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BRIEF COMMUNICATION

Evaluation of Human- and Animal-Specific Viral Markers and Application of CrAssphage, Pepper Mild Mottle Virus, and Tobacco Mosaic Virus as Potential Fecal Pollution Markers to River Water in Japan

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Abstract

Five human-specific markers were detected in 59–74% of 27 human fecal-source samples collected in Yamanashi Prefecture, Japan. Similarly, potential human-specific markers, crAssphage, pepper mild mottle virus (PMMoV), and tobacco mosaic virus were detected in 96–100% of samples, with crAssphage showing the maximum concentration of 12.03 log copies/L. However, these markers were detected in 100% (3/3) of pig fecal-source samples, suggesting their applicability as general fecal pollution markers. Microbial source tracking analysis demonstrated that the rivers are contaminated by human and pig fecal sources. CrAssphage showed higher marker concentrations in river water samples than PMMoV, suggesting the preference of crAssphage to PMMoV as a marker of fecal pollution.

Keywords CrAssphage · Microbial source tracking · Pepper mild mottle virus · Tobacco mosaic virus

Management of fecal pollution of environmental water becomes difficult when the sources of fecal pollution are unknown. Fecal indicator bacteria, such as *Escherichia coli*, are used as an indicator for monitoring water quality. However, they are discharged not only from humans but also from animals. Recently, numerous human-specific viral fecal markers, such as Aichi virus 1 (AiV-1) (Kitajima et al. 2013), human adenoviruses (HAdVs) (Heim et al. 2003), JC polyomaviruses (JCPyVs) and BK polyomaviruses (BKPyVs) (Pal et al. 2006), and noroviruses of genogroup I (NoVs-GI) (Kageyama et al. 2003), and potential human-specific viral markers, such as crAssphage (Stachler et al. 2017), pepper mild mottle virus (PMMoV) (Zhang et al. 2006; Haramoto et al. 2013), and tobacco mosaic viruses (TMV) (Balique et al. 2013), have been proposed and applied to microbial source tracking (MST) (Rosario et al. 2009; Hamza et al. 2011; Kuroda et al. 2014; Rusinol et al. 2014; Ahmed and Harwood 2017; Kitajima et al. 2018). In addition, animal-specific viral markers specific for cattle [bovine polyomaviruses (BoPyVs) (Hundesa et al. 2010; Wong and Xagoraraki 2011) and bovine noroviruses (BoNoVs) (Wolf et al. 2010)] and pig [porcine adenoviruses (PoAdVs) (Hundesa et al.

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2009) and porcine teschoviruses (PoTeVs) (Jimenez-Clavero et al. 2003)] are also used for MST (Rusinol et al. 2014; Ahmed and Harwood 2017). However, because of the geographical and dietary variations, performances of such markers should be evaluated using fecal-source samples available in a target area prior to their application in environmental water (Gawler et al. 2007; Rosario et al. 2009; Haramoto et al. 2013; Reischer et al. 2013; Stachler and Bibby 2014; Stachler et al. 2017; Yahya et al. 2017; Ahmed et al. 2018).

This study aimed to evaluate the performances of 13 host-specific viral markers (five human-, three potential human-, three cattle-, and two pig-specific), using fecal-source samples from three host types and their application to MST of river water samples collected in Yamanashi Prefecture, Japan. Human fecal-source samples [raw sewage ($n=9$) and secondary-treated sewage ($n=9$) of a wastewater treatment plant, and effluent samples of a domestic wastewater treatment tank ($n=2$)] and cattle fecal-source samples [cattle feces ($n=15$)] previously collected in 2016 (Haramoto and Osada 2018) were analyzed in this study. In addition to these samples, raw sewage and secondary-treated sewage ($n=3$ each), effluent of the domestic wastewater treatment tank, and pig fecal-source samples [pig wastewater ($n=3$)] were collected during 2017 and 2018. Furthermore, a total of 58 river water samples were collected from 21 sites (Sites 1–21) in the Fujikawa River basin, including one site (Site 21) in upstream area where human activities were not present, three times between November 2017 and December 2018.

