Frequent detection of highly diverse *Cardioviruses*, *Cosaviruses*, *Bocaviruses* and *Circoviruses* in US sewage

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ABSTRACT

Untreated sewage from twelve United States (US) cities was screened for the presence of recently characterized RNA and DNA viruses found at high prevalence in the stool of South Asian children. Genetic variants of human Cosaviruses and Cardioviruses in the Picornaviridae family and of DNA Circoviruses and human Bocaviruses were detected, expanding the known genetic diversity and geographic range of these newly identified viruses. All four virus groups were detected in less than a milliliter of sewage from multiple US cities. PCR screening of particle-protected viral nucleic acid in sewage could therefore rapidly establish the presence and determine the diversity of four newly described enteric viruses in large urban populations. More frequent and deeper sampling of sewage could be used to monitor changes in the prevalence and genetic composition of these and other novel enteric viruses.
INTRODUCTION

Screening of sewage water for human viruses has a long tradition, starting with the detection and monitoring of polioviruses (10, 11, 21, 25, 51, 61). Untreated sewage water can harbor a diverse mixture of enteric viruses (9, 18, 19, 22, 42, 54). Screening of sewage from the US, Europe, Australia, and South Africa has revealed numerous human viral pathogens including members of the family Picornaviridae (e.g., Polioviruses, Coxsackie viruses and Echoviruses), Adenoviruses, Noroviruses, Reoviruses, Rotaviruses and Picobirnaviruses (18, 34, 37, 41, 42, 45, 54, 58). In this study we tested raw sewage samples from the US for the presence of recently characterized RNA and DNA viruses, including human Cardioviruses, Cosaviruses, Bocaviruses, and Circoviruses, whose geographic distribution throughout the US is largely unknown (14, 60). Three of the four viral groups (human Cosaviruses, human Bocavirus 2, and Circoviruses) were initially discovered in stool samples from children with non-polio acute flaccid paralysis collected as part of the poliovirus eradication campaign (31, 32, 60) (Li et al, in preparation). Because these viruses were also present in equal proportions in the stool of demographically matched healthy children it is unlikely that they are highly neuro-virulent (8, 31, 32) (Li et al, in preparation), but their involvement in other diseases or their possible pathogenicity in subsets of infections remains to be determined. This study is the first to evaluate the prevalence and genetic diversity of these four novel viral groups in the sewage of US cities.

Human Cardioviruses, also known as Saffold viruses (SAFV) or Human TMEV-like cardioviruses (HTCV), are a recently characterized, highly diverse
monophyletic group of picornaviruses closely related to the rodent *Theiloviruses* (36). The first human cardiovirus sequence was reported in 2007 from the archived cell culture supernatant of fetal diploid kidney cells exhibiting unexplained cytopathic effects after inoculation with stool collected from a US child with fever of unknown origin in 1981 (28). Human *Cardioviruses* have since been found in respiratory secretions and stool samples from both healthy children and children with gastroenteritis, influenza-like symptoms, or non-polio-associated acute flaccid paralysis (AFP) in North America, Europe, and South Asia (1, 8, 14, 17, 28). Using a VP1 genetic distance criterion corresponding to neutralization serotypes in *Enteroviruses* (another genus of the family *Picornaviridae*) (43), the known human *Cardioviruses* could be organized into eight VP1 genotypes (8). With increased sampling, it is expected that additional genotypes will be identified. In the US, in addition to the 1981 strain, seven human *Cardioviruses* have been detected from stool and respiratory samples collected between 2000 and 2007 (14, 28). The highest prevalence of human *Cardioviruses* has been found in stool samples from Pakistani children under 15 years of age (9-12%) followed by children in day care in Germany (7.8%) (8, 17). A recent study measuring neutralizing antibodies to a human cardiovirus isolate belonging to the most common of eight known genotypes (gt2) showed seroconversion rates approaching 100% in 2-year-old children in Europe, Africa and South–East Asia, reflecting a high rate of early human exposure (62).

