

Eukaryotic Viruses in Wastewater Samples from the United States[∇]

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Human fecal matter contains a large number of viruses, and current bacterial indicators used for monitoring water quality do not correlate with the presence of pathogenic viruses. Adenoviruses and enteroviruses have often been used to identify fecal pollution in the environment; however, other viruses shed in fecal matter may more accurately detect fecal pollution. The purpose of this study was to develop a baseline understanding of the types of viruses found in raw sewage. PCR was used to detect adenoviruses, enteroviruses, hepatitis B viruses, herpesviruses, morbilliviruses, noroviruses, papillomaviruses, picobirnaviruses, reoviruses, and rotaviruses in raw sewage collected throughout the United States. Adenoviruses and picobirnaviruses were detected in 100% of raw sewage samples and 25% and 33% of final effluent samples, respectively. Enteroviruses and noroviruses were detected in 75% and 58% of raw sewage samples, respectively, and both viral groups were found in 8% of final effluent samples. This study showed that adenoviruses, enteroviruses, noroviruses, and picobirnaviruses are widespread in raw sewage. Since adenoviruses and picobirnaviruses were detected in 100% of raw sewage samples, they are potential markers of fecal contamination. Additionally, this research uncovered previously unknown sequence diversity in human picobirnaviruses. This baseline understanding of viruses in raw sewage will enable educated decisions to be made regarding the use of different viruses in water quality assessments.

Millions of viruses and bacteria are excreted in human fecal matter (5, 17, 82), and current methods of sewage treatment do not always effectively remove these organisms (74, 76–78). The majority of treated wastewater, as well as untreated sewage, drains into the marine environment (1) and has the potential to threaten environmental (e.g., nutrients and chemicals) (45) and public (e.g., pathogen exposure via swimming and seafood consumption) (1, 24, 28, 29, 33, 44, 57, 63) health. Currently, the U.S. Environmental Protection Agency (EPA) mandates the use of bacterial indicators such as fecal coliforms and enterococci to assess water quality (75). Although monitoring of these bacteria is simple and inexpensive, it has been shown that fecal-associated bacteria are not ideal indicators of fecal pollution.

Since fecal-associated bacteria are able to live in sediments in the absence of fecal pollution (18, 32, 55), their resuspension into the water column can result in false-positive results and mask correlations between their concentrations and the extent of recent fecal pollution. Another unfavorable characteristic of current bacterial indicators is their inability to predict or correlate with the presence of pathogenic viruses (25, 40, 41, 64, 80). Human-pathogenic viruses associated with feces are generally more robust than enteric bacteria and are not as easily eliminated by current methods of wastewater treatment (43, 80). For example, adenoviruses are more resilient to tertiary wastewater treatment and UV disinfection than are bacterial indicators of fecal pollution (74). Since bacterial indicators cannot accurately depict the risks to human health from fecal

pollution, several studies have proposed the use of a viral indicator of wastewater contamination (35, 41, 61).

While it is impractical to monitor the presence of all viral pathogens related to wastewater pollution, the development of an accurate viral indicator of sewage contamination is needed for enhanced water quality monitoring. Enteric viruses (including viruses belonging to the families *Adenoviridae*, *Caliciviridae*, *Picornaviridae*, and *Reoviridae*) are transmitted via the fecal-oral route and are known to be abundant in raw sewage. These viruses have been used to identify fecal pollution in coastal environments throughout the world (27, 35, 39, 40, 48, 50, 56, 57, 63, 64, 67–69, 71, 80). To determine which viruses are effective indicators of fecal pollution, it is first necessary to establish a broad, baseline understanding of the many diverse groups of eukaryotic viruses in raw sewage. Several studies have identified adenoviruses, noroviruses, reoviruses, rotaviruses, and other enteroviruses (e.g., polioviruses, coxsackie viruses, and echoviruses) in raw sewage in Australia, Europe, and South Africa (30, 47, 58, 76–78). However, no broad baseline data on the presence of eukaryotic viruses in raw sewage in the United States currently exist.

This study determined the presence of 10 viral groups (adenoviruses, enteroviruses, hepatitis B viruses, herpesviruses, morbilliviruses, noroviruses, papillomaviruses, picobirnaviruses, reoviruses, and rotaviruses) in raw sewage samples collected throughout the United States. All viral groups that were detected in raw sewage were then examined further to determine if they were also present in final treated wastewater effluent. These 10 viral groups were chosen because of their potential to be transmitted via the fecal-oral route, suggesting that they might be found in raw sewage. Many of these viruses (excluding adenoviruses, enteroviruses, noroviruses, reoviruses, and rotaviruses) have not been studied in sewage despite their likely presence. Picobirnaviruses have been detected in

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TABLE 1. Primer nucleotide sequences used to PCR amplify 10 viral groups

