) |
| | | Reverse primer | ATGTTTGCCAGTGATGATGAAAA | | |
| | | TaqMan probe | FAM-AGGATCCCAACACTCTACCCCACC TAAAAAGA-TAMRA | | |
| PMMoV | RNA | Forward primer | GAGTGGTTTGACCTTAACGTTTGA | 68 | Zhang et al. (2006) |
| | | Reverse primer | TTGTCGGTTGCAATGCAAGT | | Haramoto et al. (2013) |
| | | TaqMan MGB probe | FAM-CCTACCGAAGCAAATG-NFQ-MGB | | Zhang et al. (2006) |
| CrAssphage | DNA | Forward primer | CAGAAGTACAAACTCCTAAAAAACGTA GAG | 125 | Stachler et al. (2017) |
| | | Reverse primer | GATGACCAATAAACAAGCCATTAGC | | |
| | | TaqMan MGB probe | FAM-AATAACGATTTACGTGATGTAAC-NFQ-MGB | | |

*AiV-1* Aichi virus 1, *HAdVs* human adenoviruses, *BKPyVs* BK polyomaviruses, *JCPyVs* JC polyomaviruses, *PMMoV* pepper mild mottle virus, *FAM* 6-carboxyfluorescein, *MGB* minor groove binder, *NFQ* non-fluorescent quencher, *TAMRA* 5-carboxytetramethylrhodamine

The mean efficiency obtained was 73 ± 22% ($n = 114$), suggesting that there was no inhibition in the samples during qPCR.

### Basis for the Selection of the Markers

Sensitivity and specificity were the two parameters used for the selection of the best performing assays (Odagiri et al. 2015; Malla et al. 2018a). Sensitivity and specificity were calculated as sensitivity = TP/(TP + FN), where TP is the number of true-positive samples, and FN is the number of false-negative samples and specificity = TN/(TN + FP), where TN is the number of true-negative samples and FP is the number of false-positive samples.

### Application of Selected Markers to Microbial Source Tracking

Identification of the fecal contamination in the collected water samples ($n = 115$) was performed using the markers showing the best performance, following a procedure similar to that described for the validation of the host-specific *Bacteroidales* assay (Malla et al. 2018a).

### Statistical Analysis

The Kruskal–Wallis test was used to compare the ratios of crAssphage and *E. coli* concentrations among fecal-source samples. Similarly, the Kruskal–Wallis test was used to compare the ratios of PMMoV and *E. coli* concentrations among fecal-source samples. In order to perform fecal source-wise comparison of PMMoV as well as of crAssphage concentration, Kruskal–Wallis tests were used. The Pearson product-moment correlation was used to determine the relationship between *E. coli* concentration and both PMMoV and crAssphage concentrations in environmental water samples. In order to compare detection ratios and concentrations of PMMoV as well as of crAssphage across different groups of *E. coli* concentrations, $\chi^2$ test and one-way analysis of variance (ANOVA) were performed, respectively. Paired *t* tests were carried out to compare the concentrations of PMMoV and crAssphage in different environmental water samples. In order to compare positive ratios and concentrations of PMMoV as well as of crAssphage between *E. coli*-positive and *E. coli*-negative groups, $\chi^2$ test and independent *t* tests were performed, respectively. For the statistical analysis, one-tenth of the LOD were used for the negative samples. Microsoft Office Excel 2013 (Microsoft Corporation, Redmond, WA, USA) was used to perform statistical analysis, and a significant value was set at $P < 0.05$.

## Results

### Quantification of *Escherichia coli* in Fecal-Source and Water Samples

Similar to the results of a previous study (Malla et al. 2018a), *E. coli* was detected in all additional fecal-source types collected. In this study, the mean concentration of *E. coli* was highest in dog fecal samples (7.3 ± 0.6 $\log_{10}$ most probable number (MPN)/g wet feces) with the lowest in chicken manure samples (4.1 ± 1.8 $\log_{10}$ MPN/g wet feces). In addition to 97 water samples from a previous study (Malla et al. 2018b), *E. coli* was detected in 94% (17/18) of water samples collected in the current study (Table 2). Of the total 115 water samples from the two studies, *E. coli* was detected with the highest mean concentration in river

