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Detection of Human-Derived Fecal Pollution in Environmental Waters by Use of a PCR-Based Human Polyomavirus Assay▽

Shannon M. McQuaig,[1] Troy M. Scott,[2] Valerie J. Harwood,[3]*
Samuel R. Farrah,[1] and Jerzy O. Lukasik[2]

Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 33611[1]; Biological Consulting Services of North Florida, Gainesville, Florida 32609[2]; and Department of Biology, University of South Florida, Tampa, Florida 33620[3]

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Regulatory agencies mandate the use of fecal coliforms, *Escherichia coli* or *Enterococcus* spp., as microbial indicators of recreational water quality. These indicators of fecal pollution do not identify the specific sources of pollution and at times underestimate health risks associated with recreational water use. This study proposes the use of human polyomaviruses (HPyVs), which are widespread among human populations, as indicators of human fecal pollution. A method was developed to concentrate and extract HPyV DNA from environmental water samples and then to amplify it by nested PCR. HPyVs were detected in as little as 1 μl of sewage and were not amplified from dairy cow or pig wastes. Environmental water samples were screened for the presence of HPyVs and two additional markers of human fecal pollution: the *Enterococcus faecium esp* gene and the 16S rRNA gene of human-associated *Bacteroides*. The presence of human-specific indicators of fecal pollution was compared to fecal coliform and *Enterococcus* concentrations. HPyVs were detected in 19 of 20 (95%) samples containing the *E. faecium esp* gene and *Bacteroides* human markers. Weak or no correlation was observed between the presence/absence of human-associated indicators and counts of indicator bacteria. The sensitivity, specificity, and correlation with other human-associated markers suggest that the HPyV assay could be a useful predictor of human fecal pollution in environmental waters and an important component of the microbial-source-tracking "toolbox."

Identifying sources of fecal pollution in waters used for human recreation, fish breeding, or shellfish harvesting is necessary to reduce the potential for human contact with enteric pathogens. Waters contaminated with fecal matter have the capability to pose serious health risks for shellfish consumers and swimmers and major economic losses for shellfish harvesters and businesses near beaches (24, 30, 37, 45, 50, 58). Bacterial, viral, and protozoan pathogens can be introduced into waters in various ways, including leaking septic tanks, sewer malfunctions, contaminated storm drains, runoff from animal feedlots, human fecal discharge from boats, and other sources (6, 20, 46).

Enumeration of fecal coliforms, *Escherichia coli* and/or *Enterococcus* spp., has generally been used to assess microbial water quality. These microorganisms share a common feature: they all can inhabit the intestines of warm-blooded animals, including wildlife, livestock, and humans, and therefore can be excreted in the feces of these animals. Although there have been some associations between high levels of indicator bacteria and disease outbreaks (16, 17, 56), there is little or no prediction of specific sources of contamination or correlation with human pathogens when using these indicators (28, 50, 53, 55). In addition, storm water and other sources without a human component can contribute heavy loads of indicator bacteria to surface waters (25, 51). A major shortcoming of traditional indicators is their inability to distinguish human fecal pollution from wild- and domestic-animal fecal pollution. This shortcoming has given rise to microbial source tracking (MST), a collective term for methodologies using microorganisms as indicators to detect and differentiate sources of fecal pollution in waters.

The *Bacteroides* marker and *esp* marker are two previously proposed human-specific MST methodologies. The 16S rRNA of the fecal anaerobic genus *Bacteroides* has been used as an indicator of human fecal pollution in field studies on the west coast of the United States (8, 9). The enterococcal surface protein gene (*esp*) is associated with enterococci isolated from human- but not animal-derived fecal matter, and the molecular detection of this gene has been utilized as an indicator of human fecal contamination of water. A recent study found the presence of the *esp* marker in two environmental samples in which human enteric viruses were found (35).

Human polyomaviruses (HPyVs) (JC virus [JCV] and BK virus [BKV]) are unique to humans and are widespread throughout the population, and high titers have been documented in municipal sewage (12). Asymptomatic primary infection usually occurs during childhood (12, 21), followed by establishment of latent infections in the renal tissue, which can allow the viruses to persist indefinitely (18, 52). Asymptomatic viruria can occur occasionally or continuously in infected individuals (4, 32, 33, 40, 47, 62), and high titers of viral particles can be shed in urine from a healthy individual (12).

The purpose of this study was to develop a reliable method to detect human fecal pollution in environmental waters using a human-specific marker that is excreted in urine, as both malfunctioning central sewer systems and septic tanks contain a mixture of feces and urine. Therefore, a method was devel-

* Corresponding author. Mailing address: Department of Biology, SCA 110, University of South Florida, 4202 E. Fowler Ave., Tampa, FL 33620. Phone: (813) 974-1524. Fax: (813) 974-3263. E-mail: vharwood@cas.usf.edu.

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oped to concentrate and detect HPyVs in water samples, and a nested-PCR assay targeting both JCV and BKV was adapted from previously published primers (5). The nested-PCR assay was then applied to environmental samples with various microbial qualities, and the results were compared to those of assays for two other human-associated markers. To test for seasonal occurrence of human-associated markers, sampling occurred throughout a 1-year period. This method was successfully used in field studies and has the potential to be a valuable asset to MST.