Human viruses	Primer	Sequence (5'-3') ^b	Sensitivity (no. of targets)	Amplicon size (bp)
Adenoviruses	AV-A1	GCC GCA GTG GTC TTA CAT GCA CAT C	100	300
	AV-A2	CAG CAC GCC GCG GAT GTC AAA GT		
	AV-B1 ^a	GCC ACC GAG ACG TAC TTC AGC CTG		143
	AV-B2 ^a	TTG TAC GAG TAC GCG GTA TCC TCG CGG TC		
Enteroviruses	JP UP	TTA AAA CAG CCT GTG GGT TG	100	600
	ENT DOWN	ACC GGA TGG CCA ATC		
	ENT UP ^a	CCT CCG CCC CTG AAT G		154
	JP DOWN ^a	ATT GTC ACC ATA AGC GAC C		
Hepatitis B viruses	HBS-1	ATC AGG ATT CCT AGG ACC C	10,000	310
	HBS-R1	AGG ACA AAC GGG CAA CAA C		
	HBS-11 ^a	GCG GGG TTT TTC TTG TTG AC		241
	HBS-R11 ^a	GAA CCA ACA AGA AGA TGA GGC		
Herpesviruses	FP1	GAY TTY GCI AGY YTI TAY CC	10	800
	FP2	TCC TGG ACA AGC AGC ARI YSG CIM TIA A		
	RP1	GTC TTG CTC ACC AGI TCI ACI CCY TT		
	FP3 ^a	TGT AAC TCG GTG TAY GGI TTY ACI GGI GT		215–235
	RP2 ^a	CAC AGA GTC CGT RTC ICC RTA IAT		
Morbilliviruses	Up	ATG TTT ATG ATC ACA GCG GT	100	429
	Down	ATT GGG TTG CAC CAC TTG TC		
Noroviruses	P290	GAT TAC TCC AAG TGG GAC TCC AC	10,000	219
	P289	TGA CAA TGT AAT CAT CAC CAT A		
Papillomaviruses	FAP59	TAA CWG TIG GIC AYC CWT ATT	10,000	478
	FAP64	CCW ATA TCW VHC ATI TCI CCA TC		
Picobirnaviruses (genotype I)	PicoB25	TGG TGT GGA TGT TTC	1	200
	PicoB43	ART GYT GGT CGA ACT T		
Reoviruses	L1.rv5	GCA TCC ATT GTA AAT GAC GAG TCT G	100	416
	L1.rv6	CTT GAG ATT AGC TCT AGC ATC TTC TG		
	L1.rv7 ^a	GCT AGG CCG ATA TCG GGA ATG CAG		344
	L1.rv8 ^a	GTC TCA CTA TTC ACC TTA CCA GCA G		
Rotaviruses (group A)	RV1	GTC ACA TCA TAC AAT TCT AAT CTA AG	1,000	1,059
	RV2	CTT TAA AAG AGA GAA TTT CCG TCT G		
	RV3 ^a	TGT ATG GTA TTG AAT ATA CCA C		346
	RV4 ^a	ACT GAT CCT GTT GGC CAW CC		

^a Internal primer set for a nested PCR.

^b H equals A/C/T, M equals A/C, R equals G/A, S equals G/C, V equals G/C/A, W equals A/T, and Y equals T/G. Note that inosine (I) has base-pairing bias for C, A, G, and then T.

individual fecal samples (12, 70, 79, 82); however, their presence has never been analyzed in collective waste, nor have they been proposed to be potential markers of fecal pollution. This study identified potential viral indicators of fecal pollution and will have important applications to water quality monitoring programs throughout the country.

MATERIALS AND METHODS

Concentration of viruses in wastewater samples. Raw sewage and final effluent samples were collected in fall 2007 from 12 wastewater treatment facilities from the coastal United States. One raw sewage sample and one final effluent sample were collected from metropolitan areas in each of the following states: Alabama, California, Connecticut, Louisiana, Maine, Maryland, New Jersey, North Carolina, Oregon, and Washington. Two raw sewage and final effluent samples were collected from different wastewater treatment facilities in the state of Florida. All samples were vortexed prior to filtration to minimize the adhesion of viral particles to suspended solids. For each sample, 10 ml was filtered through a 0.45- μ m polyether sulfone membrane filter cartridge (Millipore, Billerica, MA).

The filtrate was concentrated to less than 200 μ l using a combination of Centriplus YM-50 and Microcon Ultracel YM-30 centrifugal concentration devices (Millipore). Nucleic acid was extracted from the filtered, concentrated sample using the QIAamp MinElute virus spin kit (Qiagen, Valencia, CA). Immediately following nucleic acid extraction, cDNA was synthesized from the extracted RNA using a First-Strand Synthesis Superscript III reverse transcription kit (Invitrogen, Carlsbad, CA) with random hexamers.