*Cosavirus*, a novel genus in the *Picornaviridae* family, consists of a highly diversified group of human viruses and includes at least four species based on the
genetic distance criteria used for Enterovirus species (24, 32). Human Cosaviruses (HCoSV) were recently identified in stool samples from children in Pakistan and Afghanistan, where they were found in roughly equal proportions, nearing 40% in both healthy children and in children with non-polio AFP (32). In contrast, Cosaviruses were detected at much lower frequencies in clinical stool samples from the United Kingdom (UK) (1/1000 samples tested) (32). A Cosavirus also was found recently in the stool of an Australian infant with acute diarrhea (24). Although Cosaviruses have been detected in South Asia, the UK, and Australia, the prevalence of this group of viruses in the US is currently unknown.

Human Bocavirus (HBoV), a common respiratory pathogen of children, is a single-stranded linear DNA virus belonging to the genus Bocavirus in the family Paroviridae, subfamily Parovirinae (3, 29, 49, 52). HBoV was first detected in 2005 in respiratory tract samples from Sweden (4) and was later found, although less frequently, in stool samples from children with gastroenteritis in South and North America, Europe, Africa, Asia and Australia (7, 13, 27, 30, 53, 59). A related species, human Bocavirus 2 (HBoV2), was recently identified in approximately 5% of stool samples from South Asian children, both healthy and with non-polio-associated AFP, and in about 0.4% of clinical stool samples from UK residents (two children and one adult) (31). HBoV2 as well as a new species, HBoV3, also were recently reported in stool samples from Australian children with diarrhea as well as from healthy children with a borderline positive association with gastro-intestinal problems (5).
Circoviruses comprise one of two genera within the Circoviridae family having small circular single-stranded DNA genomes. Circoviruses have been reported in birds and mammals and are associated with potentially fatal diseases causing lymphoid tissue damage and immunosuppression in infected animals (47, 56, 57). Circovirus pathogenicity in pigs has been extensively studied since frequent infections have a significant economic impact (12, 26, 44, 50). Porcine circovirus infections have been reported worldwide including many countries in Europe, Asia, Canada, UK, New Zealand, and the US (2). We recently identified highly divergent Circoviruses in the stool of humans from Pakistan, Nigeria and Tunisia and from chimpanzees (Li et al, under preparation). In addition, novel circovirus-like genomes have been identified in environmental samples through metagenomic sequencing; the host range and geographic distribution of these novel viruses remain to be determined (33, 48).

In order to evaluate the prevalence and genetic diversity of newly identified human Cardioviruses, Cosaviruses, Bocaviruses, and Circoviruses in the US population, we analyzed sewage samples from 12 cities in 11 states by PCR and sequencing. We show that all four viral groups could be found throughout the US and that a high degree of genetic diversity was present within each group. Untreated sewage water therefore represents a readily available source of material to rapidly assess the presence and genetic variation of newly discovered enteric viruses in large urban populations.
MATERIALS AND METHODS

Concentration of viruses in sewage samples. Raw sewage samples were collected in fall 2007 from 12 wastewater treatment facilities from the coastal United States: Alabama, California, Oregon, Washington, Louisiana, Maine, Maryland, New Jersey, North Carolina, Wisconsin, Florida (included two different treatment facilities, one from western Florida, and one from the Florida Keys). Samples were also collected at multiple times from the two sites in Florida (Table 1).

Viral particle purification, extraction of viral nucleic acid, and cDNA synthesis were performed as described earlier (54). Briefly, for each sample, 10 ml was filtered through a 0.45 µm polyether sulfone membrane filter cartridge (Millipore, Billerica, MA). The degree of viral concentration in each sample before nucleic acid extraction ranged from 2 to 167-fold, and is shown in Table 1 (54). Nucleic acid was extracted using the QIAamp MinElute Virus Spin Kit (QIAGEN, Valencia, CA), and cDNA was synthesized using the First Strand Synthesis Superscript III Reverse Transcription Kit (Invitrogen, Carlsbad, CA) with random hexamers (54). After considering the dilutions of nucleic acids, the equivalent of 76 µl of raw sewage was tested in each PCR.