MATERIALS AND METHODS

Reference sample collection. Sewage samples were collected from the University of Florida Waste Water Reclamation Facility (WWRF), Main Street WWRF, and Kanapaha WWRF (Gainesville, FL). Representative septic tank samples from individual households were collected from pump-out trucks. Composite samples of dairy cow manure were collected from the University of Florida Dairy Research Unit (Gainesville, FL). Composite pig manure samples were collected from farming operations throughout Alachua County, Florida. Surface water samples were collected from water bodies in areas characterized by low-density human populations, which were expected to receive little human impact (Gainesville and Tampa, FL), and were designated low-impact water samples. A total of 14 septic tank samples, 36 sewage samples, 18 dairy cow waste samples, 7 composite pig waste samples, and 12 low-impact water samples were collected from August 2004 to April 2005.

Experimental sample collection. In northern Florida, 10 surface water sites were sampled on an approximately monthly basis from November 2004 through October 2005. Five sites were tributaries to the St. Johns River situated in urban areas in Duval County (designated D), and five sites were located in rural Nassau County (designated N). Each site was analyzed on seven occasions for fecal coliforms, *E. coli* and enterococci, and for the presence of the *esp* marker, the *Bacteroides* marker, and HPyVs. In Gainesville, Florida, surface water samples from small urban tributaries were collected by Gainesville Regional Utilities from October 2004 through June 2005 and analyzed for fecal coliforms, enterococci, and the presence of the *esp* maker and HPyVs. A total of 86 samples were collected from 39 sites. In addition, the presence of human-associated *Bacteroides* spp. was assessed on 7 June ($n = 10$ sites) and 21 June ($n = 12$ sites) 2005 and once in October 2005 ($n = 12$ sites). All samples were collected in sterile bottles, transported to the laboratory on ice, and stored at 4°C until they were processed (within 24 h of collection).

Sample preparation for HPyV analysis. Approximately 100 ml of dairy cow or pig waste samples (slurries consisting of waste from multiple animals) was passed through a series of prefiltration steps. To reduce the viral adsorption to the prefilter, the pH of the samples was raised to 9.5 using 1 M NaOH. The samples were then vacuum filtered through a 47-mm-diameter prefilter (Millipore Reinforced; catalog no. RW0304700; Fisher Scientific, Pittsburgh, Pa). The pH of the filtrate was adjusted to 3.5 using 2.0 N HCl, and the filtrate was then filtered through a 0.45-μm-pore-size, 47-mm-diameter nitrocellulose filter (GE nitrocellulose; Fisher Scientific, Pittsburgh, Pa.). Multiple filters were used where necessary to prevent clogging, and all filters were placed in one tube and stored at −20°C for further processing (see below). All filters were processed within 24 h.

Aliquots of 100 μl of human sewage samples were inoculated into 100 ml dechlorinated tap water and analyzed for HPyVs. To promote viral adsorption to the nitrocellulose filters, all of the samples were adjusted to pH 3.5 using 2.0 N HCl prior to filtration. The viruses were concentrated onto a 0.45-μm-pore-size, 47-mm-diameter nitrocellulose filter using vacuum filtration. Each filter(s) was placed into a 30-ml polypropylene tube. Viruses were eluted from the filters with 2 ml beef extract (pH 9.3). DNA was extracted from the resulting eluate using the QIAamp Blood Midi Kit (QIAGEN, Inc., Valencia, CA) as described below, and HPyVs were detected by the nested PCR assay as described below. The entire DNA aliquot was subjected to nested PCR.

Approximately 600 ml of environmental water samples was prefiltered to prevent filter clogging. To reduce the viral adsorption to the prefilter, the pH of the environmental water samples was raised to 9.5 using 1 M NaOH. The samples were then vacuum filtered through a 47-mm-diameter prefilter. The pH of the filtrate was adjusted to 3.5 using 2.0 N HCl, and 600 ml was filtered through a 0.45-μm-pore-size, 47-mm-diameter nitrocellulose filter. The filter was placed in a 30-ml polypropylene tube. Viruses were eluted from the filters with 2 ml beef extract (pH 9.3). DNA was extracted from the resulting eluate using the QIAamp Blood Midi Kit (QIAGEN, Inc.) to a final volume of 90 μl as described below, and HPyVs were detected using 4 μl of the extracted DNA in the PCR assay described below. Water samples that yielded positive PCR results in the initial analysis were not tested further, but initially negative samples were subjected to at least four more PCRs, resulting in the analysis of a total of 20 μl extracted DNA for negative samples. This corresponds to a water sample volume of 133 ml.

