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Pepper Mild Mottle Virus as an Indicator of Fecal Pollution▽

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Accurate indicators of fecal pollution are needed in order to minimize public health risks associated with wastewater contamination in recreational waters. However, the bacterial indicators currently used for monitoring water quality do not correlate with the presence of pathogens. Here we demonstrate that the plant pathogen *Pepper mild mottle virus* (PMMoV) is widespread and abundant in wastewater from the United States, suggesting the utility of this virus as an indicator of human fecal pollution. Quantitative PCR was used to determine the abundance of PMMoV in raw sewage, treated wastewater, seawater exposed to wastewater, and fecal samples and/or intestinal homogenates from a wide variety of animals. PMMoV was present in all wastewater samples at concentrations greater than 1 million copies per milliliter of raw sewage. Despite the ubiquity of PMMoV in human feces, this virus was not detected in the majority of animal fecal samples tested, with the exception of chicken and seagull samples. PMMoV was detected in four out of six seawater samples collected near point sources of secondary treated wastewater off southeastern Florida, where it co-occurred with several other pathogens and indicators of fecal pollution. Since PMMoV was not found in nonpolluted seawater samples and could be detected in surface seawater for approximately 1 week after its initial introduction, the presence of PMMoV in the marine environment reflects a recent contamination event. Together, these data demonstrate that PMMoV is a promising new indicator of fecal pollution in coastal environments.

Existing wastewater treatment practices are not always effective at removing the large number of pathogens (bacteria, protists, and viruses) present in human feces (17, 42, 47–49, 51). Therefore, wastewater discharges into the environment can have a negative impact on human health. Recreational waters throughout the United States are monitored for the presence of fecal pollution as a means of limiting public exposure to pathogens in areas impacted by wastewater discharges (44). The presence of pathogenic viruses in aquatic environments is an important parameter to consider in the evaluation of water quality. However, the bacterial indicators currently used to detect fecal contamination, such as fecal coliforms and enterococci, often do not correlate with the presence of feces-associated viruses and other pathogens (5, 10, 26, 33, 37, 51). In response, several researchers have proposed the use of viral indicators as a more effective method for monitoring wastewater contamination and the associated risks to public health (11, 14, 31).

To date, the majority of the proposed viral indicators of fecal pollution are enteric viruses transmitted via the fecal-oral route (4). Enteric viruses present in raw sewage (including members of the families *Adenoviridae*, *Caliciviridae*, *Picornaviridae*, and *Reoviridae* and of the genus *Anellovirus*) have been used in several previous studies to identify fecal pollution in the environment (7, 8, 11, 12, 13, 18, 19, 27, 28, 32–36, 38, 50, 51). Of the enteric viruses that have been used as indicators, only the adenoviruses were ubiquitously found in raw sewage samples collected throughout the United States (41). Picobirnaviruses and *Torque teno virus* are abundant in raw sewage from some regions and thus have also been proposed as indicator viruses (15, 41). However, one potential problem with the use of human viruses as indicators is that their abundance in wastewater depends on the degree of infection and shedding in the human population at any given time.

In addition to viruses infecting humans, other viruses shed in feces may be useful for indicating wastewater pollution. The plant pathogen *Pepper mild mottle virus* (PMMoV) was the most abundant virus found in a metagenomic survey of RNA viruses from human feces (52). PMMoV is a positive-sense, single-stranded RNA virus that belongs to the *Tobamovirus* genus and infects hot, bell, and ornamental peppers (*Capsicum* spp.) (9). The nonenveloped, rod-shaped PMMoV virions are extremely stable (9) and have been demonstrated to retain their infectivity for plants after passage through the human gut (52). PMMoV originates from processed pepper products (e.g., hot sauce and curry) and is excreted in human feces at concentrations of 1 million to 1 billion viruses per g (dry weight) (52). Since the presence of PMMoV in human feces is dietary in origin, this plant pathogen may be more abundant in the healthy human population than viruses that cause human disease.