PCR for eukaryotic viral groups. PCR or reverse transcription-PCR for each of the targeted viral groups was executed using previously published primers and conditions (Table 1). All PCR mixtures had a total volume of 50 μ l and contained 2 μ l of target DNA, 1 \times REDTaq PCR buffer (10.0 mM Tris-HCl [pH 8.3], 50.0 mM KCl, 1.1 mM MgCl₂, 0.01% gelatin; Sigma-Aldrich, St. Louis, MO), 0.25 mM each deoxynucleoside triphosphate, 1 μ M of each primer, and 1 U REDTaq DNA polymerase (Sigma-Aldrich), unless otherwise noted.

Adenoviruses. Nested PCR was used to amplify the hexon gene of 47 different adenovirus serotypes (2). Five microliters of the product from the first round of PCR was used as a template for the second reaction. Both rounds of PCR had an additional 0.4 mM MgCl₂ in the reaction mixture. Both adenovirus PCR conditions were 4 min at 94°C, followed by 40 cycles of 92°C for 30 s, 60°C for 30 s, and 72°C for 1 min and a final incubation step at 72°C for 5 min.

Enteroviruses. Nested PCR capable of detecting at least 25 different enteroviruses was used to amplify the 5'-untranslated region of the enterovirus genome (34). An additional 1.8 mM MgCl₂ and an additional 1.4 mM MgCl₂ were added to the first- and second-round PCR mixtures, respectively. The first-round PCR conditions were 40 cycles of 95°C for 30 s, 57.7°C for 30 s, and 72°C for 45 s, followed by 5 min at 72°C. One microliter of amplified PCR product was added to the second round of PCR, which was amplified by 40 cycles of 95°C for 30 s, 56.5°C for 30 s, and 72°C for 30 s, followed by 5 min at 72°C.

Hepatitis B virus. Nested PCR was used to amplify the S gene of hepatitis B viruses (53). Both rounds of PCR had an additional 0.4 mM MgCl₂ added to the mixture, and the PCR conditions were as follows: 5 min at 95°C, followed by 30 cycles of 95°C for 30 s, 55°C for 40 s, and 72°C for 40 s. Two microliters of product from the first reaction was used as a template in the final PCR.

Herpesviruses. Nested PCR was used to amplify the DNA polymerase genes of eight human herpesvirus strains (11). The second round of PCR used 5 µl of the first-round product. The conditions for both rounds of PCR were 2 min at 94°C, followed by 55 cycles of 94°C for 20 s, 46°C for 30 s, and 72°C for 30 s, followed by incubation at 72°C for 10 min.

Morbilliviruses. The phosphoprotein gene of morbilliviruses was amplified by PCR (10). The reaction mixture contained an additional 0.4 mM MgCl₂, and reaction conditions were 35 cycles of 94°C for 1.5 min, 25°C for 2 min, and 72°C for 2 min, followed by incubation at 72°C for 7 min.

Noroviruses. The RNA polymerase genes of noroviruses were amplified by PCR (51). The reaction mixture contained an additional 0.4 mM MgCl₂ and 100 µg/ml bovine serum albumin. The PCR conditions were as follows: 3 min at 94°C, followed by 40 cycles of 94°C for 30 s, 49°C for 80 s, and 72°C for 1 min, and a final extension step of 72°C for 10 min.

Papillomaviruses. The L1 gene of papillomaviruses was amplified by PCR that was capable of detecting 87% of human papillomavirus strains (36). The reaction mixture contained an additional 1.4 mM MgCl₂ and was incubated for 10 min at 94°C, followed by 45 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, followed by incubation at 72°C for 5 min.

Picobirnaviruses. A portion of genomic segment 2 of genotype 1 picobirnaviruses was amplified by PCR (70). The picobirnavirus PCR mixture had an additional 0.9 mM MgCl₂ and was incubated for 2 min at 94°C, followed by 40 cycles of 94°C for 1 min, 49°C for 2 min, and 72°C for 3 min and then incubation at 72°C for 7 min.

Reoviruses. Nested PCR was used to amplify the L1 gene of reoviruses (54). The first PCR mixture contained an additional 1.4 mM MgCl₂, and 1 µl of the first-round PCR product was added to the second PCR mixture, which contained an additional 0.9 mM MgCl₂ and had a total volume of 25 µl. Both PCR mixtures were incubated for 1 min at 94°C, followed by 35 cycles of 94°C for 20 s, 50°C for 30 s, and 72°C for 30 s and then incubation at 72°C for 10 min.