Fecal-source and water samples were concentrated using an electronegative membrane-vortex method (Haramoto et al. 2011, 2012), with some modifications, as described previously (Malla et al. 2019), except for cattle feces. Briefly, 20 mL of 2.5 M $MgCl_2$ was added to 2 L each of river water and secondary-treated sewage samples, and 1 mL of 2.5 M $MgCl_2$ was added to 100 mL each of raw sewage, effluent of a domestic wastewater treatment tank, and pig wastewater samples and filtered using a mixed cellulose-ester membrane filter (pore size of 0.8 µm and diameter of 90 mm; Merck Millipore, Billerica, MA, USA). Subsequently, viruses were eluted using 15 mL of elution buffer, followed by centrifugation and filtration using a disposable membrane filter unit (pore size of 0.45 µm and diameter of 25 mm; Advantec, Tokyo, Japan). The filtrate was further concentrated using a Centriprep YM-50 ultrafiltration device (Merck Millipore). Cattle feces were concentrated using 1% fecal suspension prepared using phosphate buffered saline, as described previously (Malla et al. 2019).

Viral DNA and RNA were extracted using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) and QIAamp Viral RNA Mini Kit (QIAGEN), respectively, as described previously (Malla et al. 2019). cDNA was prepared using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol. As recommended (Haramoto et al. 2018), F-specific coliphage MS2 was added to the sample as a molecular process control, prior to RNA extraction. The calculated extraction-reverse transcription-quantitative PCR (qPCR) efficiency was $94.5 \pm 15.6\%$ ($n=26$). qPCR mixtures for AiV-1 (Kitajima et al. 2013), HAdVs (Heim et al. 2003), JCPyVs and BKPyVs (Pal et al. 2006), NoVs-GI (Kageyama et al. 2003), crAssphage (Stachler et al. 2017), PMMoV (Zhang et al. 2006; Haramoto et al. 2013), TMV (Balique et al. 2013), two BoPyV assays (Hundesa et al. 2010; Wong and Xagoraraki 2011), BoNoVs (Wolf et al. 2010), PoAdVs (Hundesa et al. 2009), and PoTeVs (Jimenez-Clavero et al. 2003) were prepared, as mentioned previously (Malla et al. 2019). All the unknown and standard samples, and negative controls were run in duplicate using a Thermal Cycler Dice Real Time System TP800 (Takara Bio, Kusatsu, Japan).

As shown in Table 1, all of the five human-specific markers tested exhibited sensitivities below 75%. However, these markers exhibited high specificities (83–100%). Previous studies reported specificity of 100% and sensitivities of 100% for HAdVs (Hundesa et al. 2009), 81–100% for JCPyVs (Rusinol et al. 2014), 22–100% for JCPyVs and BKPyVs (McQuaig et al. 2009), and 40–82% for NoVs-GI (Wolf et al. 2010), depending on the fecal-source types tested. Human-specific markers were not detected in pig fecal-source samples, except for NoVs-GI (100%, 3/3). A previous study reported the detection of NoVs in pigs in Japan (Nakamura et al. 2010). HAdVs were selected as a human-specific marker for MST of river water samples because of higher marker concentrations in positive samples (5.45 ± 0.92 log copies/L) than AiV-1 (5.24 ± 1.21 log copies/L), although the difference was not significant (independent t test, $P > 0.05$). Although potential human-specific markers, crAssphage, PMMoV, and TMV exhibited high sensitivities of 96–100%, these markers were also detected in 100% (3/3) of pig fecal-source samples. In addition, PMMoV and TMV were detected in 7–40% of cattle fecal-source samples, suggesting that these markers are less reliable to be human-specific in the study area. However, it should be noted that the number of pig fecal-source samples tested was very low. High sensitivities of crAssphage and PMMoV have been reported previously (Ahmed et al. 2018; Stachler et al. 2017, 2018; Kongprajug et al. 2019; Malla et al. 2019); however, cross-reactions of these markers with non-human fecal-source samples have also been reported (Rosario et al. 2009; Hamza et al. 2011; Ahmed et al. 2018; Malla et al. 2019). A previous study also reported low specificity of crAssphage using a different set of primers and a probe (Garcia-Aljaro et al. 2017). High cross-reactions of PMMoV and TMV in pig fecal-source samples could be attributed to the dietary origins of these viruses. Thus, the combination of multiple markers should be applied to MST

Table 1 Detection of human- and potential human-specific markers in fecal-source samples