**Table 2** Positive percentage and concentrations of crAssphage and PMMoV in different water source types

| Source types | No. of samples tested | *E. coli*[a] | | CrAssphage | | PMMoV | |
|---|---|---|---|---|---|---|---|
| | | No. of positive samples (%) | Conc.[b] ($\log_{10}$ MPN/100 mL) (mean ± SD) | No. of positive samples (%) | Conc.[b] ($\log_{10}$ copies/L) (mean ± SD) | No. of positive samples (%) | Conc.[b] ($\log_{10}$ copies/L) (mean ± SD) |
| Shallow dug well | 51 | 44 (86) | 2.3 ± 1.2 | 38 (75) | 3.9 ± 0.3 | 39 (76) | 3.0 ± 1.0 |
| Shallow tube well | 21 | 10 (48) | 1.7 ± 1.5 | 16 (76) | 4.0 ± 0.5 | 13 (62) | 3.1 ± 1.1 |
| Deep tube well | 18 | 11 (61) | 1.7 ± 1.4 | 11 (61) | 4.0 ± 0.4 | 5 (28) | 2.6 ± 0.7 |
| Stone spout | 7 | 6 (86) | 2.5 ± 1.7 | 6 (86) | 4.2 ± 0.6 | 3 (43) | 3.7 ± 0.7 |
| Spring | 10 | 9 (90) | 1.3 ± 1.0 | 7 (70) | 3.7 ± 0.3 | 3 (30) | 2.9 ± 0.5 |
| River | 8 | 8 (100) | 5.7 ± 1.2 | 6 (75) | 6.8 ± 0.8 | 8 (100) | 6.0 ± 0.9 |
| Total | 115 | 88 (77) | 2.4 ± 1.7 | 84 (73) | 4.1 ± 0.9 | 71 (62) | 3.3 ± 1.4 |

[a]Including the data previously reported (Malla et al. 2018b)

[b]Mean concentration of positive samples

*SD* standard deviation

water samples ($5.7 \pm 1.2$ $\log_{10}$ MPN/100 mL) and the lowest mean concentration in spring water samples ($1.3 \pm 1.0$ $\log_{10}$ MPN/100 mL).

## Performances of HAdVs, AiV-1, BKPyVs, JCPyVs, PMMoV, and crAssphage as human-specific markers

As summarized in Table 3, of the six human-specific markers tested, only PMMoV (100%) and crAssphage (90%) showed sufficiently high sensitivity for the tested human sewage samples, whereas BKPyVs, JCPyVs, HAdVs, and AiV-1 showed lower sensitivities of 0, 30, 40, and 40%, respectively. PMMoV showed high cross-reactivity with pig (100%) and duck feces (80%) and 40–60% cross-reactivity with other non-target hosts. Similarly, crAssphage was detected in 90% of the ruminant manure samples and 40–63% of dog, chicken manure, and duck fecal samples. AiV-1, HAdVs, BKPyVs, and JCPyVs showed high specificity (> 80%). Taking into account the low specificity of PMMoV and crAssphage and the low sensitivity of HAdVs, AiV-1, BKPyVs, and JCPyVs, none of these tested markers were reliable to be specific to humans in this study. However, there is no evidence that HAdVs, AiV-1, BKPyVs, and JCPyVs are not specific.

Previous studies have used abundance ratios between discriminating and non-discriminating markers as a useful tool to distinguish human from non-human pollution sources (Sauer et al. 2011; Garcia-Aljaro et al. 2017). As both crAssphage and PMMoV showed high sensitivities (90–100%), the abundance ratio was calculated between crAssphage and *E. coli* concentrations and between PMMoV and *E. coli* concentrations. As shown in Fig. 1, the ratios of crAssphage to *E. coli* concentrations were not significantly different between sewage ($-0.5 \pm 1.4$ $\log_{10}$ copies/g wet feces) and non-human samples ($-1.4$ to $1.7$ $\log_{10}$ copies/g wet feces) (Kruskal–Wallis test, $P > 0.05$), except for chicken fecal sources ($-2.0 \pm 0.8$ $\log_{10}$ copies/g wet feces). Similarly, the ratios of PMMoV to *E. coli* concentrations were not significantly different between sewage ($-0.3 \pm 1.7$ $\log_{10}$ copies/g wet feces) and non-human samples ($-1.6$ to $0.5$ $\log_{10}$ copies/g wet feces (Kruskal–Wallis test, $P > 0.05$), except for chicken and dog fecal sources.