Rotaviruses. Nested PCR was used to amplify the VP7 gene of group A rotaviruses (38). The initial PCR mixture had an additional 0.4 mM MgCl₂ and was incubated for 1 min at 94°C, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by incubation at 72°C for 3 min. The second reaction mixture, containing an additional 2.4 mM MgCl₂ and 2 µg/ml bovine serum albumin, was incubated for 1 min at 94°C, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s and then incubation at 72°C for 3 min.

Detection and sequencing of PCR positives. All PCR products were visualized using agarose gel (2%) electrophoresis with ethidium bromide. The presence of inhibitors in each sample was examined by spiking samples with a positive control and analyzing 1:10 serial dilutions to determine if the sample inhibited amplification. To verify the identity of positive assays, PCR products were sequenced. Positive PCR products with one distinct band were purified using the UltraClean PCR Clean-Up kit (Mo Bio Laboratories, Inc., Carlsbad, CA). If more than one band was present, the UltraClean Gelspin kit (Mo Bio Laboratories, Inc.) was utilized to gel purify the positive PCR product of the correct size. All purified positive PCR products were sequenced with their respective forward primers. Sequences were trimmed using Sequencher (Gene Codes Corporation, Ann Arbor, MI), and the identities of the positive PCR products were confirmed by comparing the sequences against the GenBank nonredundant database using BLASTN (3, 4).

Wastewater viral concentration efficiency. Known quantities of adenoviruses were added to a raw sewage sample and a final effluent sample to determine the efficiency of the methods employed to concentrate viruses. Twenty milliliters of raw sewage and final effluent was autoclaved for 15 min at 121°C to eliminate adenovirus particles while minimizing the alteration of particulates in the sample. Ten milliliters of these wastewater samples were spiked with adenovirus 20 at a final concentration of 8×10^5 viruses per milliliter. The concentration of adenovirus 20 (strain AV-931; ATCC VR-1097) particles suspended in cell culture supernatant was determined through SYBR gold staining and epifluo-

rescent microscopy (66). No aggregation of adenovirus 20 particles was observed. The remaining (unspiked) 10 ml of autoclaved raw sewage and final effluent served as a negative control throughout the viral concentration, nucleic acid isolation, and viral detection processes. Viruses were concentrated from all four samples as described above. PCR for adenoviruses was performed on 1:10 serial dilutions of DNA extracted from spiked and control samples using nested PCR as described above.

Determination of assay sensitivity. A standard curve of target DNA was created for each assay to allow determinations of assay sensitivity. Positive PCR products were purified using the UltraClean PCR Clean-Up kit (Mo Bio Laboratories, Inc.), and the amount of nucleic acid in the purified product was quantified using a NanoDrop ND-1000 apparatus (NanoDrop Technologies, Wilmington, DE). In the case of nested PCRs, the positive control from the first round of amplification was used to create the standard curve. The number of targets was back calculated using the measured concentration of DNA and amplicon size using the following equation, where *Y* is the concentration of DNA measured by the NanoDrop instrument and *Z* is the length of the amplicon:

$$\frac{Y \text{ ng}}{1 \mu\text{l}} \times \frac{1 \text{ g}}{10^9 \text{ ng}} \times \frac{1 \text{ mol bp}}{660 \text{ g}} \times \frac{6.02 \times 10^{23} \text{ bp}}{1 \text{ mol bp}} \times \frac{1 \text{ target}}{Z \text{ bp}} = \frac{\text{no. of targets}}{\mu\text{l}}$$

The purified PCR positive control was then serially diluted to 1 target/µl. The entire dilution series underwent the appropriate PCR or nested PCR for each viral group, with the exception that only 1 µl of target DNA was added to the reaction mixture. Sensitivity determination was performed at the same time as the sewage and effluent samples were assayed. PCR products were visualized on a 2% agarose gel stained with ethidium bromide, and the lowest concentration in which a band could be visualized defined the sensitivity of each assay.

Characterizing diversity of picobirnaviruses in raw sewage. In order to gain a deeper understanding of the diversity of genotype I picobirnaviruses in raw sewage, positive PCR products from each state were cloned into pCR4-TOPO (Invitrogen), and transformants were screened for inserts by PCR with primers M13F and M13R. The UltraClean PCR Clean-Up kit (Mo Bio Laboratories, Inc.) was used to purify PCR products of the proper size, which were then sequenced with primer M13F. All sequences were trimmed using Sequencher (Gene Codes Corporation), and the identities of the positive PCR products were confirmed by comparing the sequences against the GenBank nonredundant database using BLASTN (3, 4).

All sequences with insignificant identities (E values of >0.001 and/or identities of less than 50 bp in length) to known picobirnaviruses were excluded from phylogenetic analyses. All of the remaining sequences were dereplicated to 99% sequence identity with gaps using the online program FastGroup II (81). The dereplication process and the representative sequences for each group were visually confirmed. FastGroup II was also used to calculate Chao1 (22, 23) and to perform rarefaction analyses (42, 46). The phylogenetic relationships of the picobirnaviruses detected in raw sewage to known picobirnaviruses were identified through aligning dereplicated raw sewage sequences with known sequences from GenBank using ClustalX v.1.8 (73). The resulting alignment was verified visually for accuracy and then imported into MEGA v. 4 for phylogenetic analyses (72).