This study analyzed the presence of PMMoV in raw sewage and treated wastewater samples collected from wastewater treatment facilities throughout the coastal United States. To determine if PMMoV is a human-specific indicator useful for

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TABLE 1. List of treatment facilities sampled during this study and their respective treatment processes

State	Date of collection (mo/day/yr)	Treatment		
		Primary	Secondary	Tertiary
Alabama	11/5/2007	Grit removal via screen	Aeration basin, secondary clarifier	Chlorination
California	10/18/2007	Sedimentation	Activated sludge system	Gravity filters, chlorination
Connecticut	11/14/2007	Sedimentation	Activated sludge system	Chlorination
Florida	11/30/2007	None	Aeration basin, secondary clarifier	Sand filters, chlorination
Florida Keys	9/29/2008[a]	Grit removal via screen	Activated sludge system	Fabric filters, UV irradiation, chlorination
Louisiana	11/12/2007	None	Activated sludge system	Final clarifiers, chlorination
Maine	11/5/2007	Sedimentation	Activated sludge system	Sand filters, chlorination
Maryland	11/24/2007	Sedimentation	Rotating biological contactors	Chlorination
New Jersey	11/13/2007	Grit removal via screen	Activated sludge system	Deep bed filters, chlorination
North Carolina	11/13/2007	Sedimentation	Activated sludge system	Multimedia filters, chlorination
Oregon	11/13/2007	Sedimentation	Activated sludge system	Chlorination
Washington	11/8/2007	Sedimentation	Activated sludge system	Chlorination

[a] The Florida Keys treatment plant was sampled approximately every 3 days over a 2-week period (9/29/2008 to 10/11/2008).

tracking the source of fecal pollution, fecal samples from numerous animals were tested for this virus. Finally, the presence of PMMoV in marine environments exposed to wastewater was determined and compared to that of other microbial indicators. The results of this work demonstrate that PMMoV is a promising indicator of fecal pollution.

MATERIALS AND METHODS

Detection of PMMoV. Quantitative PCR (qPCR) targeting the replication-associated protein was used to quantify the abundance of PMMoV. For this purpose, RNA was extracted from purified PMMoV virions (provided by Scott Adkins, U.S. Department of Agriculture, Agricultural Research Service [USDA-ARS]) by using the QIAamp MinElute virus spin kit (Qiagen, Valencia, CA). After the extraction of nucleic acid, cDNA was synthesized using the First Strand Synthesis Superscript III reverse transcription kit (Invitrogen, Carlsbad, CA) with random hexamer primers, and 2 μl of this cDNA was used for qPCR. In order to create a standard curve, PMMoV cDNA was purified using the UltraClean PCR clean-up kit (Mo Bio Laboratories, Inc.), and the amount of cDNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The number of targets in this positive-control PMMoV cDNA was determined using the measured cDNA concentration (Y) and the PMMoV genome size (i.e., 6,357 nucleotides) according to the following equation:

$$\frac{Y\ \text{ng}}{1\ \mu\text{l}} \cdot \frac{1\ \text{g}}{10^9\ \text{ng}} \cdot \frac{1\ \text{mol nt}}{330\ \text{g}} \cdot \frac{6.02 \times 10^{23}\ \text{nt}}{1\ \text{mol nt}} \cdot \frac{1\ \text{target}}{6{,}357\ \text{nt}} = \frac{\text{number of targets}}{\mu\text{l}}$$

The PMMoV cDNA was then serially diluted to 1 target/μl for use as a standard curve in each qPCR assay.

The 50-μl qPCR mixture contained 2 μl of target cDNA, 1× TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems, Foster City, CA), 400 nM each primer (PMMV-FP, 5′-GAG TGG TTT GAC CTT AAC GTT GA-3′; PMMV-RP, 5′-TTG TCG GTT GCA ATG CAA GT-3′), and 125 nM TaqMan probe (PMMV-Probe) (5′-CCT ACC GAA GCA AAT G-3′) (52). The qPCR mixture was incubated at 95°C for 10 min, followed by 45 cycles of 95°C for 30 s, 53°C for 1 min, and 72°C for 1 min; finally, the mixture was incubated at 72°C for 10 min. To account for any PCR inhibition, each sample, both undiluted and diluted 1:10, was assayed in duplicate. The qPCR assay was capable of detecting 100 copies of PMMoV per reaction. It should also be noted that all detection limits reported are based on purified target DNA in pure water and not in an environmental water matrix.

Preparation of wastewater samples. Ten-milliliter samples of raw sewage and treated wastewater were gathered from 12 wastewater treatment facilities throughout the coastal United States (Table 1) and were concentrated according to the methods of Symonds et al. (41). One raw sewage sample and one treated-wastewater sample each were gathered from Alabama, California, Connecticut, Louisiana, Maine, Maryland, New Jersey, North Carolina, Oregon, and Washington. Two sets of samples were collected from different wastewater treatment facilities in the state of Florida (one in mainland Florida and one in the Florida Keys). To determine the daily variability in PMMoV abundance, six samples of raw sewage and treated wastewater were collected over a 2-week period from the wastewater treatment plant in the Florida Keys.