The nested PCR products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide. The PCR reactions were treated with ExoSap-IT exonuclease (USB, Cleveland, OH) and then directly sequenced by dideoxy-sequencing.
PCR for virus detection and characterization.

Human Cardioviruses. A nested PCR reaction targeting a 265 bp (base pair) region in the conserved 2C (helicase) region was used to test for SAFV. Primers HelF1 and HelR1 were used in the first round, and primers HelF2 and HelR2, in the second round (Table 2). PCR conditions were as previously described (8).

Human Cosavirus. A nested PCR reaction targeting 316 bp in the 5' UTR region was used to test for HCoSV. Primers DKV-N5U-F1 and DKV-N5U-R2 were used in the first round of PCR and primers DKV-N5U-F2 and DKV-N5U-R3 in the second round, as previously described (32).

Human Bocavirus. A nested PCR reaction targeting 455 bp within the VP1 region was used to test for HBoV1 and HBoV2 related viruses (Kapoor et al, in preparation). Primers BocaF1 (5'-CGCCGTGGCTCCTGCTCT-3') and BocaR1 (5'-TGTTCGCCATCACAAAAGATGTG-3') were used in the first round and BocaF2 (5'-GGCTCCTGCTCTAGGAAATAAAGAG-3') and BocaR2 (5'-CCTGCTGTTAGGTCTCTGTATATGTATGT-3') in the second round. In the first round of PCR, the reaction mix included 1X ThermoPol reaction buffer with 2mM MgSO₄ (New England Biolabs), 0.25mM of each dNTP, 20 pmol of each primer (BocaF1 and BocaR1), 0.75 µl of Taq DNA polymerase (New England Biolabs), and 1 µl of template DNA, in a 50µl mix. Amplification cycles were as follows: denaturation at 95°C for 2 min, 6 cycles of (95°C – 35 sec, 58°C – 45 sec, 72°C – 45 sec) with a decrease of 0.5 ºC per cycle in annealing temperature, 34 cycles of (95°C – 30 sec, 54°C – 30 sec, 72°C – 45 sec), and a final extension step at 72°C for 7 min. In the
second round, the reaction mix included 1X Thermopol reaction buffer with 2mM MgSO₄ (New England Biolabs), 0.25mM of each dNTP, 20 pmol of each primer (BocaF2 and BocaR2), 0.75 µl of Taq DNA polymerase (New England Biolabs), and 1.5 µl of first-round product as template in a 50 µl mix. The cycle for the second round was as follows: denaturation at 95°C for 2 min, 10 cycles of (95 °C – 35 sec, 60°C – 45 sec, 72°C – 45 sec) with a decrease of 1 °C per cycle in annealing temperature, 30 cycles of (95°C – 30 sec, 58°C – 30 sec, 72°C – 45 sec), and a final extension at 72°C for 7 min.

Circoviruses. A nested PCR reaction targeting a 500 bp region of the replicase gene was used to test for Circoviruses. The first-round PCR primers were CV-F1 (GTIMGIMGITTYTGYTTYACITGG) and CV-R1 (CCICCYTTIAYYTGIACYTTRTA) and the second-round primers were CV-F2 (GGIGARGARYTIGCICCIACIAC) and CV-R2 (GGICCCCARTARATAIACYTC). In the first-round, the PCR reaction mix included 1X ThermoPol reaction buffer with 2mM MgSO4 (New England Biolabs), 0.25mM of each dNTP, 50 pmol of each primer, 0.75 µl of Taq DNA polymerase (New England Biolabs), and 2 µl of template DNA, in a 50µl mix. The reactions were carried out with the following cycling profile: 95 °C for 5 min, 40 cycles with 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, and a final incubation for 10 min at 72 °C. The second round PCR conditions and annealing temperature were identical to first round, except that annealing was set at 56°C. 1µl of first-round PCR product was used as template for second PCR round.
**Phylogenetic analysis.** For each group of viruses, the sequences generated in this study were aligned to available sequences of related viruses from GenBank. Alignments were performed using CLUSTALW with default settings (23) and then used to generate phylogenetic trees using neighbor-joining with bootstrap values calculated from 1000 replicates in MEGA 4 (35). Nucleotide sequences were used for *Cardioviruses, Bocaviruses,* and *Cosaviruses,* and amino acids alignments were used for the more diverse *Circoviruses.*