The phylogenetic analysis of cloned picobirnavirus PCR products from raw sewage was executed in a manner similar to that used for previous picobirnavirus studies (12, 70). Prior to creating a phylogenetic tree, the average pairwise Jukes-Cantor distance was calculated to determine the appropriateness of creating a neighbor-joining tree. Since the average pairwise Jukes-Cantor distance was less than 1.0 (0.356), a neighbor-joining phylogenetic tree was calculated using a Jukes-Cantor model, and bootstrap analyses were performed with 2,000 replicates. Branches with insignificant bootstrap values ($\leq 50\%$) were condensed to create the final phylogenetic tree.

Nucleotide sequence accession numbers. The nucleotide sequences of 207 genotype I picobirnavirus amplicons from raw sewage have been deposited in the GenBank database under accession numbers EU938707 to EU938913.

RESULTS

Wastewater viral concentration efficiency. The efficiencies of the centrifugal concentration devices used to concentrate raw sewage and final effluent were evaluated by spiking autoclaved wastewater samples with a final concentration of 8.48×10^5 adenovirus particles/ml. Spiked and unspiked samples were processed as described in Materials and Methods. PCR was

TABLE 2. Viruses detected in raw sewage and final effluent samples collected from throughout the coastal United States

Virus	% Positive (no. of positive samples/ total no. of samples) ^a	
	Raw sewage	Final effluent
Adenoviruses	100 (12/12)	25 (3/12)
Enteroviruses	75 (9/12)	8.3 (1/12)
Hepatitis B viruses	0	ND
Herpesviruses	0	ND
Morbilliviruses	0	ND
Noroviruses	58.3 (7/12)	8.3 (1/12)
Papillomaviruses	0	ND
Picobirnaviruses (genotype 1)	100 (12/12)	33 (4/12)
Reoviruses	0	ND
Rotaviruses (group A)	0	ND

^a Positive results for targeted viruses were those with a PCR product of the expected size and BLASTN hit in GenBank with an E value of ≤ 0.001 . ND, not determined.

performed on 1:10 serial dilutions of the extracted DNA and compared to an identical dilution series of DNA extracted directly from adenovirus particles. Using DNA extracted directly from adenovirus particles, the detection limit of the assay was determined to be 100 viruses. For both the spiked raw sewage and spiked final effluent samples, adenoviruses could be detected in a 10^{-3} dilution of extracted DNA, which was equivalent to ~ 100 viruses. Negative (unspiked) controls were processed in parallel to the spiked samples, and the lack of PCR product in these controls ensured the elimination of preexisting adenovirus particles. Although this experiment was only semiquantitative, it demonstrated that the methods used here to concentrate wastewater samples were successful in concentrating viruses without major losses.

Viruses identified in wastewater samples. The sensitivities of the assays used to identify the viruses under investigation ranged from 1 target to 100,000 targets and were identical to those reported previously (Table 1). The presence of 10 types of viruses was analyzed in raw sewage and final effluent samples from 12 wastewater treatment facilities located in 11 different states (Table 2). Adenoviruses and picobirnaviruses were found in 100% of the raw sewage samples. Enteroviruses were detected in 9 of 12 (75%) raw sewage samples, and noroviruses were detected in 7 of 12 (58%) raw sewage samples. Hepatitis B viruses, herpesviruses, morbilliviruses, papillomaviruses, reoviruses, and rotaviruses were not detected in any of the raw sewage samples; however, it is possible that they were present in concentrations below the detection limit of their assays (Table 1). Since adenoviruses, enteroviruses, noroviruses, and picobirnaviruses were detected in raw sewage, their presence was determined in final effluent samples collected from each wastewater treatment plant at the same time as raw sewage collection. Adenoviruses and picobirnaviruses were identified in 25% and 33% of the final effluent samples, respectively, while enteroviruses and noroviruses were identified in only one final effluent sample each.

Diversity of picobirnaviruses in raw sewage. Since picobirnaviruses were found in all of the raw sewage samples, and limited knowledge exists regarding this virus, the diversity of picobirnaviruses in raw sewage was analyzed. A total of 288 (~ 22 products per location) cloned picobirnavirus PCR prod-

ucts (200 bp) of the RNA-dependent RNA polymerase gene were sequenced by Agencourt (Beverly, MA), and 207 (72%) of these sequences had significant identities (E value of ≤ 0.001 and identities over 50 bp) to known picobirnaviruses in the GenBank database. The significant BLASTN sequence identities to known human and porcine picobirnaviruses ranged from 83% to 100% over regions ranging from 56 bp to 194 bp.