For each 10-ml sample, a 0.45-μm-pore-size polyether sulfone membrane filter cartridge (Millipore, Billerica, MA) was used to remove bacteria and larger particles. Using the Centriplus YM-50 and Microcon Ultracel YM-30 centrifugal concentration devices (Millipore), the filtrate was concentrated to less than 200 μl. Nucleic acid was extracted and cDNA synthesized using the methods mentioned above, and 2 μl of this cDNA was used for qPCR.

Efficiency of isolation and extraction methods. Known quantities of PMMoV were added to a treated-wastewater sample in order to determine the effectiveness of the methods employed to isolate viral cDNA. Ten milliliters of treated wastewater was spiked with a final concentration of 2.52×10^{10} PMMoV copies/ml. Another 10-ml aliquot of treated wastewater served as a control throughout the viral concentration, nucleic acid isolation, reverse transcription, and viral detection processes. Isolation of viral cDNA and qPCR for PMMoV were performed for both samples as described above.

Analysis of PMMoV and other fecal indicators in seawater exposed to wastewater discharge. Seawater samples influenced by wastewater discharge were collected and assayed in order to determine the concentrations of PMMoV and other microbial water quality markers. For this purpose, seven seawater samples were collected aboard the NOAA Nancy Foster Ship during a 6-day cruise along the southeast coast of Florida in February 2008. Samples were collected as part of a large multi-institutional research collaboration led by the NOAA Florida Area Coastal Environment (FACE) program, utilizing many different analyses synoptically in order to better characterize the discharge of these outfalls to the coastal environment (broader data from the FACE study will be published separately). Sample sites consisted of six treated-wastewater ocean outfalls from the following locations (in order from north to south): South Central at Boynton-Delray (26°27.7314′N, 80°02.5275′W), Boca Raton (26°20.9756′N, 80°03.2357′W), Broward (26°15.1243′N, 80°03.7353′W), Hollywood (26°01.1500′N, 80°05.1594′W), Miami North (25°55.1786′N, 80°05.1706′W), and Miami Central (25°44.6224′N, 80°05.1642′W). These six outfalls (from north to south) have average daily discharge flow rates of 12, 11, 36, 40, 80, and 105 million gallons per day, respectively (23). Samples were also collected from Gulf Stream surface water to serve as an unpolluted offshore deep-water site control (26°17.3793′N, 79°54.0812′W). In addition to PMMoV quantification, all samples were assayed for 15 other microbial water quality markers (culturable enterococci, total enterococcal 23S rRNA genes, the human-specific *Enterococcus faecium esp* gene, culturable *Bacteroides*, human-specific *Bacteroides* detected by the BacHum-UCD assay, the human-specific *Bacteroides* HF8 gene cluster, the human-specific *Methanobrevibacter smithii nifH* gene, norovirus, *Cryptosporidium* oocysts, *Giardia* cysts, the human adenovirus hexon gene, the *Campylobacter jejuni hipO* gene, the *Salmonella* sp. *ipaB* gene, the coagulase-negative *Staphylococcus aureus clfA* gene, and the *Escherichia coli* O157:H7 *rfb* gene).

(i) PMMoV. For the PMMoV analyses, 50-ml water samples were collected in sterile polypropylene centrifuge tubes and were stored at 4°C until processing at the end of the cruise. Viral cDNA was isolated from 30 ml of seawater collected from each ocean outfall and the deep-water control using the methods described above for wastewater, and the concentration of PMMoV was quantified by qPCR.

(ii) Enterococci and *Bacteroides*. Culture-based assays for enterococci and *Bacteroides* were conducted aboard the ship during the cruise. Enterococci were enumerated from replicate 1:10 and 1:20 dilutions of samples by the commercial Idexx EnteroLert chromogenic substrate assay using QuantiTray 2000 trays according to the EPA-approved standard protocol (45). *Bacteroides* were enumerated from 100-ml samples filtered onto 0.45-μm-pore-size Whatman cellulose nitrate filters and were incubated on BBE agar plates under anaerobic conditions (1).