DNA extraction. Preliminary studies were employed to test the abilities of the QIAamp DNA Blood Midi Kit, QIAamp DNA Stool Mini Kit, QIAamp Viral RNA Kit, and QIAamp MinElute Virus Spin Kit (QIAGEN, Inc.) to extract HPyV DNA from the filtered preparations of human sewage described above and the reproducibility of the results. Only preparations from the QIAamp DNA Blood Midi Kit consistently yielded DNA that tested positive for HPyVs. Therefore, the QIAamp DNA Blood Midi Kit was used for viral-DNA extraction throughout the study. Two milliliters of beef extract (pH 9.3) was added to each filter-containing tube, which was vortexed for approximately 30 s, followed by the addition of 200 μl QIAGEN proteinase and 2.4 ml of buffer AL. The tubes were rotated (Dynal Rotator; Invitrogen, Inc., Carlsbad, CA) for 10 min at 70°C. The lysate was then added to the QIAGEN spin column to bind the DNA. The viral DNA was isolated according to the manufacturer's instructions (QIAGEN, Inc., Valencia, CA). DNA was eluted from the QIAGEN spin column by two rounds of addition of 45 μl of nuclease-free reagent grade water, resulting in a final volume of 90 μl. The DNA was stored at −20°C.

Nested-PCR detection of HPyVs. Previously published primers specific for the homologous T antigen of both JCV and BKV were used to amplify HPyV DNA (Fwd, 5′-AGT CTT TAG GGT CTT CTA CC-3′, and Rev, 5′-GGT GCC AAC CTA TGG AAC AG-3′) (5). The PCR mixtures were prepared using 45 μl of Platinum Blue PCR SuperMix (Invitrogen, Inc., Carlsbad, CA), 200 nM of each primer, and 4 μl of DNA template. The final reaction volume was adjusted to 50 μl using reagent grade water (64). The PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 45 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 20 s, and then a final elongation at 72°C for 2 min (Eppendorf Mastercycler Thermocycler; Eppendorf International, Hamburg, Germany). Nested PCR was used to increase the sensitivity of the assay in environmental water samples. The nested protocol was utilized for all results reported and was run under the same reaction conditions described above, with 1 μl of the first reaction mixture used as the template.

The PCR products were separated by agarose gel electrophoresis (1.5%). DNA was viewed using GelStar nucleic acid stain (Biowhittaker, Inc., Walkersville, MD) under UV light. Bands were identified visually by comparison to a pGEM HindIII digest (Promega, Inc., San Luis Obispo, CA) and a positive control. The presence of HPyVs in a sample was recorded when bands appeared at 172 bp, which corresponded to the PCR product of the BK virus positive control (ATCC VR-837).

Sequencing of the HPyV PCR product. Two select bands from raw sewage samples that were visually identified (see above) were excised from the agarose gel and purified using a QIAquick Gel Extraction kit (QIAGEN, Inc., Valencia, CA). Cloning was carried out using a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Amplicons were ligated into the pCR 2.1 vector and transformed into competent *E. coli* TOP10F′ cells. Transformed cells were plated on Luria-Bertani agar supplemented with 100 $\mu g \cdot ml^{-1}$ ampicillin, IPTG (isopropyl-β-D-thiogalactopyranoside), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Clones containing inserts were identified by blue/white screening. Plasmids were extracted using a plasmid miniprep kit (QIAGEN, Inc.) according to the manufacturer's instructions.

DNA sequencing was carried out at the Michigan State University DNA Sequencing Facility (East Lansing, MI). The sequences obtained from the raw sewage sample were combined with data from previously published sequences of human polyomavirus T antigen. The sequences were aligned and compared using Bioware Jellyfish Software.

Sensitivity and specificity of the assay. To test the range and sensitivity of detection of the assay, 100 ml of dechlorinated tap water was inoculated with known titers of BKV (ATCC VR-837), which were determined by direct enumeration of SYBR gold stained preparations with an epifluorescence microscope. The seeded viral concentrations were 223 ± 77, 160 ± 43, or 16 ± 4 BK virus particles. The viruses were concentrated, and DNA was extracted as described above (see "Sample preparation for HPyV analysis" and "DNA extraction"), resulting in a final volume of 90 μl. Of this volume, 4 μl was used as the template for nested PCRs. The sensitivity of the method was also tested in wastewater influent. Aliquots of sewage were heated at 99°C for 5 min to ensure viral-capsid lysis. Volumes ranging from 0.1 to 4 μl were used as templates in nested PCRs as described above.

Specificity was tested against DNAs extracted from dairy cow and pig waste

TABLE 1. Geometric means of fecal coliform, *E. coli*, and enterococcus counts from the Jacksonville area study and numbers of positive PCRs for human markers of fecal pollution

Site	No. of times sampled	Geometric mean (range) (CFU · 100 ml^{-1}) of:			No. of samples positive by PCR/no. of samples tested		
		Fecal coliforms	*E. coli*	Enterococci	*esp*	HPyVs	*bac*[a]
D1	7	339 (85–680)	165 (18–453)	541 (210–1,083)	4/7	3/7	3/7
D2	7	243 (64–1,733)	126 (20–553)	345 (147–827)	2/7	3/7	3/7
D3	7	120 (42–400)	58 (7–300)	147 (32–570)	1/7	3/7	1/7
D4	7	92 (27–310)	41 (5–207)	54 (27–240)	0/7	2/7	0/7
D5	7	109 (45–343)	69 (20–300)	129 (33–690)	1/7	1/7	0/7
N1	7	28 (13–150)	14 (1–110)	10 (4–96)	1/7	2/7	2/7
N2	7	27 (10–67)	16 (6–32)	11 (4–49)	1/7	3/7	3/7
N3	7	26 (4–68)	10 (0.3–46)	14 (7–33)	0/7	2/7	0/7
N4	7	36 (9–220)	29 (6–150)	53 (5–697)	0/7	2/7	0/7
N5	7	47 (6–123)	33 (6–119)	38 (14–270)	2/7	2/7	2/7

[a] *bac*, *Bacteroides* 16S rRNA marker.