## Performances of crAssphage and PMMoV as Fecal Pollution Markers

The overall positive ratios for PMMoV and crAssphage were 61% (74/122) and 41% (50/122), respectively, in the fecal samples. In addition to being detected in all sewage samples, interestingly, PMMoV was detected in all pig feces, 80% of duck feces, 60% of ruminant manure samples, 58% of ruminant feces, 53% of dog feces, 40% of chicken feces, and 50% of chicken manure samples (Table 3). The mean concentrations of PMMoV were similar in sewage ($6.7 \pm 1.4$ $\log_{10}$ copies/g wet feces) and pig fecal samples ($6.9 \pm 0.5$ $\log_{10}$ copies/g wet feces), whereas the mean concentrations ranged from 4.6 to 5.0 $\log_{10}$ copies/g wet feces in fecal samples of non-target hosts, except for duck samples ($6.1 \pm 0.7$ $\log_{10}$ copies/g wet feces). The PMMoV concentrations were not significantly different among the various types of fecal sources (Kruskal–Wallis test, $P > 0.05$). Likewise, crAssphage was detected in 63% of dog feces, 90% of ruminant manure, 40% of chicken manure, 10% of chicken feces, and 10% of pig feces. The crAssphage concentrations were $6.7 \pm 0.3$ $\log_{10}$ copies/g wet feces in sewage samples, $6.5 \pm 0.1$ $\log_{10}$ copies/g wet feces in ruminant manure, and $6.5 \pm 0.3$ $\log_{10}$ copies/g wet feces in dog feces, whereas chicken feces and chicken manure samples had identical concentrations (5.9 $\log_{10}$ copies/g wet feces), with the concentrations among these various fecal-source types not being significantly different (Kruskal–Wallis test, $P > 0.05$). Cross-reactivity of PMMoV and crAssphage with all types of fecal sources and the absence of significantly different concentrations among most of the fecal sources (Kruskal–Wallis test, $P > 0.05$) suggested the applicability of crAssphage and PMMoV as general fecal pollution markers.

## Applicability of PMMoV and crAssphage to Environmental Water Samples as Fecal Pollution Markers

Due to low sensitivity of HAdVs, AiV-1, BKPyVs, and JCPyVs to human fecal-source samples, these markers were not selected for further testing in environmental water samples to identify human fecal contamination. On the other hand, PMMoV and crAssphage were selected for further study as fecal pollution markers. In this study, 115 water samples collected from different water source types during the dry and wet seasons of 2016 were analyzed for possible fecal contamination using PMMoV and crAssphage. PMMoV and crAssphage were detected in 62% (71/115) and 73% (84/115) of tested samples, with mean concentrations of $3.3 \pm 1.4$ and $4.1 \pm 0.9$ $\log_{10}$ copies/L, respectively (Table 2). The positive ratios of PMMoV ranged from 28 to 100% in water sources. Unlike PMMoV, the positive ratios of crAssphage in all water source types ranged from 61 to 86%. Except for the river water samples, the concentrations of crAssphage were significantly higher than those of PMMoV in each water source type (paired *t* test, $P < 0.05$).

The performances of PMMoV and crAssphage were compared between *E. coli*-positive and *E. coli*-negative groups (Table 4). PMMoV was detected with a higher positive ratio ($\chi^2$ test, $P < 0.05$) and higher concentrations (independent *t* test, $P < 0.05$) in the *E. coli*-positive group (69%, 61/88; $2.7 \pm 1.7$ $\log_{10}$ copies/L) than in the *E. coli*-negative group (37%, 10/27; $1.4 \pm 0.8$ $\log_{10}$ copies/L). On