(iii) *Methanobrevibacter smithii*. To detect human-specific *M. smithii*, 1-liter water samples were filtered through a 47-mm-diameter, 0.4-μm-pore-size polycarbonate filter (GE Osmonics, Minnetonka, MN), and DNA was extracted using the Generite DNA-Easy kit (Generite, North Brunswick, NJ). A TaqMan-based qPCR assay that included a competitive internal positive control (CIPC) was developed to detect the *nifH* gene of *M. smithii* (C. Johnston, J. Ufnar, J. Griffith, J. Gooch, and J. Stewart, unpublished data) by using a modification of the previously published targeted gene sequence (43). Briefly, the optimized reaction was performed in a total volume of 25 μl containing 1× PCR buffer (50 mM KCl, 20 mM Tris-HCl [pH 8.4]), 5 mM $MgCl_2$, 800 μM deoxynucleotide triphosphates, 800 nM primers, 240 nM concentrations of the *M. smithii*-specific and CIPC probes, 225 copies of the CIPC template, 2.3 U *Taq* DNA polymerase, and 3 μl of the *M. smithii* quantification standard or DNA extract. Thermal cycling and fluorescence detection were carried out in the iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). The number of *M. smithii nifH* gene targets was interpolated from the standard curve generated from the quantification standards in relation to their threshold cycle (C_T). The detection limit for the *M. smithii* reaction was 5 targets.

(iv) Norovirus. For the detection of norovirus, 1-liter water samples were filtered through 0.45-μm-pore-size nitrocellulose type HA filters (Millipore), and the filters were stored frozen. The filters were then disrupted with a FastPrep bead beater (MP Biomedicals, Solon, OH), and RNA was extracted using the RNeasy plant minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Norovirus sequences were detected using a TaqMan real-time reverse transcription-PCR (RT-PCR) assay (20). A CIPC was included with each assay to assess PCR inhibition and to correct for inhibited samples when appropriate (J. Gregory, L. Webster, B. Robinson, J. Griffith, and J. Stewart, unpublished data). The detection limit of the norovirus assay was 25 targets.

(v) Protozoan oocysts and cysts (*Cryptosporidium* and *Giardia*). For the analysis of encysted protozoans, 100-liter seawater samples were immediately filtered and eluted aboard the ship with the Idexx FiltaMax system according to the EPA-approved standard protocol. Eluates were stored refrigerated and were transferred within 48 h to BCS Laboratories, Inc. (Miami, FL), which is a NELAP-certified laboratory for the analysis of *Cryptosporidium* and *Giardia*. Eluates were analyzed by EPA standard method 1623 (immunomagnetic separation/immunofluorescence) (46).

(vi) *Enterococcus faecium esp* gene, *Campylobacter jejuni hipO* gene, *Salmonella* sp. *ipaB* gene, coagulase-negative *Staphylococcus aureus clfA* gene, *E. coli* O157:H7 *rfb* gene, human adenovirus hexon gene, total enterococci, human-specific *Bacteroides*, and human-specific *Bacteroides* HF8 gene cluster. One-liter seawater samples were filtered onto Whatman cellulose nitrate filters (pore size, 0.45 μm) and were immediately stored frozen until the end of the cruise. Total-community DNA was extracted from the filter using a modified version of the commercial FastPrep DNA spin kit (MP Biomedicals/Qbiogene), where filters were first bead beaten in "lysing matrix E" bead tubes (MP Biomedicals/Qbiogene) with the FastPrep kit's lysis buffer in the FastPrep instrument at a speed setting of 6.5 for 45 s; then they were centrifuged, and the lysate was transferred to a sterile microcentrifuge tube, after which DNA was purified from the lysate according to the manufacturer's instructions. Endpoint PCR analyses were performed using published assays for the human source *Enterococcus faecium esp* gene (39), the *Campylobacter jejuni hipO* gene (24), the *Salmonella ipaB* gene (22), the *Staphylococcus aureus clfA* gene (29), the *Escherichia coli* O157:H7 *rfb* gene (30), and the human adenovirus hexon gene (16). qPCR assays were performed for total enterococci and human-specific *Bacteroides* (both the BacHum-UCD marker and the HF8 gene cluster) on an MJ Research Chromo4 real-time system in 25-μl reaction volumes using the QuantiTect probe master mix (Qiagen, Inc.) with 0.5 μM each primer and 0.4 μM probe per reaction. Previously described primers and probes were utilized for the qPCR analysis for the human-specific *Bacteroides* BacHum-UCD marker (21) and total enterococcal 23S rRNA genes (40). The qPCR analysis of the *Bacteroides* HF8 gene cluster marker utilized primers HF183F and Bac708R (2) in conjunction with the BacHum-193p TaqMan probe (21). For all qPCR assays, samples were run in triplicate wells (with one well spiked with inhibition control) with the following cycling parameters: 95°C for 15 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. A fluorescent plate read was performed at the end of each extension. Detection limits for each of the enterococcal and *Bacteroides* qPCR source-tracking assays were determined to be approximately 4 gene copies, using purified genomic DNA from cultures of *Enterococcus faecium* and *Bacteroides dorei*, respectively. To determine extraction/purification efficiency, each filter was spiked with 1×10^5 cells of *Lactococcus lactis* before lysis and extraction. To control for environmental sample inhibition of qPCR, environmental sample extracts spiked with *Lactococcus* extraction controls were run in triplicate (including 1 replicate spiked with inhibition control). The qPCR assays were run on an MJ Research Chromo4 real-time qPCR thermocycler in a 25-μl reaction volume by using the QuantiTect probe master mix (Qiagen, Inc.) with 0.9 μM each primer (forward, 5′-GCTGAAGGTTGGTACTTGTA-3′; reverse, 5′-TCAGGTCGGCTATGTATCAT-3′) and 0.3 μM probe (5′–6-carboxyfluorescein–TGGATGAGCAGCGAACGGGTGA–BHQ–3′) per reaction and following the cycling conditions mentioned above. Variations in the C_T value of the *Lactococcus* control indicate the extraction efficiency plus any potential inhibition, while variations in the C_T value greater than 1.4 C_T of the inhibition control of the third replicate target well indicate sample inhibition. Any inhibited samples were diluted, and the assay was repeated. Uninhibited samples were corrected for extraction/purification efficiency according to the *Lactococcus* extraction control. The potential for any background *Lactococcus* signal was assessed by comparison to replicate filters unspiked with the *Lactococcus* control. No background *Lactococcus* signal was observed for any of the cruise samples.