Fecal sources	No. of samples tested	Human-specific markers										Potential human-specific markers					
		AiV-1		BKPyVs		HAdVs		JCPyVs		NoVs-GI		CrAssphage		PMMoV		TMV	
		No. of positive samples (%)	Conc.[a] (min–max)	No. of positive samples (%)	Conc. (min–max)	No. of positive samples (%)	Conc. (min–max)	No. of positive samples (%)	Conc. (min–max)	No. of positive samples (%)	Conc. (min–max)	No. of positive samples (%)	Conc. (min–max)	No. of positive samples (%)	Conc. (min–max)	No. of positive samples (%)	Conc. (min–max)
Raw sewage	12	10 (83)	4.33–7.00	12 (100)	5.94–8.30	9 (75)	5.03–7.21	12 (100)	3.95–7.53	7 (58)	4.24–7.51	12 (100)	10.98–12.03	12 (100)	7.99–10.35	12 (100)	5.04–7.29
Secondary-treated sewage	12	9 (75)	3.24–4.97	4 (33)	4.43–5.64	10 (83)	4.12–5.42	5 (42)	4.48–4.96	9 (75)	3.16–5.08	12 (100)	7.45–8.62	12 (100)	6.99–8.40	12 (100)	2.98–5.23
Effluent of a domestic wastewater treatment tank	3	1 (33)	3.89	0 (0)	NA[b]	0 (0)	NA	3 (100)	6.00–6.81	1 (33)	6.24	3 (100)	9.54–11.00	3 (100)	8.80–9.19	2 (67)	5.50–6.57
Pig wastewater	3	0 (0)	NA	0 (0)	NA	0 (0)	NA	0 (0)	NA	3 (100)	5.87–7.32	3 (100)	8.67–9.25	3 (100)	6.61–7.62	3 (100)	5.01–6.04
Cattle feces	15	0 (0)	NA	0 (0)	NA	0 (0)	NA	2 (13)	5.33–6.63	0 (0)	NA	0 (0)	NA	6 (40)	3.94–6.52	1 (7)	5.98
Parameters		No. of samples judged correctly/no. of samples tested (%)															
Sensitivity (%)		20/27 (74)		16/27 (59)		19/27 (70)		20/27 (74)		17/27 (63)		27/27 (100)		27/27 (100)		26/27 (96)	
Specificity (%)		18/18 (100)		18/18 (100)		18/18 (100)		16/18 (89)		15/18 (83)		15/18 (83)		9/18 (50)		14/18 (78)	

[a]Unit, log copies/L, except for cattle feces which is calculated as log copies/g-wet feces

[b]*NA* not applicable

Table 2 Detection of animal-specific markers in fecal-source samples

Fecal sources	No. of samples tested	Cattle						Pig			
		BoNoVs		Hundesa-BoPyVs[a]		Wong-BoPyVs[b]		PoAdVs		PoTeVs	
		No. of positive samples (%)	Conc.[c] (min–max)	No. of positive samples (%)	Conc. (min–max)	No. of positive samples (%)	Conc. (min–max)	No. of positive samples (%)	Conc. (min–max)	No. of positive samples (%)	Conc. (min–max)
Raw sewage	12	0 (0)	NA[d]	0 (0)	NA	0 (0)	NA	0 (0)	NA	0 (0)	NA
Secondary-treated sewage	12	0 (0)	NA	0 (0)	NA	0 (0)	NA	0 (0)	NA	0 (0)	NA
Effluent of a domestic wastewater treatment tank	3	0 (0)	NA	0 (0)	NA	0 (0)	NA	0 (0)	NA	1 (33)	8.74
Pig wastewater	3	0 (0)	NA	0 (0)	NA	0 (0)	NA	3 (100)	7.20–7.95	3 (100)	8.48–9.51
Cattle feces	15	6 (40)	7.18–9.20	0 (0)	NA	0 (0)	NA	0 (0)	NA	0 (0)	NA
Parameters		No. of samples judged correctly/no. of samples tested (%)									
Sensitivity (%)		6/15 (40)		0/15 (0)		0/15 (0)		3/3 (100)		3/3 (100)	
Specificity (%)		30/30 (100)		30/30 (100)		30/30 (100)		42/42 (100)		41/42 (98)	

[a]Hundesa et al. 2010

[b]Wong and Xagoraraki 2011

[c]Unit, log copies/L, except for cattle feces which is calculated as log copies/g-wet feces

[d]*NA* not applicable

of water samples to obtain a high level of confidence on the pollution source (Balleste et al. 2010).