**Table 3** Positive percentage and mean marker concentration of human-specific markers in fecal-source samples

| Fecal sources | No. of samples tested | *E. coli*[a] | | AiV-1 | | HAdVs | | BKPyVs | | JCPyVs | | PMMoV | | CrAssphage | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. of positive samples (%) | Conc.[b] (mean ± SD) | No. of positive samples (%) | Conc.[c] (mean ± SD) | No. of positive samples (%) | Conc.[c] (mean ± SD) | No. of positive samples (%) | Conc.[c] (mean ± SD) | No. of positive samples (%) | Conc.[c] (mean ± SD) | No. of positive samples (%) | Conc.[c] (mean ± SD) | No. of positive samples (%) | Conc.[c] (mean ± SD) |
| Sewage | 10 | 10 (100) | 7.0 ± 1.0 | 4 (40) | 6.0 ± 0.2 | 4 (40) | 6.1 ± 0.3 | 0 (0) | NA | 3 (30) | 6.2 ± 0.4 | 10 (100) | 6.7 ± 1.4 | 9 (90) | 6.7 ± 0.3 |
| Dog | 30 | 30 (100) | 7.3 ± 0.6 | 2 (7) | 6.6 ± 0.3 | 5 (17) | 6.5 ± 0.1 | 1 (3) | 6.8 | 1 (3) | 6.4 | 16 (53) | 4.8 ± 0.3 | 19 (63) | 6.5 ± 0.3 |
| Chicken | 30 | 30 (100) | 6.9 ± 0.8 | 0 (0) | NA | 0 (0) | NA | 0 (0) | NA | 0 (0) | NA | 12 (40) | 4.6 ± 0.3 | 3 (10) | 5.9 ± 0.1 |
| Chicken manure | 10 | 9 (90) | 4.1 ± 1.8 | 0 (0) | NA | 1 (10) | 6.4 | 1 (10) | 6.7 | 0 (0) | NA | 5 (50) | 5.0 ± 1.1 | 4 (40) | 5.9 ± 0.2 |
| Pig | 10 | 10 (100) | 6.3 ± 0.6 | 0 (0) | NA | 1 (10) | 6.4 | 0 (0) | NA | 0 (0) | NA | 10 (100) | 6.9 ± 0.5 | 1 (10) | 5.7 |
| Ruminant | 12 | 12 (100) | 5.6 ± 0.6 | 0 (0) | NA | 0 (0) | NA | 0 (0) | NA | 2 (17) | 6.2 ± 0.1 | 7 (58) | 4.7 ± 0.7 | 1 (8) | 5.7 |
| Ruminant manure | 10 | 10 (100) | 5.7 ± 1.0 | 0 (0) | NA | 5 (50) | 6.5 ± 0.1 | 1 (10) | 7.0 | 0 (0) | NA | 6 (60) | 4.9 ± 0.3 | 9 (90) | 6.5 ± 0.1 |
| Duck | 10 | 10 (100) | 6.0 ± 1.6 | 0 (0) | NA | 2 (20) | 6.5 | 0 (0) | NA | 0 (0) | NA | 8 (80) | 6.1 ± 0.7 | 4 (40) | 6.5 ± 0.1 |
| Parameters | | No. of samples judged correctly/no. of samples tested (%) | | | | | | | | | | | | | |
| Sensitivity (%) | | NC | | 4/10 (40) | | 4/10 (40) | | 0/10 (0) | | 3/10 (30) | | 10/10 (100) | | 9/10 (90) | |
| Specificity (%) | | NC | | 110/112 (98) | | 98/112 (88) | | 109/112 (97) | | 109/112 (97) | | 48/112 (43) | | 71/112 (63) | |

*SD* standard deviation, *NA* not applicable, *NC* not calculated

[a]Including the data previously reported (Malla et al. 2018a)