Persistence of the PMMoV qPCR signal in seawater. Incubation experiments were conducted to determine how long PMMoV could be detected in seawater after its initial introduction. Thirty-one 10-ml samples of seawater collected off the seawall in Bayboro Harbor (Saint Petersburg, FL) were spiked with 500 μl of raw sewage in 15-ml conical clear plastic tubes. These containers were incubated in situ off the seawall in Bayboro Harbor (Saint Petersburg, FL) from 24 June to 22 July 2008. One 10-ml seawater sample was collected prior to spiking, as a control. Before the containers were placed off the seawall, the sewage-spiked seawater was mixed for 10 min, and three initial (time zero) 10-ml samples were collected. Three conical tubes were collected as triplicate samples after 1, 2, 3, 4, 5, 6, 7, 14, 21, and 28 days of incubation. During this period, the seawater surface temperature ranged between 31°C and 33°C (NOAA CO-OPS station 8726520). Nucleic acids were isolated and cDNA synthesized from the viruses in each 10-ml sample on the same day as it was collected, and the concentration of PMMoV was determined by qPCR.

Preparation and analysis of animal fecal samples. In order to determine if PMMoV is specific to human-derived fecal waste, several samples of animal feces and/or intestinal homogenates were tested for PMMoV. The animal feces tested included samples from laughing gulls ($n = 17$) (Florida and California), a chicken ($n = 1$) (Idaho), cows ($n = 4$; includes a pooled sample from a dairy farm) (Idaho and Puerto Rico), horses ($n = 4$; includes a pooled sample from a race track) (Idaho and Puerto Rico), a sheep ($n = 1$) (Idaho), coyotes ($n = 3$) (Florida), a raccoon ($n = 1$) (Florida), feral pigs ($n = 5$) (Florida), a wild hog ($n = 1$) (Florida), pigs ($n = 12$; all pooled samples) (Canada), and dogs ($n = 9$) (Florida). With the exception of the pig feces, the AllPrep DNA/RNA MiniKit (Qiagen, Valencia, CA) was used to extract nucleic acids from 15 mg of feces according to the manufacturer's instructions. RNA from pig feces was provided by Marie-Josée Gagné (Agriculture and Agri-Food Canada). cDNA was synthesized and qPCR performed for PMMoV as described above for wastewater.