Accession numbers of sequences used are as follows: GQ243574-GQ243692
RESULTS

Detection of human *Cardioviruses, Cosaviruses, Bocaviruses* and *Circoviruses* in raw sewage samples from different states. The presence of human *Cardioviruses, Cosaviruses, Bocaviruses,* and *Circoviruses* was analyzed in sewage waters (Table 1). At least one of the four viral groups was detected in 9 out of 11 states. *Cardioviruses* were found in 9 of 21 (43%) samples, *Cosaviruses* in 8 out of 21 (38%) samples, *Circoviruses* in 6 out of 14 samples (43%), and *Bocaviruses,* the most frequently detected virus, in 17 of 21 samples (81%).

**Human Cardioviruses.** In neighbor-joining trees, all the human cardiovirus helicase regions amplified from US sewage clustered with genotype 2 VP1 sequences (Fig 1A). Genotype 2 human *Cardioviruses* have been seen previously in the US and Canada (SAFV-UC1 and SAFV-Can112051-06) (1, 8, 14). Only a single US sequence belonging to genotype 1, which had been cultured from a Californian child in 1981 (28), did not cluster with the cardiovirus helicase sequences derived from the US sewage samples. Unexpectedly, five out of six cardiovirus sequences that were derived from sewage samples collected in a treatment facility over a two-week period were identical (Table 1 and Fig. 1A SAFL-FL2-3 to SAFL-FL2-7).

**Human Cosaviruses.** Direct sequencing of PCR product from a subset of cosavirus amplicons resulted in ambiguous sequences due to mixed nucleotide bases,
indicating that multiple genetic variants had been simultaneously amplified. In these
cases, the PCR product was cloned and sequenced. Sequencing of multiple plasmids
confirmed the co-amplification of distinct sequence variants. A subset of bocavirus
and circovirus amplicons were similarly subcloned into plasmids prior to sequencing.
According to the phylogenetic analysis, the majority of cosavirus 5’UTR sequences (18
of 29, or 62%) could be classified within Cosavirus species D (Fig. 1B)(32). However
some sequences were more closely related to Cosavirus species A and the recently
identified species F (Kapoor et al, in preparation). In addition, some sequences were
identified in samples from Florida and New Jersey that did not cluster with known
Cosavirus species (see ? in figure 1B). Further genetic analyses of other loci will be
required to determine if these divergent cosavirus-related sequences represent new
species of human Cosaviruses.

Human Bocaviruses. Phylogenetic analysis of the VP1 region indicated that
4/67 sequences (6%) were closely related to HBoV, 45/67 (67%) were related to
HBoV2, and 18/67 (26%) were related to the recently identified HBoV3 (5)(Fig. 1C).
None of the sequence was related to the new HBoV4 species recently identified in
African stool samples (Kapoor et al, in preparation). Most of the sequences in US
sewage clustered with HBoV2 genotype A, a variant previously reported only in
Pakistan, UK, and recently, Australia (5, 31) (Kapoor et al, in preparation).
Circoviruses. A tBLASTx analysis revealed that 9 out of 13 sequences obtained from PCR reactions that target the circovirus replicase gene were 35-58% identical to viruses from the Circoviridae family. In neighbor-joining trees, these sequences clustered with porcine and bird Circoviruses. (Fig. 1, D). In contrast, the remaining four circovirus-like sequences fell outside of the circovirus clade, but within a larger cluster including the plant geminiviruses and nanoviruses. Definitive classification of the viruses with the divergent circovirus-like replicase sequences will require amplification and sequencing of their full genomes. For three of these four circovirus-like sequences, the twenty best tBLASTx matches were to circoviruses, but for one sequence (WA-1) the best tBLASTx hits were to both bird circoviruses and plant pathogens from the Nanoviridae family (i.e., Faba bean necrotic yellow virus, Milk vetch dwarf virus, and Banana bunchy top virus). It is possible that this group of divergent circovirus-like replicase sequences represents a new viral family. In our neighbor-joining tree, the sequences are not included in either the circovirus or nanovirus clades, so these viruses might be animal or plant viruses.
DISCUSSION