[b]Unit, $\log_{10}$ MPN/g wet feces

[c]Unit, $\log_{10}$ copies/g wet feces

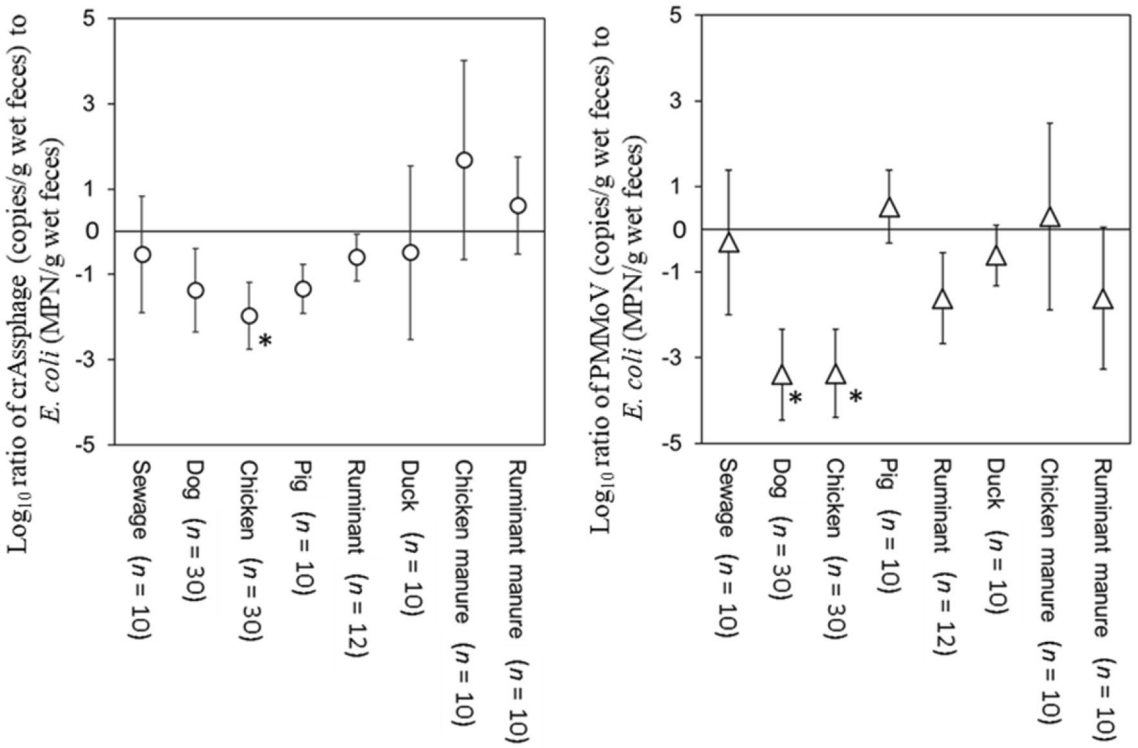


**Fig. 1** $Log_{10}$ ratio of relationship between crAssphage or PMMoV (copies/g wet feces) and *E. coli* (MPN/g wet feces) for the different fecal-source samples (mean ± standard deviation). n, number of tested samples. *Significant difference in concentration between sewage samples. Although chicken manure showed a high variation in concentration because of one negative sample, there was no significant difference in concentration with the sewage samples

**Table 4** Relationship of crAssphage and PMMoV frequency with *E. coli* concentration in environmental water samples

| Conc. range of *E. coli* groups[a] | CrAssphage | | | | PMMoV | | | |
|---|---|---|---|---|---|---|---|---|
| | No. of positive samples/no. of tested samples (%) | Significant grouping for positive samples[b] | Conc.[c] (mean ± SD) | Significant grouping for conc.[b] | No. of positive samples/no. of tested samples (%) | Significant grouping for positive samples[b] | Conc.[c] (mean ± SD) | Significant grouping for conc.[b] |
| Negative | 20/27 (74) | 1 | 3.4 ± 0.6 | 1 | 10/27 (37) | 1 | 1.4 ± 0.8 | 1 |
| 0–1.13 | 17/22 (77) | 1 | 3.5 ± 0.6 | 1 | 11/22 (50) | 1 | 1.8 ± 1.1 | 1 |
| 1.14–2.00 | 16/22 (73) | 1 | 3.5 ± 0.7 | 1 | 10/22 (46) | 1 | 1.7 ± 1.1 | 1 |
| 2.01–3.51 | 14/22 (64) | 1 | 3.4 ± 0.8 | 1 | 19/22 (86) | 2 | 2.6 ± 1.0 | 2 |
| >3.51 | 17/22 (77) | 1 | 4.6 ± 1.7 | 2 | 21/22 (96) | 2 | 4.6 ± 1.7 | 3 |

*SD* standard deviation

[a]Unit, $log_{10}$ MPN/100 mL

[b]Same number means there was no significant difference in either positive percentage or concentration among different *E. coli* groups represented by same number