For intestinal samples, chicken and turkey intestinal homogenates were prepared in 1× phosphate-buffered saline (courtesy of Michael Day, USDA-ARS). Chicken homogenates included pooled and individual chicken samples collected from six different states: Arkansas ($n = 1$), California ($n = 4$), Delaware ($n = 1$), Georgia ($n = 5$), Missouri ($n = 1$), and North Carolina ($n = 3$). Turkey homogenates included a series of samples collected from turkeys in North Carolina ($n = 5$). Nucleic acids were extracted from 15 mg of homogenized intestinal tissue from each sample by using the All Prep DNA/RNA MiniKit (Qiagen); cDNA was synthesized; and PMMoV abundance was determined by qPCR. To ensure the quality of the extracted cDNA, animal fecal samples were spiked with and assayed for PMMoV as a positive control.

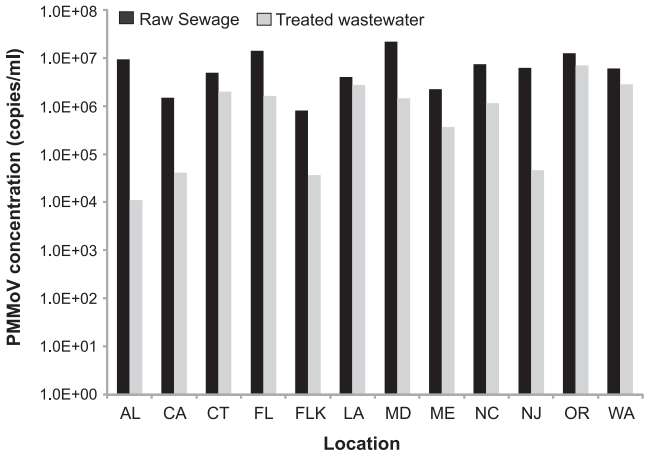

FIG. 1. PMMoV concentrations in raw-sewage and treated-wastewater samples collected throughout the United States. Duplicate measurements for each sample range within 0.2 log unit. FLK, Florida Keys.

RESULTS

Identification of PMMoV in wastewater samples. Wastewater samples were collected from 12 treatment facilities in 11 coastal states from the United States to determine the prevalence of PMMoV. The recovery efficiency of the methods used to isolate PMMoV cDNA ranged from 63 to 77% based on the spiking of a treated-wastewater sample with a known concentration of PMMoV virions. The detection limit of the qPCR assay was 100 targets. PMMoV was detected in all 12 raw-sewage and treated-wastewater samples analyzed in this study regardless of the treatment level (Table 1). The concentration of PMMoV ranged from 1.50×10^6 to 2.16×10^7 copies/ml in raw sewage and from 1.10×10^4 to 7.00×10^6 copies/ml in treated wastewater (Fig. 1). During repeated sampling at the Florida Keys wastewater treatment plant over a 2-week period, PMMoV concentrations ranged from 8.04×10^5 to 1.9×10^6 copies/ml in raw sewage and from 2.02×10^4 to 1.01×10^6 copies/ml in treated wastewater.

PMMoV in seawater exposed to wastewater discharge. PMMoV was not detected in the offshore Gulf Stream seawater sample, indicating that PMMoV was not found in seawater from this region in the absence of fecal pollution. To determine if PMMoV was present in seawater samples exposed to wastewater discharge, several samples were collected near point sources of secondary treated waste. Four of the six surface boil seawater samples collected from ocean outfalls in southeastern Florida contained detectable concentrations of PMMoV (Table 2). PMMoV co-occurred with several indicators commonly used to identify sewage contamination, as well as with several pathogens of concern. The detection of PMMoV coincided with the detection of total enterococci, the human-specific *Enterococcus faecium esp* gene, culturable *Bacteroides*, human-specific *Bacteroides*, norovirus, and the coagulase negative *Staphylococcus aureus clfA* gene, as well as with the abundances of *Cryptosporidium* oocysts and *Giardia* cysts.

The stability of PMMoV in seawater was evaluated in order to determine if the detection of this virus in coastal environments indicated a recent contamination event. After its initial introduction, PMMoV could be detected for approximately 7 days in seawater at temperatures ranging from 31 to 33°C (Fig. 2). Based on the qPCR data, the half-life for PMMoV in seawater at this temperature was 1.54 days.

PMMoV in animal feces and intestines. PMMoV was not detected by qPCR in fecal samples from cows, horses, sheep, coyotes, raccoon, pigs, or dogs. Intestinal homogenates from turkeys were also negative for PMMoV. However, PMMoV was detected in chicken and seagull samples at concentrations ranging from 5.24×10^2 to 2.2×10^4 copies/mg and 5.84×10^2 to 9.55×10^2 copies/mg, respectively (Table 3).