(slurries consisting of waste from multiple animals). Samples were filtered and processed as described above.

Enumeration of indicator organisms. In the Gainesville samples, fecal coliform concentrations were assessed by the most-probable-number/multiple-tube fermentation technique (2). For each sample, three tubes containing A-1 broth (Fisher Scientific) were inoculated with 0.1 ml, 1.0 ml, and 10 ml of the water sample. The tubes were incubated at 35 ± 0.5°C for 3 h and then transferred to a water bath at 44 ± 0.5°C for 21 h (2). In the northern-Florida samples, fecal coliform concentrations were determined by membrane filtration using mFC agar (2), with incubation at 44 ± 0.5°C for 24 h. *E. coli* organisms were enumerated by membrane filtration on modified mTEC agar, with incubation at 35°C for 2 h to resuscitate any injured or stressed cells, followed by incubation at 44.5°C for 22 h in a water bath (61). Enterococci were enumerated by membrane filtration on mEI agar, with incubation at 41 ± 0.5°C for 48 h (60).

Detection of *esp* marker. Enterococci were cultured from 300-ml sample volumes by membrane filtration. The filters were incubated for 18 to 24 h on mEI agar (Sigma-Aldrich, Inc., St. Louis, MO) (60). Filters containing enterococci were suspended in azide dextrose broth, vortexed vigorously, and incubated for 3 h at 41°C. DNA extractions were performed on the culture using a QIAGEN Stool DNA extraction kit according to the manufacturer's instructions (QIAGEN, Inc., Valencia, CA). The DNA was resuspended in 100 μl nuclease-free reagent grade water and stored at −20°C.

The forward PCR primer used in this study was specific for the *Enterococcus faecium esp* gene (5′-TAT GAA AGC AAC AGC ACA AGT T-3′) (49). A conserved reverse primer (5′-ACG TCG AAA GTT CGA TTT CC-3′) was used for all reactions (26). PCRs were performed in a 20-μl mixture containing 1× PCR buffer (pH 8.7), 1.5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 0.3 μM of each primer, 2.5 U of DNA polymerase (HotStarTaq; QIAGEN, Inc., Valencia, CA), and 2 μl of template DNA. Amplification was performed with an initial step at 95°C for 15 min (to activate *Taq* polymerase), followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. The PCR products were separated on a 1.5% agarose gel stained with GelStar nucleic acid stain (BioWhittaker, Inc., Walkersville, MD). The presence of the *esp* marker in a sample was recorded when bands appeared at 680 or 681 bp, representing both variants of the *E. faecium esp* gene. *E. faecium* C68 was used as the positive control.

Detection of *Bacteroides* 16S rRNA. One hundred milliliters of each water sample was filtered through a 0.45-μm-pore-size, 47-mm-diameter filter to collect bacterial cells for molecular analysis. The filters were then suspended in QIAGEN Stool Lysis Buffer and vortexed vigorously. DNA was extracted from the resulting lysate (QIAamp DNA Stool Kit; QIAGEN, Inc., Valencia, CA) according to the manufacturer's instructions.

Primers specific for an uncultured member of the genus *Bacteroides* derived from human feces (Fwd, 5′-AACGCTAGCTACAGGCTT-3′, and Rev, 5′-CA ATCGGAGTTCTTCGTG-3′) were used in the PCR (8). PCRs were performed in a 20-μl mixture containing 1× PCR buffer (pH 8.7), 1.5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 0.3 μM of each primer, 2.5 U of DNA polymerase (HotStarTaq; QIAGEN, Inc., Valencia, CA), and 2 μl of template DNA. Amplification consisted of 35 cycles at 94°C for 30 s, an annealing temperature of 58°C for 30 s, and 72°C for 1 min, followed by a final 6-min extension at 72°C. The PCR products were separated on a 1.5% agarose gel stained with GelStar. Positive controls were amplified from DNA extracted from untreated sewage.