[c]Unit, $log_{10}$ copies/L

the other hand, positive ratios ($\chi^2$ test, $P > 0.05$) and concentrations (independent *t* test, $P > 0.05$) of crAssphage were not significantly different between *E. coli*-negative (74%, 20/27; 3.4 ± 0.6 $log_{10}$ copies/L) and *E. coli*-positive groups (72%, 64/88; 3.8 ± 1.2 $log_{10}$ copies/L). In the environmental water samples tested, *E. coli* concentrations were moderately positively correlated with crAssphage concentrations (Pearson correlation coefficient ($r$) = 0.43, $P < 0.05$) but highly positively correlated with PMMoV concentrations (Pearson correlation coefficient, $r = 0.70$, $P < 0.05$). The

samples were separated into five groups based on *E. coli* concentration, and the positive ratios and mean concentrations of crAssphage and PMMoV were examined across the groups (Table 4). PMMoV and crAssphage concentrations were significantly higher in the highest *E. coli* concentration group compared with the other groups [one-way analysis of variance (ANOVA), $P < 0.05$]. PMMoV had significantly higher positive ratios in the groups with high *E. coli* concentrations ($\geq 2.01$ log MPN/100 mL) compared with the other groups ($\leq 2.00$ $\log_{10}$ MPN/100 mL) ($\chi^2$ test, $P < 0.05$). On the other hand, the positive ratios of crAssphage did not differ significantly among all the groups, regardless of the *E. coli* concentration ($\chi^2$ test, $P > 0.05$).

## Discussion

The objectives of this study were to evaluate the performances of HAdVs, AiV-1, BKPyVs, JCPyVs, PMMoV, and crAssphage as human-specific markers of fecal contamination. The four enteric viruses showed poor sensitivities (0–40%), whereas PMMoV and crAssphage showed high cross-reactivity with other non-target hosts, resulting in specificities of 45–63%. On the basis of these results, the viruses were considered not useful for human fecal detection in the study area.

Previous MST studies had reported high sensitivity (100%) of HAdVs for sewage samples and high specificity (100%) for animal feces and slaughterhouse wastewater samples (Heim et al. 2003; Fong et al. 2005; Hundesa et al. 2006, 2009; Ahmed et al. 2010a). However, the current study reported poor sensitivity with sewage although specificity was high, which is in line with the result reported previously (Harwood et al. 2013). Likewise, BKPyVs and JCPyVs had previously been recommended as strong human-specific markers (Rachmadi et al. 2016) with high sensitivity (100%) in raw wastewater samples and high specificity (100%) with other animal fecal samples (Rusinol et al. 2014; McQuaig et al. 2009; Ahmed et al. 2010b). However, in the current study, BKPyVs and JCPyVs showed sensitivities of 0% and 30% with sewage samples, respectively. A multi-laboratory comparative study also reported low sensitivities of human polyomaviruses (8–10%) (Harwood et al. 2013). In contrast, a previous study had reported a high prevalence of BKPyVs and JCPyVs in sewage samples from widely divergent urban areas (Biofill-Mas et al. 2000), which could be partially linked to variation between geographic regions. However, such discrepancies in sensitivity could be also due to difference in LOD of same tested assay among different studies. AiV-1 also showed poor sensitivity (40%) to sewage samples in the current study although previous studies had reported a higher detection ratio (up to 100%) in samples from a sewage pipe and wastewater treatment plants (Haramoto and Kitajima 2017; Shrestha et al. 2018). A study done in Tunisia reported a low sensitivity of AiV (6%, 15/250) in raw and treated sewage samples using different set of primers (Sdiri-Loulizi et al. 2010). Enteric viruses are excreted in higher concentrations in human and animal feces of individuals showing clinical syndromes than in the healthy population (Biofill-Mas et al. 2000). Therefore, such poor performance of all four potential human-specific markers could be linked to geographical variation and the nature of the sewage samples collected in this study. In the current study, the sewage samples were collected from the fecal collection tanks of mobile public toilet vans to collect pure human fecal samples. Unlike wastewater, a public toilet van is used by 200–300 people in a day and the feces are disposed of daily, whereas wastewater is the waste from households (> 10,000 households). Thus, it is recommended to analyze the pooled samples in future surveys.

PMMoV showed greater sensitivity (100%) in sewage in the current study compared with the results from enteric viruses in previous studies (Rosario et al. 2009; Hamza et al. 2011). The high prevalence of PMMoV in sewage in our study area is consistent with the results reported previously in the USA (Rosario et al. 2009; Kitajima et al. 2014), Nepal (Shrestha et al. 2018), and Singapore (Zhang et al. 2006), which could indicate its universal distribution (Hamza et al. 2011; Haramoto et al. 2018; Kitajima et al. 2018; Symonds et al. 2018). PMMoV was detected in fecal samples from ruminants, dogs, chicken, pig, duck, and animal manure samples in the current study (Table 3). In contrast, a previous study had reported that PMMoV was not detected in cow, sheep, horse, pig, or dog feces, but, as in our results, detection was reported in chicken feces (Rosario et al. 2009). PMMoV was frequently detected in the pig (100%) and duck fecal samples (80%) in the current study, which could be because PMMoV is assumed to be dietary in origin and pigs are usually fed with leftovers of human food, whereas the duck feces were collected near the pig farms. Although PMMoV showed 100% sensitivity to human sewage, it could not be considered to be a human-specific marker because of its high positive ratios and high concentrations in fecal samples from other animals. Alternatively, the performance of PMMoV was examined in the current study as an indicator of general fecal pollution, as previously suggested (Rosario et al. 2009).