TABLE 2. Concentrations of PMMoV compared to those of several pathogens and standard indicators in seawater samples[a]

Indicator (unit)	Location						
	Gulf Stream (control)	South Central	Boca Raton	Broward	Hollywood	Miami North	Miami Central
PMMoV (copies/100 ml)	−	−	−	3.57×10^6	6.00×10^6	1.80×10^6	4.09×10^4
Culturable enterococci (MPN/100 ml)	<1	<1	1	<1	6.3	2	1.09×10^2
Total enterococci (copies/100 ml)	3.3	3.2	4.4	10.7	2.27×10^2	2.53×10^2	1.99×10^2
Human-specific *Enterococcus faecium esp* gene	−	−	−	−	+	+	+
Culturable *Bacteroides* (CFU/100 ml)	<1	4	3	<1	10	37	2.21×10^2
Human-specific *Bacteroides* (copies/100 ml)	4.2	27.6	21	2.08×10^2	5.78×10^3	3.72×10^3	2.79×10^3
Human-specific *Bacteroides* HF8 gene cluster (copies/100 ml)	−	−	−	49	1.94×10^2	66.2	63.9
Human-specific *Methanobrevibacter smithii* (copies/100 ml)	−	7.2×10^2	2.7×10^4	3.7×10^4	3.0×10^5	1.3×10^5	3.4×10^5
Norovirus (copies/100 ml)	−	−	2.3	6.3	2.35×10^2	3.47×10^2	11
Cryptosporidium (oocysts/100 liters)	<0.5	4	<0.5	9.8	88.3	15.7	210.5
Giardia (cysts/100 liters)	<0.5	5.3	<0.5	16.9	115.1	121.2	159.9
Human adenovirus hexon gene		−	−	−	+	+	−
Campylobacter jejuni hipO gene	−	−	−	−	−	+	−
Salmonella sp. *ipaB* gene	−	−	−	+	−	+	−
Coagulase-negative *Staphylococcus aureus clfA* gene	−	−	−	+	+	+	+
E. coli O157:H7 *rfb* gene	−	−	−	−	−	−	−

[a] The samples included one offshore Gulf Stream control and surface boils from six ocean outfalls near southeast Florida (listed from north to south). MPN, most probable number; −, the gene was not detected; +, the gene was detected.

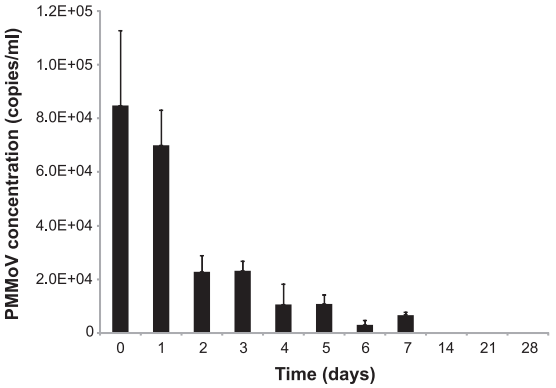

FIG. 2. PMMoV concentrations measured over time during incubation experiments to determine the stability of PMMoV in coastal seawater. Error bars represent 1 standard deviation for triplicate samples.

DISCUSSION

The primary goal of this study was to assess the utility of PMMoV as an indicator of fecal pollution in the coastal marine environment. For this purpose, the abundance of PMMoV was determined in raw sewage and treated wastewater, as well as in seawater samples collected in areas exposed to wastewater. Large quantities of PMMoV were identified in all of the raw-sewage and treated-wastewater samples collected throughout the United States. In this study, PMMoV was found at concentrations between 1.50×10^6 and 2.16×10^7 copies/ml of raw sewage. These concentrations are higher than the maximum concentrations reported in the literature for human viruses that have been proposed as indicators of fecal pollution. For example, qPCR has shown that raw sewage can contain concentrations as high as 1.16×10^5 copies of adenovirus, 6.6×10^4 copies of *Torque teno virus*, 1.8×10^4 copies of norovirus, and 8.9×10^3 copies of polyomavirus per milliliter (3, 6, 16, 25). The extremely high concentrations of PMMoV detected in human sewage suggest that this virus would be a good indicator of human fecal pollution. One of the advantages of using PMMoV instead of human enteric viruses to indicate fecal pollution is that the presence of PMMoV in wastewater is independent of active human infection. Repeated sampling of a single treatment plant demonstrated only limited variability in the levels of PMMoV in raw sewage over a 2-week period, which consistently averaged 1 million copies per milliliter. However, PMMoV concentrations in treated wastewater at the same treatment plant varied by more than 2 orders of magnitude during the same period, suggesting day-to-day variation in wastewater treatment efficiency. Seasonal variability was not examined in this study; however, since PMMoV is dietary in origin and is not dependent on active human infection, no large seasonal variations are expected. Before PMMoV can be used as a fecal indicator in other parts of the world with different dietary preferences, studies will need to determine the prevalence of PMMoV in sewage from each geographic region, as well as the baseline presence of PMMoV in local recreational waters.