Statistical analysis. Means of $\log_{10}$-transformed indicator organism concentrations at sites sampled on at least four dates were compared by analysis of variance. Significance for all tests was assessed at an α of <0.05. The upper limit of detection for fecal coliforms was 2,400 CFU · 100 ml^{-1}. Samples containing more than 2,400 CFU · 100 ml^{-1} were recorded as >2,400 CFU · 100 ml^{-1}, and for statistical analysis, these samples were assigned the value of 2,400 CFU · 100 ml^{-1}. Observations of *esp*, HPyV, and *Bacteroides* markers were converted to binary data, and the relationship of the concentration of the bacterial indicator and the presence or absence of each human-associated marker was assessed. Binary logistic regression models (SPSS version 12.0) were used to assess the relationship of bacterial indicators with the presence of molecular markers specific for human fecal pollution (27). Nagelkerke's *R* square, which can range from 0.0 to 1.0, denotes the effect size (the strength of the relationship); stronger associations have values closer to 1.0. Relationships were considered significant when the *P* value for the model chi square was <0.05 and the confidence interval for the odds ratio did not include 1.0. Greater odds ratios indicate a higher probability of change in the dependent variable with a change in the independent variable.

RESULTS

Sensitivity and specificity of the assay. Throughout the year, HPyVs were consistently detected in DNAs extracted from 100-μl volumes of septic tank and sewer samples. All human-derived waste samples were positive for HPyVs, and HPyVs were not detected in any low-impact water samples (receiving no known human impact), dairy cow waste samples, or pig waste samples. DNA sequencing showed that sequences of presumptive HPyV PCR products amplified from raw sewage were highly similar to previously published sequences. One amplicon (GenBank no. DQ768800) aligned with 100% similarity to BK polyomavirus strain As (GenBank no. M23122), while the other (GenBank no. DQ768801) aligned with 92% similarity to BK polyomavirus isolate BKV HC-u9 (GenBank no. AY628236).

Dechlorinated tap water samples (100 ml) were inoculated with a range of BKV concentrations. The method was able to detect BK virus DNA in 100-ml samples inoculated with as few as ~150 BK virus particles, but not with ~15 viruses. These results corresponded to detection of ~7 virus particles in a PCR. Moreover, HPyVs were consistently detected in as little as 1 μl of raw sewage, but not in 0.1-μl volumes.

TABLE 2. Geometric means of total coliform, fecal coliform, and enterococcus counts from the Gainesville area study and number of positive PCRs for human markers of fecal pollution

Site	No. of times sampled	Single observation or geometric mean (range) for:			No. of samples positive by PCR/no. of samples tested[a]		
		Total coliforms (CFU · 100 ml^{-1})	Fecal coliforms (MPN[b] · 100 ml^{-1})	Enterococci (CFU · 100 ml^{-1})	*esp*	HPyVs	*bac*
S3	6	14,003 (7,000–68,000)	1,244 (540–2,400)	3,231 (1,020–15,600)	4/6	4/6	1/3
S4	1	10,000	350	7,400	0/1	0/1	0/1
S4b	1	22,000	920	1,400	1/1	0/1	0/1
S7	5	6,357 (2,000–12,000)	786 (350–1,600)	2,325 (1,200–5,640)	5/6	6/6	0/2
S8	2	8,000 (2,000–32,000)	593 (220–1,600)	938 (400–2,200)	1/2	2/2	0/2
S9	3	4,642 (2,000–10,000)	412 (240–540)	1,726 (600–3,200)	3/3	3/3	0/2
S11	4	6,000 (3,000–9,000)	598 (280–920)	2,726 (1,360–4,000)	3/4	2/4	NA
S12	5	8,074 (3,000–44,000)	861 (220–2,400)	3,672 (2,000–4,800)	1/5	1/5	2/3
S13P	4	8,384 (4,000–19,000)	737 (540–1,100)	1,119 (700–1,600)	1/4	1/4	0/3
S13R	1	13,000	540	2,160	0/1	0/1	NA
S16	1	4,000	920	6,210	0/1	0/1	NA
S17M	2	2,000 (1,000–4,000)	920	1,824 (1,060–3,140)	0/2	0/2	NA
S18	2	15,588 (9,000–27,000)	567 (350–920)	1,200	0/2	0/2	NA
S20	2	5,916 (5,000–7,000)	540	3,751 (3,140–4,480)	0/2	0/2	NA
S21	1	23,000	1,600	1,200	1/1	0/1	0/1
S22	2	5,196 (3,000–9,000)	470 (240–920)	603 (200–1,820)	0/2	1/2	0/1
S23	4	5,144 (4,000–7,000)	494 (130–920)	3,984 (2,000–7,960)	1/4	1/4	0/1
S31	2	7,746 (6,000–10,000)	2,400	3,031 (2,240–4,100)	0/2	0/2	0/0
S37	5	6,128 (4,000–18,000)	866 (170–2,400)	2,363 (1,980–3,200)	2/5	2/5	2/2
S38	6	12,479 (5,000–23,000)	980 (170–2,400)	3,649 (2,080–5,800)	6/6	6/6	2/2
S39	2	17,292 (13,000–23,000)	350	1,833 (1,600–2,100)	1/2	1/2	0/1
S41	1	18,000	1,600	2,000	0/1	0/1	0/1
S41P	1	8,000	540	1,120	0/1	0/1	NA
S42	2	2,000 (1,000–4,000)	639 (170–2,400)	3,015 (2,840–3,200)	0/2	0/2	NA
S43	1	5,000	1,600	3,700	0/1	0/1	NA
S45M	2	5,477 (5,000–6,000)	705 (540–920)	1,862 (1,520–2,280)	0/2	0/2	NA
S45P	2	4,472 (4,000–5,000)	917 (350–2,400)	1,874 (760–4,620)	0/2	0/2	NA
S46	2	5,292 (4,000–7,000)	748 (350–1,600)	2,406 (2,160–2,680)	0/2	0/2	NA
S100	1	100,000	2,400	41,200	1/1	1/1	1/1
S101	1	62,000	2,400	17,800	1/1	1/1	1/1
S102	1	80,000	2,400	12,800	1/1	1/1	1/1
S103	1	36,000	2,400	6,800	1/1	1/1	1/1
S104	1	34,000	2,400	10,400	0/1	0/1	0/1
S105	1	66,000	2,400	6,000	1/1	1/1	0/1
S106	1	45,000	2,400	8,000	1/1	1/1	1/1
S107	1	30,000	2,400	800	0/1	0/1	0/1
SSE	2	10,954 (10,000–12,000)	1,486 (920–2,400)	3,245 (2,460–4,280)	0/2	0/2	NA
SWD	1	3,000	540	1,080	1/1	1/1	NA
SWU	2	1,000	540	880	2/2	2/2	NA