We report here the detection and genetic diversity of Cardioviruses, Cosaviruses, Bocaviruses, and Circoviruses in untreated sewage water from different US cities. Human Bocaviruses were the most commonly detected viruses and were found in all but two of the analyzed states (WI and LA) (Table 1). HBoV1 has a very broad geographical distribution when respiratory samples are analyzed but is less commonly detected in stool (3, 7, 13, 27, 29, 30, 49, 52, 53, 59). In this study HBoV1 was detected in only one city in Florida, while HBoV2 and HBoV3 were detected in sewage from all bocavirus-positive cities (Figure 1C). This result might reflect enhanced human gut tropism for HBoV2 and HBoV3 relative to HBoV1. To our knowledge, this is the first report of HBoV2 or HBoV3 in the US. HBoV2 genotype A dominated the HBoV2 sequences from US sewage, while HBoV2 genotype B dominated in Africa (Kapoor et al, in preparation). A divergent sequence (HBoV?-CA-1-C1) was also detected which could reflect another genotype of HBoV3 or possibly another species of HBoV.

Human Cosaviruses were detected in 38% of the samples analyzed (Table 1). This is the first documentation of Cosaviruses in the US. Sequencing of the conserved 5’UTR region showed that some variants clustered with the known species A and D, while others formed separate clusters unrelated to currently known species (Figure 1B). The predominance of species D distinguishes the US from what has been observed in other countries (Pakistan, Nigeria, and Tunisia), where species A predominates (32) (Kapoor et al, in preparation).
The human cardiovirus helicase sequences in sewage were found to cluster weakly with two of the three available US-derived sequences (1, 14) (Figure 1A). Since the cardiovirus helicase sequences found in the US are distinct from sequences obtained from Pakistani samples, it is likely that, as for cosaviruses species and HBoV2 genotypes, the prevalence of human cardiovirus genotypes differs among geographic regions. Further work is needed to obtain the VP1 genotypes of these strains from US sewage to determine if they correspond to genotype 2, like the recently acquired US isolates (1, 14).

The observation that 5 of 6 samples collected days apart in Bradenton, Florida yielded identical human cardiovirus sequences was unexpected in light of the diverse sequences acquired from each of the other positive sites. Possible explanations for the genetically homogeneous Cardioviruses sampled in this town include differences in the geographic distribution of cardiovirus strains, a recent spread in that Florida community of the repeatedly sampled strain, and even the contribution to the local sewage of Cardioviruses from a single individual shedding very high levels of virus.

Circoviruses are currently known to infect birds and swine, and as of yet, no human Circoviruses have been demonstrated. Circoviruses present in US sewage water were phylogenetically diverse, falling into two main groups distinct from known Circoviruses. Nine sequences clustered into four groups related to pig and bird Circoviruses, but sufficiently distinct to represent possible new species of Circoviruses. Four other replicase sequences fell outside of the known animal circovirus clade in our neighbor-joining trees, but were included in a larger clade with
both Circoviruses and Geminiviruses. The N-terminal region of circovirus replicase proteins has been shown to be related to those of nanoviruses, while its C-terminal region is more closely related to the 2C RNA binding protein of picornaviruses, indicating a possible chimeric origin from plant and animal viruses (20). Whether these sequences represent novel Circoviruses, Nanoviruses, or a new family of replicase-containing viruses will require further analyses, including their full genome sequencing.