In addition to being abundant in raw sewage, PMMoV was also found in all of the treated-wastewater samples examined. This demonstrates that PMMoV cannot be used to differentiate between contamination with raw sewage versus input of treated wastewater. Instead, PMMoV serves as a conservative viral tracer of fecal pollution that can be used to represent microconstituents and pathogens that may not be removed effectively through wastewater treatment processes. Further work is needed to understand the correlation between the detection of PMMoV and the infectivity of human pathogens throughout the wastewater treatment process.

The high concentration of PMMoV in wastewater suggests that this virus could be useful for detecting wastewater contamination in recreational waters. PMMoV was found in four out of six surface boils from oceanic wastewater outfalls on the southeastern coast of Florida. PMMoV was not detected in the two northernmost wastewater outfalls, which have lower daily discharge flow rates and tested negative for many of the water quality markers (Table 2). The co-occurrence of PMMoV with many other sewage-associated indicators and pathogens in oceanic wastewater outfalls shows that PMMoV is a promising indicator of human fecal pollution in the marine environment. PMMoV may also be used to indicate fecal contamination in freshwater samples; however, the natural abundance of PMMoV and the persistence of positive signals in these environments will need to be determined.

Experiments were also performed to determine the stability of PMMoV in seawater in order to understand if the detection of PMMoV in the environment reflects a recent or past pollution event. Detectable concentrations of PMMoV were present for approximately 1 week after its initial introduction in seawater at temperatures ranging from 31 to 33°C (Fig. 2). An additional experiment conducted in seawater at temperatures ranging from 22 to 27°C showed similar results, with detectable amounts of PMMoV present for approximately 1 week (data not shown). This demonstrates that detection of PMMoV in the marine environment reflects a recent contamination event. Future work needs to determine if a correlation exists between the presence of PMMoV and disease risks in polluted environments.

An ideal indicator of fecal pollution would also be able to distinguish the source of the contamination. Since PMMoV is dietary in origin, we hypothesized that this virus might be specific to human sewage. To determine if PMMoV could

TABLE 3. Concentrations of PMMoV in pooled and individual chicken and seagull samples collected from locations throughout the United States

Source of sample	Location (type of sample[a])	No. of birds	10^2 PMMoV copies/mg (range)
Chicken	Arkansas (I)	1	ND[b]
Chicken	California (I)	4	ND
Chicken	Delaware (I)	1	ND
Chicken	Georgia (I)	5	100–110
Chicken	Missouri (I)	1	5.24–14.6
Chicken	North Carolina (I)	3	ND
Chicken	Idaho (F)	1	199–216
Seagull	Florida (F)	12	ND
Seagull	California (F)	5	5.84–9.55

[a] I, intestinal homogenate; F, feces.
[b] ND, not detected; the level of PMMoV was below the detection limits of the assay.

distinguish between sources of fecal pollution, the abundance of PMMoV in the feces of several animal species was examined. PMMoV was not detected in the feces of the majority of animals tested (e.g., pigs, horses, dogs, and cows); however, PMMoV was detected in samples from chickens and seagulls. Therefore, the presence of PMMoV in environmental samples does not necessarily represent human fecal contamination. However, PMMoV is consistently found in high concentrations in human sewage, in contrast to the less frequent detection of lower concentrations in the bird feces; thus, high concentrations in recreational waters may reflect a human source. Baseline levels of any indicator should be investigated before its implementation in new areas.

In conclusion, this study has shown that PMMoV is a promising indicator of fecal pollution in the marine environment. As a plant pathogen, PMMoV is different from other proposed viral indicators of fecal contamination in that its presence in sewage is dietary in origin and is not dependent on active human infection. Further research is necessary to understand how the presence of PMMoV correlates with the infectivity of enteric pathogens of concern throughout the wastewater treatment process and in aquatic environments exposed to fecal pollution.

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