Prior to alignment, a total of 70 groups were created from the 207 raw sewage picobirnavirus sequences using FastGroup II (81), which was set to group sequences with 99% identity with gaps. While the majority of the groups were composed of one or two sequences, seven groups contained three or more sequences. The dereplicated sequences along with the top BLASTN hits from GenBank were aligned with ClustalX v.1.8 (73), and phylogenetic analyses were carried out with MEGA v. 4 (72). Since the average pairwise Jukes-Cantor distance was 0.356, a neighbor-joining tree was created using the Jukes-Cantor model with a bootstrap replication of 2,000. The final phylogenetic tree with insignificant (bootstrap value of < 50) branches condensed is displayed in Fig. 1.

Some of the picobirnavirus sequences from raw sewage had the greatest identities to porcine picobirnaviruses in the GenBank database, and this grouping is illustrated in the phylogenetic tree. The raw sewage picobirnavirus sequences do not have any discernible geographic distribution. The blocks on the phylogenetic tree representing more than one sequence show that identical (or $> 99\%$ identical) sequences were recovered from multiple states. Furthermore, several unique sequences were identified from each state. For example, 8 of 10 sequences from North Carolina were unique ($< 99\%$ identical).

In addition to dereplicating sequences, FastGroup II (81) was also used to calculate the Chao1 value, a richness estimator (22, 23), and to plot a rarefaction curve, which displays the number of unique sequences versus the number of clones sequenced (42, 46). Chao1 predicted a minimum of 200 unique picobirnaviruses in the raw sewage, but only 70 were sampled in this study; thus, more clones need to be sequenced to adequately describe the diversity of picobirnaviruses. Since the rarefaction curve is not yet approaching an asymptote (data not shown), more clones need to be sequenced in order to ensure a complete analysis of picobirnavirus diversity in raw sewage throughout the United States.

DISCUSSION

Viruses detected in wastewater. This study used PCR to determine the presence of 10 types of viruses in raw sewage and final effluent collected from 12 wastewater treatment facilities in the United States. Four different types of viruses (adenoviruses, enteroviruses, noroviruses, and picobirnaviruses) were detected in at least 50% of the raw sewage samples. Interestingly, all of the viruses identified in wastewater were nonenveloped viruses, and these viruses tend to be more stable in the environment (31). Rotaviruses and reoviruses were not detected in any of the U.S. raw sewage samples even though their presence in raw sewage has been documented in other countries (30), and they have been used in prior fecal pollution studies (6, 7, 16, 19, 52, 62, 67). It is plausible that