In conclusion, newly characterized enteric viruses (Human Bocaviruses, Cardioviruses, Cosaviruses) as well as distant relatives of animal viruses (Circoviruses) could be readily detected in untreated sewage waters from different US cities. An abundance of viral diversity can therefore be readily accessed in untreated sewage. Since the nucleic acids derived from less than 100 µl equivalent of untreated sewage were examined in each PCR, it is conceivable that testing of larger volumes would have revealed an even greater prevalence of these enteric viruses. As has been previously shown, sewage water therefore represents a convenient material to test large populations for the presence of enteric viruses (9-11, 18, 19, 21, 22, 25, 42, 54). Modeling studies have indicated that a single poliovirus toilet “flush” could contain enough enterovirus to be detected in the sewage system for up to four days (46). Studies of untreated sewage waters collected at different times might show seasonal fluctuation and yield further insight into the epidemiology of these viruses. Similarities have also been detected in the poliovirus strains in sewage and the stools of sampled children (15).
More detailed genetic analyses of the viruses in untreated sewage might also show how rapidly new variants can colonize populations in different cities. Such a phenomenon might have been reflected in the identical cardioviruses sampled over several weeks from the same city in Florida. Long standing endemic infections would be expected to yield viral populations with more genetic diversity among strains, due to introduction of new strains and evolution of local strains.

Efficient and reproducible sampling of the complex viral nucleic acid populations in sewage will need to be demonstrated before solid conclusions regarding the frequency of particular viral genetic variants can be drawn (25). Increasing the starting volume of sewage water, and therefore the subsequent nucleic acid input, might provide a better and deeper sampling of the replicating viral populations. In the future, quantitative PCR assays can also be developed to measure fluctuations in viral loads in sewage. Detailed analyses of PCR amplicons using ultra-deep sequencing technologies may also allow a more accurate view into the composition and change in the frequency of circulating variants (39, 40, 60). Untreated sewage therefore provides a readily available source to test for the presence of newly identified viruses and to monitor their genetic diversity on a population level scale. Monitoring viral concentration in sewage might also be used for surveillance and, if done in real-time, to provide early warning signs of impending outbreaks (16, 38, 51).
Figure legend

Figure 1. Phylogenetic analysis of amplicons derived from untreated sewage waters. A) Human *Cardioviruses* (SAFV) based on partial helicase sequences, available genotypes based on VP1 are included in brackets (gt), (B) Human *Cosaviruses* (HCoSV) based on partial 5’UTR sequences, (C) Human *Bocaviruses* (HBoV) based on partial VP1 sequences, (D) *Circoviruses* (SewCV) based on partial replicase sequences. Sequences from sewage samples are marked by black dots. Genbank accession numbers of non-sewage derived sequences are included in taxa names.

Taxa are labeled with name of virus followed by letter or number of the species (for HCoSV and HBoV) then by US state, location within state (1 or 2), time points (FL1-1 to FL1-3 and FL2-1 to FL2-7 only) and origin of sequences (P for direct PCR sequencing, C for plasmid clone).
## TABLE 1. Detection of enteric viruses in sewage samples

<table>
<thead>
<tr>
<th>State</th>
<th>Sample</th>
<th>Collected</th>
<th>Concentration grade (55)</th>
<th>Cardio virus</th>
<th>Cosa virus</th>
<th>Boca virus</th>
<th>Circo virus</th>
<th>% of positive samples</th>
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<td>2</td>
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<td>93</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>43</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+(9)</td>
<td>+(3)</td>
<td>81</td>
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<td>-</td>
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<td>43</td>
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<td>-</td>
<td>+</td>
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<td>43</td>
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<td>+</td>
<td>+(3)</td>
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</table>

*+/− sample positive/negative by nested PCR for the tested virus.

The number in parentheses indicates the number of viral sequences derived from plasmids after subcloning of mixed sequences amplicons. NT: not tested due to unavailability of viral particle associated cDNA/DNA.
Bibliography

6. B-REF7. Incisions were made in the abdominal wall and the internal organs imaged as in the legend to figure 1. Following the 5 min. integration time, the anesthetized mice were euthanized.