[a] *bac*, *Bacteroides* 16S rRNA marker. NA, PCR analysis not performed. The numbers of sampling events varied with sites.
[b] MPN, most probable number.

Northern-Florida study results. The geometric means of indicator organism concentrations and the frequencies of detection of human-associated markers at each site are presented in Table 1. Twenty-six of 70 (37.1%) surface water samples from Duval and Nassau Counties were positive for at least one human-associated indicator of fecal pollution. The *esp* marker was detected in 17% of samples, HPyVs were detected in 33% of samples, and the *Bacteroides* marker was detected in 20% of samples (Table 1). A positive PCR for HPyVs occurred in 100% ($n = 9$) of the samples that contained both the *esp* and *Bacteroides* markers. The HPyV marker was present in 83% (10 of 12) of the samples positive for the *esp* marker and in 93% (13 of 14) of the samples positive for the *Bacteroides* marker. The *Bacteroides* marker was present in 75% (9 of 12) of the samples positive for the *esp* marker, and the *esp* marker was present in 64% (9 of 14) of the samples positive for the *Bacteroides* marker.

The Florida Department of Environmental Protection standard for class III recreational waters (a one-time grab sample) is ≤800 CFU · ml^{-1} fecal coliforms. The U.S. Environmental Protection Agency has determined that there is a significantly increased risk of gastroenteritis (8 illnesses in 1,000 swimmers) in recreational waters when one-time grab samples contain *E. coli* concentrations in excess of 235 CFU · 100 ml^{-1} and *Enterococcus* concentrations in excess of 61 CFU · 100 ml^{-1} (59). Based on these parameters, a total of 69 samples met regulatory standards for fecal coliform concentrations, 35 samples met standards for *E. coli* concentrations, and 38 met standards for enterococcus concentrations. Of the 69 samples that met standards for fecal coliforms, 26 (38%) contained at least one of the three human-associated indicators of fecal pollution, 14 (20%) contained two markers, and 9 (13%) contained all three markers. Of the samples that met standards for *E. coli*, 10 (29%) contained at least one of the

TABLE 3. Relationships between bacterial indicators and human-associated markers of fecal pollution analyzed using binary logistic regression[a]

Analyte	North Florida study		Gainesville area study		Compiled data	
	R square	Odds ratio	*R* square	Odds ratio	*R* square	Odds ratio
FC vs *esp*	0.143	1.003	0.015	1.000	0.107	1.001
FC vs HPyVs	0.044	1.002	0.012	1.000	0.033	1.000
FC vs *bac*	0.087	1.002	0.107	1.001	0.105	1.001
E. coli vs *esp*	**0.192**	1.007	NA	NA	NA	NA
E. coli vs HPyVs	0.064	1.004	NA	NA	NA	NA
E. coli vs *bac*	**0.109**	1.005	NA	NA	NA	NA
ENT vs *esp*	0.049	1.002	0.102	1.000	0.163	1.000
ENT vs HPyVs	0.003	1.000	0.091	1.000	0.073	1.000
ENT vs *bac*	0.025	1.001	0.204	1.000	0.116	1.000
esp vs HPyVs	**0.435**	17.308	**0.712**	94.062	**0.558**	38.824
bac vs HPyVs	**0.535**	59.800	**0.212**	9.167	**0.391**	30.545
bac vs *esp*	**0.458**	31.800	**0.248**	11.000	**0.371**	15.238

[a] NA, *E. coli* analysis was carried out only in the North Florida study. Boldface values indicate significant correlation ($\alpha < 0.05$ for χ^2; confidence interval for odds ratio does not include 1.0). FC, fecal coliforms; *bac*, human-associated 16S rRNA *Bacteroides* marker; ENT, enterococci.

three human-associated indicators of fecal pollution, 5 (14%) contained two markers, and 1 (3%) contained all three markers. Of the samples that met standards for enterococcus concentrations, 14 (37%) contained at least one of the three human-associated indicators of fecal pollution, 6 (16%) contained two markers, and 3 (8%) contained all three.