Although a novel bacteriophage, crAssphage, has been recommended for use as a human marker (Stachler and Bibby 2014; Garcia-Aljaro et al. 2017; Ballesté et al. 2019; Kongprajug et al. 2019), the current study revealed high sensitivity (90%), but low specificity (63%) of crAssphage, indicating its poor performance as a human-specific marker in the studied area. Previous studies performed in USA (Stachler et al. 2017) and Australia (Ahmed et al. 2018a, b) using the same set of primers and probe have reported

sensitivity of 100% and specificity of 93–98%. However, crAssphage was detected in the current study in fecal samples from all animal species tested (dog, duck, chicken, pig, and ruminant) with higher detection levels in duck and ruminant manure. On the other hand, in a study done using the same primers and probe in Southeast Asia, it revealed sensitivity of 100% and specificity of 99% using feces from swine, cattle, chicken, duck, goat, sheep, buffalo, and fish, with cross-detection only occurring for one composite swine sample (Kongprajug et al. 2019). These differences between the results of the two studies could be due to difference in climate, food habit, and the rearing practice of animals. For example, dog feces were mainly collected from the stray dogs kept in temporary shelter which might have consumed food and water contaminated with human feces. In addition, due to huge scarcity of water in the valley, groundwater is one of the major sources of drinking water for animals, which is reported to be mostly contaminated with human and ruminant feces (Malla et al. 2018b). Because of the similar food habit, they might have developed some similarity in gut microbiome. A diverse group of crAss-related phages could be present in human gut (Yutin et al. 2018). The different crAssphages or genomic regions used in designing the crAssphage assay, geographic variation, dietary habit, and climate could be important factors for the difference in performance of this marker across different geographical areas. A previous study performed in Spain had also reported high cross-reactivity (61%) of crAssphage in samples collected from sources contaminated with feces of different animals (Garcia-Aljaro et al. 2017). However, the primers and probe used in this previous study (Garcia-Aljaro et al. 2017) were different from the current study. There is also a possibility that different genomic regions can have different homologies to closely related phages in other animals' guts, while some regions may be human-specific (Stachler et al. 2018). This study reported a high prevalence of crAssphage in fecal samples from Nepal. CrAssphage has been recommended to be used as a human-specific marker in conjunction with other markers such as a human-specific *Bacteroidales* marker, HF183 (Ahmed et al. 2018a). A previous study had utilized the technique of comparing the ratio of crAssphage to *E. coli* concentrations to discriminate between human and non-human fecally polluted water samples of different origins (Garcia-Aljaro et al. 2017). Unlike their results (Garcia-Aljaro et al. 2017), the ratios of crAssphage to *E. coli* concentrations in the current study were not significantly different between sewage and animal fecal samples, further indicating the unsuitability of crAssphage to identify the human fecal contamination in the study area.

Previous studies had reported low abundance and concentration of PMMoV in a limited number of animal species (Rosario et al. 2009; Hamza et al. 2011). In the current study, however, PMMoV was detected in all pig fecal samples and in 80% of duck feces with high mean concentrations of $6.9 \pm 0.5$ and $6.1 \pm 0.7$ $\log_{10}$ copies/g wet feces, respectively. This difference in concentration could be due to difference in moisture content in fecal-source samples. In addition, PMMoV was abundant in fecal samples from other animals tested. Similarly, crAssphage was abundant in most of the animal feces tested, with mean concentrations ranging from $5.9 \pm 0.1$ to $6.5 \pm 0.3$ $\log_{10}$ copies/g wet feces. Both PMMoV and crAssphage had significant positive correlations with *E. coli* concentration in environmental water samples. Furthermore, concentrations of both PMMoV ($4.6 \pm 1.7$ $\log_{10}$ copies/L) and crAssphage ($4.6 \pm 1.7$ $\log_{10}$ copies/L) were significantly higher in the group with the highest *E. coli* concentrations ($> 3.51$ $\log_{10}$ MPN/100 mL) compared with those with lower *E. coli* concentrations ($\leq 3.51$ $\log_{10}$ MPN/100 mL). The current study showed great abundance and high concentrations of both PMMoV and crAssphage in fecal samples from animals. The relationship between *E. coli* and both PMMoV and crAssphage in environmental water samples further supports the potential use as general fecal pollution markers.