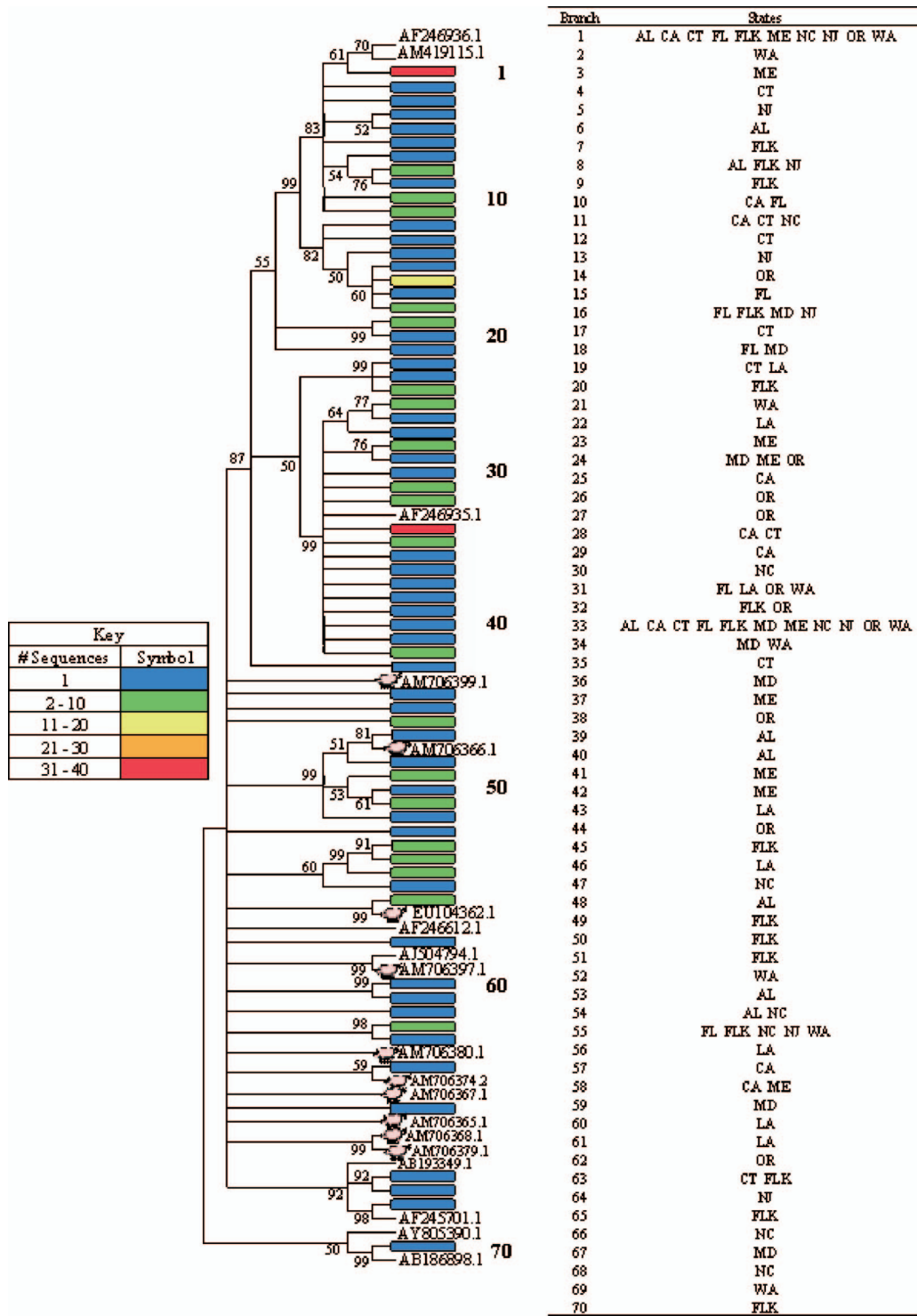


FIG. 1. Condensed neighbor-joining (Jukes-Cantor model) phylogenetic tree of a ~200-bp segment of the picobornavirus RNA-dependent RNA polymerase gene from 207 U.S. raw sewage sequences. Each branch represents a sequence or a group of sequences (99% identical with gaps) depending upon the block color. Every colored block is numbered from top to bottom, and the number corresponds to the adjacent table, which explains the locations represented by each block (Alabama [AL], California [CA], Connecticut [CT], Florida [FL], Florida Keys [FL K], Louisiana [LA], Maine [ME], Maryland [MD], North Carolina [NC], New Jersey [NJ], Oregon [OR], and Washington [WA]). Reference sequences of human and porcine picobornaviruses from previously published studies are identified by their GenBank accession numbers, and porcine picobornaviruses are indicated with a pig (8, 9, 13, 20, 59, 70, 79; J. Buesa, R. Montava, C. Tellez, and J. M. Ribes, unpublished data).

these viruses are not abundant in the United States or were simply not abundant on the day of sampling.

Adenoviruses and picobirnaviruses are potentially useful viral indicators of fecal pollution since they were found in 100% of raw sewage samples collected throughout the United States. Since these viruses were also detected in 25% and 33% of final effluent samples, respectively, future studies will need to determine how the presence of these viruses correlates with important human pathogens. Enteroviruses were detected in 75% of the raw sewage samples, and noroviruses were detected in 58% of the raw sewage samples; thus, the use of these viruses as markers of fecal pollution could potentially underestimate the extent of fecal contamination. The results of this study support prior findings regarding the prevalence of adenoviruses and enteroviruses in raw sewage and final effluent (15, 26, 37, 47, 58, 74, 77, 78). Furthermore, the use of adenoviruses and enteroviruses to indicate fecal contamination has been demonstrated by numerous studies in a variety of environments (reviewed in references 35 and 49) and experimentally tested in coastal ecosystems (35, 39, 40, 56, 64, 69, 80). This study is the first to demonstrate the widespread prevalence of picobirnaviruses in raw sewage and to suggest that these viruses can potentially be used as indicators of sewage contamination.

Since many viral types under investigation were not detected in this study, it is important to acknowledge the limitations of this research. It is expected that the viral load in the human population differs for each virus and that the abundance of these viruses fluctuates daily and seasonally in raw sewage. The samples analyzed in this study represent only a single time point at each treatment facility; therefore, it is possible that the types of viruses found could vary if samples were collected at different times of the year. In addition, the presence of viruses in the final effluent will also vary in response to fluctuations in treatment efficiency. Furthermore, the results of this study may have been biased by the differential recovery efficiency of the concentration and nucleic acid isolation methods for different viral groups. For example, viruses adhering to particles may have been lost in the filtration step. It is also possible that inefficient reverse transcription or the degradation of RNA could have skewed the results. Further studies need to be completed to verify the absence of undetected or underdetected viruses.