As summarized in Table 2, all the three cattle-specific markers tested exhibited high specificity (100%, 30/30). In contrast, they showed low sensitivity (0%, 0/15), except for BoNoVs which showed sensitivity of 40% (6/15). Previous studies reported specificity of 100% for BoPyVs using raw human wastewater and pig feces, while sensitivities of 0–6%, similar to the results of this study, were obtained using bovine or cow feces. However, sensitivities of > 90% were obtained using manure and slaughterhouse wastewater samples (Hundesa et al. 2010; Wong and Xagoraraki 2011). Sensitivity and specificity of 100% each were reported for BoNoVs (Wolf et al. 2010). Pig-specific markers, PoAdVs and PoTeVs, showed sensitivity of 100% (3/3) and specificity of 98–100%. High sensitivities (87–100%) and specificity (100%) of PoAdVs and PoTeVs were reported previously (Jimenez-Clavero et al. 2003; Hundesa et al. 2009). In this study, PoTeVs (8.92 ± 0.53 log copies/L) were selected for further MST of river water samples because of higher marker concentrations than PoAdVs (7.63 ± 0.38 log copies/L) in pig fecal-source samples.

As mentioned earlier, HAdVs were selected as a human-specific marker, whereas crAssphage, PMMoV, and TMV were selected as general fecal pollution markers for MST of river water samples. PoTeVs and BoNoVs were used as pig- and cattle-specific markers, respectively. As shown in Table 3, crAssphage, PMMoV, and TMV were detected in 97% (56/58), 97% (56/58), and 69% (40/58) of river water samples, respectively, with significantly higher concentrations of crAssphage (8.18 ± 0.55 log copies/L) compared to PMMoV (6.75 ± 0.81 log copies/L) (paired *t* test, $P < 0.05$). This result indicated that crAssphage has higher chance of detection in river water samples than PMMoV. CrAssphage was detected at all sites, except for two samples collected at the upstream site (Site 21). On the other hand, either PMMoV or PoTeVs were detected in the two samples, suggesting the effect of the presence of wild boars (*Sus scrofa*) in the forest area. PoTeVs were detected significantly more frequently in the samples collected at three sites (Sites 4, 12, and 20) located downstream of a pig farming area (89%, 8/9) compared to those of other sites (4%, 2/49) (χ^2 test, $P < 0.05$), demonstrating the high impact of pig wastewater on microbial quality of river water. In contrast, BoNoVs were not detected in any of the river water samples tested. HAdVs were detected at 11 (52%) out of 21 sites in which crAssphage was also detected, suggesting that these sites are vulnerable to human fecal pollution. Site 4, the most downstream site, was previously tested for human-, ruminant-, and pig-specific *Bacteroidales* markers, where all nine samples, except for one sample for the pig-specific marker, were positive for these markers (Haramoto and Osada 2018). A similar trend was observed in this study for human (HAdVs) and pig (PoTeVs) viral markers, but BoNoVs were not detected in any of three samples tested, which may require further studies to compare the performances among different types of markers .

This study demonstrated that the tested human- and potential human-specific markers either showed low sensitivity or high cross-reactions with other non-human hosts in the study area. None of the tested cattle-specific markers was suitable, whereas PoTeVs were found suitable for tracking pig fecal contamination in the study area. All the river water samples were judged fecally contaminated, suggesting for proper sewage and farm waste management. Further studies are recommended to evaluate the performances of these markers by increasing the number of host types, including wild animals and sample types and size in the study area. In addition, other cattle-specific markers should be tested using cattle feces and wastewater from cattle farm and applied to MST.

Table 3 Detection host-specific markers in river water samples

Markers	No. of positive samples/ no. of samples tested (%)	Concentration among positive samples (log copies/L) (min–max)
HAdV	28/58 (48)	3.64–4.76
CrAssphage	56/58 (97)	6.32–9.31
PMMoV	56/58 (97)	4.60–8.95
TMV	40/58 (69)	3.07–5.37
PoTescho	10/58 (17)	2.58–5.19
BoNoVs	0/58 (0)	Not applicable

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