Overall, the positive ratio of crAssphage (73%, 84/115) was higher than that of PMMoV (62%, 71/115) in the tested water samples. However, PMMoV was detected in all eight (100%) river water samples tested. A high positive ratio of PMMoV (75–100%) in river water samples had been reported in previous studies conducted in Japan, Nepal, and Vietnam (Haramoto et al. 2013; Kuroda et al. 2014; Shrestha et al. 2018; Tandukar et al. 2018), supporting the ubiquitous distribution of PMMoV in surface water samples.

Although both PMMoV and crAssphage are proposed in the current study to be general fecal pollution markers, their behavior varied in *E. coli*-negative environmental water samples. More importantly, crAssphage was detected in 74% (20/27) of the culture-based *E. coli*-negative samples, indicating that *E. coli* is not suitable to confirm the absence of fecal markers in the water samples tested in this study. A previous study had also reported the unsuitability of *E. coli* to confirm the absence of waterborne pathogens in groundwater samples in the Kathmandu Valley (Haramoto 2018). This result could be due in part to the low survivability of *E. coli* in water samples (Wait and Sobsey 2001). The positive ratio and concentrations of PMMoV were significantly higher in the *E. coli*-positive group compared with the *E. coli*-negative group, whereas the positive ratio ($> 70\%$) and concentrations ($> 3.4$ $\log_{10}$ copies/L) of crAssphage were comparable between the *E. coli*-negative and *E. coli*-positive groups. The detection of *E. coli* in crAssphage-negative samples could be explained by the use of a large volume of water for a culture method (100 mL), whereas only 2.5 µL of the extracted DNA was used for qPCR, an aliquot that was equivalent to 0.05–0.74 mL of the original water sample. This could be also due to the limit of

detection of the assay. In addition, previous studies have reported that the detection of *E. coli* does not always represent the presence of viruses (Baggi et al. 2001; Hamza et al. 2011; Haramoto et al. 2013). The independent relationship between crAssphage and *E. coli* suggests that the analysis of crAssphage along with *E. coli* may provide information on the contamination of water by bacteria and viruses of fecal origins. The positive ratio of PMMoV was higher than that of crAssphage in fecal samples, whereas in environmental water samples, crAssphage had a higher positive ratio than did PMMoV. High concentrations of crAssphage in environmental samples also decreased the challenge associated with the dilution of marker abundance in natural waters. Although PMMoV is considered to be more conservative than enteric viruses (Hamza et al. 2011), our result would suggest the greater stability of crAssphage in environmental water samples. Further studies on crAssphage are recommended including its relationship with pathogenic viruses in environmental water samples.

## Conclusion

In summary, this study evaluated the performance of previously recommended human-specific viral markers using sewage and animal fecal samples from the Kathmandu Valley for tracking human fecal contamination in environmental water sources. HAdVs, AiV-1, BKPyVs, and JCPyVs had low sensitivities despite exhibiting high specificities, suggesting the unsuitability of these enteric viruses to identify human fecal contamination in the study area. PMMoV and crAssphage had low specificities in spite of having high sensitivities, suggesting that these markers are unsuitable to be used as human MST markers in the study area. However, their great prevalence and high concentrations in fecal as well as environmental samples and their positive relationships with *E. coli* concentrations in environmental water samples make them potential markers for general fecal contamination. CrAssphage was independent of the detection of *E. coli*, and its concentrations were comparable between *E. coli*-positive and *E. coli*-negative samples, suggesting that the analysis of crAssphage along with *E. coli* could provide more accurate information on the contamination of water by feces. In addition, crAssphage was more abundant in environmental water samples than was PMMoV, suggesting its greater chance of detection. Further research is needed to investigate the relationship between crAssphage and pathogenic viruses.

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## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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