Similarly, it is possible that undetected viruses were present at concentrations below the assay detection limits on the day of sampling. To detect smaller concentrations of these viruses, whole-genome amplification using GenomiPhi V2 (GE Healthcare, Piscataway, NJ) was performed according to the manufacturer's instructions prior to viral PCR for each raw sewage sample and each final effluent sample as a means of increasing the concentration of target nucleic acid. Whole-genome amplification increases the total amount of DNA up to 600-fold (reviewed in reference 14), thus increasing the sensitivity of each assay by more than 2 orders of magnitude. After executing whole-genome amplification, papillomaviruses were identified in 2 of 12 raw sewage samples, and enteroviruses were detected in an additional 2 raw sewage samples, raising the total rate of enterovirus detection to 11 of 12 samples. This suggests that papillomaviruses and enteroviruses were present in those samples but at a lower abundance.

Diversity of picobirnaviruses in raw sewage. Picobirnaviruses are currently an unclassified group of viruses even though they have recently been proposed to belong to the family *Picobirnaviridae* (9). As their name suggests, these viruses have a bisegmented double-stranded RNA genome (21, 70). Picobirnavirus particles are fairly small (35 nm), nonenveloped, and spherical. They have been found in the feces of a wide range of mammals including humans (9, 12, 60, 70, 79). They have not been successfully cultured in the laboratory, and their pathogenesis is unknown (21). These viruses have been implicated as possible enteric pathogens and have occasionally been associated with gastroenteritis (12, 21). This is the first study to examine the presence and diversity of picobirnaviruses in raw sewage collected throughout the United States.

Several important conclusions can be drawn from the phylogenetic analysis of the picobirnavirus sequences from raw sewage. First, it appears that a complete understanding of the sequence diversity of picobirnaviruses remains unknown given the large number of single sequences. This is further supported by the Chao1 and rarefaction analyses. The high level of diversity of genotype I human picobirnaviruses, based on the RNA-dependent RNA polymerase gene, has been previously reported (8, 12). Similarly, extensive diversity has been observed in genotype I porcine picobirnaviruses, and their existence as quasispecies has been postulated (9). To fully understand the sequence diversity of genotype I human picobirnaviruses in raw sewage, it would be necessary to sequence more clones until an asymptote is reached in the rarefaction curve.

While the majority of picobirnavirus sequences from raw sewage grouped with known human sequences, some raw sewage sequences grouped most closely with porcine picobirnaviruses. Since the presence of porcine picobirnaviruses in human raw sewage is doubtful and because strong sequence identity between human and porcine picobirnaviruses was previously observed (9), the close grouping of a few raw sewage picobirnavirus sequences to known porcine picobirnavirus sequences is likely the result of the conserved nature of the RNA-dependent RNA polymerase gene.

Potential of adenoviruses, enteroviruses, noroviruses, and picobirnaviruses as indicators of water quality. To protect public health, it is necessary to identify a practical method for assessing fecal pollution in recreational waters. This is often accomplished through the detection of indicators, which are used to approximate the presence of pathogens. The current bacterial indicators of fecal contamination are not good indicators of wastewater pollution or human health risk in the marine environment (18, 32, 40, 41, 55, 80). The results of this study suggest that adenoviruses, enteroviruses, noroviruses, and picobirnaviruses have potential as viral indicators of fecal pollution because they were detected in the majority of raw sewage samples collected throughout the United States. Since they were detected in 100% of raw sewage samples, adenoviruses and picobirnaviruses are the most promising viral indicators of fecal pollution. Numerous previous studies (34, 35) have used adenoviruses to monitor water quality; however, this is the first study to suggest picobirnaviruses as possible indicator viruses. To assess the potential of picobirnaviruses as indicators to monitor water quality, future studies will need to determine how the presence, abundance, and stability of these

viruses correlate with pathogens of concern throughout the wastewater treatment process and in contaminated coastal environments. It will also be necessary to determine if these viruses occur naturally in the environment in the absence of fecal pollution. Our preliminary data indicated that picobirnaviruses were not naturally present at detectable levels in seawater (data not shown); however, further research is needed.

In conclusion, adenoviruses, enteroviruses, noroviruses, and picobirnaviruses were found in the majority of raw sewage samples collected from 12 wastewater treatment facilities throughout the coastal United States. However, adenoviruses and picobirnaviruses were the only viruses detected in 100% of the raw sewage samples. Adenoviruses and picobirnaviruses were also detected in 25% and 33% of final effluent samples, respectively. While adenoviruses, enteroviruses, and noroviruses are known human pathogens and were proposed as markers of fecal pollution in previous studies, the results of this research demonstrate the potential use of picobirnaviruses as an indicator of fecal pollution. Further research will be needed to determine if these candidate viruses have the necessary characteristics of a microbial water quality indicator.

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