Binary logistic regression was used to assess the predictive relationship between bacterial indicators and the presence or absence of human-associated indicators (*esp*, HPyVs, and 16S rRNA *Bacteroides*) (see Table 3, North Florida). Nagelkerke's *R* square measures the predictive power (effect size) of the model. The strength of the relationship ranges from 0.0 to 1.0, and stronger associations have values closer to 1.0. The odds ratio compares the probability that a change in the independent variable will lead to a change in the dependent variable; larger values indicate a stronger relationship between the variables. Fecal coliform concentrations showed no correlation with any of the human-associated markers. *E. coli* counts correlated weakly but significantly with both the *esp* marker and the *Bacteroides* marker, but no correlation was found with the presence of HPyVs. There was no correlation between the concentration of enterococci and the presence of *esp*, HPyVs, or the *Bacteroides* marker. The presence and absence of the human-associated markers were all strongly correlated.

Gainesville area study results. The geometric means of indicator organism concentrations and the frequencies of detection of human-associated markers are shown in Table 2. As observed in the northern-Florida study, there was a strong correlation among the human-associated markers; however, there was no correlation with concentrations of indicator bacteria and human-associated markers (Table 3, Gainesville area study). Forty-three of 86 (50%) surface water samples from Gainesville, FL, were positive for at least one of the indicators of human-associated fecal pollution. The *esp* marker and HPyVs were detected in 45% of the samples, and the *Bacteroides* marker was detected in 35% of the samples. A positive PCR for HPyVs occurred in 91% ($n = 10$) of the samples that contained both the *esp* and *Bacteroides* markers. The HPyV marker was present in 90% (35 of 39) of the samples positive for the *esp* marker and in 92% (11 of 12) of the samples positive for the *Bacteroides* marker. The *Bacteroides* marker was present in 50% (11 of 22) of the samples positive for the *esp* marker, and the *esp* marker was present in 92% (11 of 12) of the samples positive for the *Bacteroides* marker.

Based on regulatory-agency guidelines, a total of 35 samples did not exceed the recommended fecal coliform concentrations. All sites exceeded standards for enterococcus concentrations. Of the 35 sites that met the standards for fecal coliforms, 18 (51%) contained at least one of the two human-associated indicators of fecal pollution, and 11 (31%) contained both the *esp* marker and HPyVs. Thirty-four sites were analyzed for the presence of the *Bacteroides* 16S rRNA marker. The *Bacteroides* marker was detected in 4 of 13 (31%) of the samples that met the criteria for fecal coliform concentrations.

Binary logistic regression was used to assess the predictive relationship between bacterial indicators and the presence or absence of human-associated indicators (*esp*, HPyVs, and 16S rRNA *Bacteroides*). Fecal coliform and enterococcus concentrations were not correlated with the *esp* marker, HPyVs, or the *Bacteroides* marker. There was a very strong correlation between the presence of the *esp* marker and HPyVs. The presence of the *Bacteroides* marker was moderately correlated with the presence of both HPyVs and the *esp* marker.

The results from both the northern-Florida study and the Gainesville area study were compiled, and the predictive relationship between indicators and human-specific indicators of the total data was analyzed using binary logistic regression (Table 3, compiled data). A total of 156 samples from 49 sites were analyzed to assess correlations between fecal coliform concentrations and the presence of the *esp* marker or HPyVs, between enterococcus concentrations and the presence of the *esp* marker or HPyVs, and between the presence of the *esp* marker and the presence of HPyVs. A total of 104 samples from 33 sites were analyzed to assess correlations between fecal coliform concentrations and the presence of the *Bacteroides* marker, between enterococcus concentrations and the presence of *Bacteroides* marker, and between the presence of the *Bacteroides* marker and the presence of the *esp* marker or HPyVs. Overall, there was no correlation with fecal coliforms or enterococcus concentrations compared to all three human-associated markers. The presence of the *esp* marker and that of HPyVs were strongly correlated, while the presence of the *Bacteroides* marker and HPyVs and that of the *esp* marker were moderately correlated. The chi-square value for all reported *R*-square values was <0.05.

DISCUSSION

This is the first documented study in the United States using a virus secreted in urine as an indicator of sewage in environmental waters. The International Committee on Taxonomy of Viruses recognizes 13 different polyomaviruses, two of which are human specific (JCV and BKV) (14). Various studies have utilized primers specific for either JCV or BKV (7, 12, 62); however, to increase the sensitivity of the assay, both viruses were exploited as targets by using primers specific to a homologous region of the T antigens of both JCV and BKV (5).

Nested PCR was implemented to increase the sensitivity of the method. The sensitivity of the assay used here (~7 viruses/reaction) compares favorably with those in other reports, which range from 5 to 520 viruses/reaction (11, 12). Furthermore, the levels of HPyVs detected in sewage are comparable to observations made in other countries, i.e., $\sim 10^3$ JC viruses · ml^{-1} (11, 12).

Of the 13 recognized polyomaviruses, bovine polyomavirus is the only virus specific to livestock. To ensure that the primers used in this study did not amplify the polyomavirus found in bovines, samples of dairy waste were tested with the HPyV-specific primers. HPyVs were not detected in any of the samples. Moreover, HPyVs were not detected in any composite porcine-manure samples. Primer specificity for HPyVs was also confirmed with sequence analysis of the PCR product from raw sewage. Before application of the method to environmental samples, water bodies located in watersheds with very-low-density human populations were tested for the presence of HPyVs, which were not found in waters devoid of human impact.

The surface waters sampled in this study were all freshwater sites in Florida. Several of the sites were in the vicinity of lift stations, old sewer lines, and septic tanks, creating the potential for contamination by human sewage, while others were in areas in which human fecal impact was not expected. High levels of indicator organisms were observed in many samples in the absence of human-associated markers of fecal pollution, while the markers were detected in other samples when indicator organism concentrations were low. The result was that there was little or no correlation between indicator organisms and the markers, yet the human-associated markers were highly correlated with one another, strongly suggesting true detection of contamination from human sources. A hypothetical factor that may contribute to the low-indicator but detected-marker scenario is that while the indicator bacteria were assayed based on the standard system of culturable counts, detection of the *Bacteroides* and HPyV markers was based exclusively on PCR assays, and the *esp* test utilizes an enrichment step that could contribute to the growth of injured or stressed cells. Injured and stressed indicator bacteria grow poorly on the selective differential media stipulated by regulatory requirements (10); therefore, one may detect the markers with PCR assays when culturable indicator organism concentrations are low. Conversely, high indicator organism counts in the absence of marker detection are not surprising, given that there are many sources of these bacteria other than human fecal pollution (1, 39, 57, 63), including the contribution of storm water and agricultural runoff to fecal loading (31, 41, 42, 51).

Binary logistic regression showed that the three human-associated markers of fecal pollution were significantly correlated in these environmental water samples, with *R*-square values of up to 0.712 and odds ratios of up to 94.1. For comparison, in assays that routinely yielded very similar values, the *R*-square value for coliphages in reclaimed water assayed on two different *E. coli* hosts was 0.762 (27). Many factors probably contribute to the imperfect correlation between the human-associated markers, including the stochastic factors associated with environmental sampling and the sample volumes employed for the various assays. Furthermore, differences among the human-associated microorganisms used in these assays could well influence their detection in environmental samples, including transport characteristics and survival kinetics (54). *Enterococcus faecium* (*esp* marker) is a fermentative, aerotolerant bacterium, *Bacteroides* spp. (16S rRNA *Bacteroides* marker) are obligately anaerobic bacteria, and HPyVs are double-stranded DNA viruses. It has been documented that the indicator organisms, as well as aerotolerant bacteria, obligately anaerobic bacteria, and viruses, are susceptible to environmental stresses (e.g., UV radiation, temperature, and salinity) to various degrees (3, 15, 36, 38, 48, 54). Further study will be necessary to determine the dominant factors that influence the persistence and detection of these organisms in environmental waters.

Comparison of bacterial indicator counts and the presence or absence of human-associated indicators of fecal pollution showed weak correlations at best. Interestingly, three Gainesville area sites were located near a vagrant camp and were positive for both the *esp* marker and the presence of HPyVs. All contained acceptable levels of fecal coliforms but were in excess of enterococcus standards. The lack of correlation between indicator organisms and human-associated markers observed in this study is not a novel outcome. Bower et al. compared the presence or absence of the human-specific *Bacteroides* marker with *E. coli* levels (13). The *Bacteroides* marker was detected by PCR during combined sewer overflow events with *E. coli* levels of <235 CFU · 100 ml^{-1}, and there were instances in which the human-specific *Bacteroides* marker was absent when *E. coli* levels were in excess of EPA-recommended levels (13).

The results presented in this study support the hypothesis that conventional indicator organisms are poor predictors of human fecal pollution, particularly in subtropical waters. Indicator concentrations below acceptable standards for microbiological water quality were found in areas known to harbor human fecal contamination. Conversely, high concentrations of indicators were found in areas devoid of human impact. *Enterococcus* concentrations exceeded regulatory standards in a majority of the samples. The potential for enterococcus regrowth and prolonged survival in sediments in warmer climates may be an underlying factor that contributed to the high levels (3, 19).

The human-associated, PCR-based assays of fecal pollution published to date rely on detection of bacterial genes (8, 22, 49) or bacteriophages (29, 34, 43) or on pathogenic viruses (23, 43, 44). Pathogenic viruses are carried by a low percentage of the human population, while HPyVs are shed by more than 50% of immunocompetent individuals (47). It has been documented that individuals experiencing asymptomatic virurria can shed up to 1.5×10^9 virus particles in a day (12), and therefore, high titers of HPyVs are generally found in urban sewage. Thus, HPyVs are widely distributed, abundant viruses, making them attractive surrogates for pathogenic human viruses transmitted by waterborne routes and a useful addition to the MST "toolbox."

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