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BRIEF REPORT

# Occurrence and genetic diversity of human cosavirus in influent and effluent of wastewater treatment plants in Arizona, United States

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**Abstract** Human cosavirus (HCoSV) is a novel member of the family *Picornaviridae*. We investigated the prevalence and genetic diversity of HCoSV in influent and effluent wastewater in Arizona over a 12-month period, from August 2011 to July 2012. HCoSV sequences were identified in six (25 %) influent samples and one (4 %) effluent sample, with the highest concentration of $3.24 \times 10^5$ and $1.54 \times 10^3$ copies/liter in influent and effluent, respectively. The strains were characterized based on their 5' untranslated region and classified into species A and D, demonstrating that genetically heterogeneous HCoSV were circulating with a clear temporal shift of predominant strains in the study area.

**Keywords** Cosavirus · qPCR · Wastewater · Nested PCR · Phylogenetic analysis

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## Introduction

Human cosavirus (human *co*mmon *s*tool *a*ssociated picornavirus, HCoSV) is a novel member of the family *Picornaviridae* and the genus *Cosavirus* [1]. HCoSV was first identified in 2008 in the feces of South Asian children with non-polio acute flaccid paralysis (AFP) and healthy controls [1]. The genus is currently divided into six genetically distinct species (A-F) with a total of more than 30 genotypes [2]. Similar to cardioviruses, which are close relatives of picornaviruses, HCoSV has been frequently detected in the feces of symptomatic as well as asymptomatic subjects, and thus their role in human enteric disease remains unclear [3]. This is partly because there have been only a limited number of epidemiological studies on this emerging virus.

Examining domestic wastewater has been considered an effective approach to understand the actual prevalence and epidemiology of enteric viruses, and a number of studies targeting various types of virus in wastewater have been carried out [4, 5]. In one study conducted in the United States, HCoSV was detected in 38 % of raw sewage samples tested [6]. More recently, Haramoto and Otagiri reported the detection of HCoSV in wastewater and river water in Japan, demonstrating a high prevalence in tested samples (raw sewage, 71 %; secondary-treated sewage, 29 %; river water, 29 %) with the highest concentration of $2.80 \times 10^6$ copies/liter in raw sewage [7]. Moreover, our recent work revealed the presence of another emerging picornavirus potentially associated with infantile gastroenteritis and AFP, namely, salivirus/klassevirus, in wastewater samples collected from Arizona, the United States [8]. These recent findings prompted us to carry out a year-long investigation on the occurrence and genetic diversity of HCoSV in wastewater in Arizona. In the present

study, we quantified HCoSV genomes in wastewater using quantitative PCR (qPCR) and phylogentically characterized the virus strains based on their partial 5' untranslated region (UTR) sequences.

Wastewater sampling was conducted monthly from August 2011 to July 2012 at two wastewater treatment plants (plant A and B, utilizing activated sludge and trickling filter, respectively) located in southern Arizona. A total of 48 grab samples were collected, which consisted of 12 influent and 12 effluent samples each from two plants. Viruses in the wastewater samples (100 mL influent and 1,000 mL effluent) were concentrated using an electronegative filter (cat. no. HAWP-090-00; Merck Millipore, Darmstadt, Germany) and a Centriprep YM-50 device (Merck Millipore) to obtain a final volume of 650 μL as described previously [9]. More details of the characteristics of each plant and sampling and virus concentration procedures are described elsewhere [10]. Viral nucleic acid was extracted and purified using a ZR Viral DNA/RNA Kit (Zymo Research, Irvine, CA), according to the manufacturer's instructions. The reverse transcription (RT) reaction was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

The presence of HCoSV genomes was first examined by TaqMan-based qPCR [11], using a LightCycler® 480 Real-Time PCR Instrument II (Roche Diagnostics, Mannheim, Germany). The genome copy numbers of HCoSV were determined based on a standard curve prepared with ten-fold serial dilutions of plasmid DNA containing the partial 5' UTR sequence of strain HCoSV-A1 (GenBank accession number: J438902). The qPCR reactions were performed in duplicate (i.e., two PCR tubes were used for each sample), and the sample was considered positive only when a fluorescent signal with sufficient intensity was obtained from both tubes and the average crossing point (*Cp*) value was not more than 40, as recommended by the guidelines described elsewhere [12].

The nested PCR assay using DKV-N5U-F1, -R2, -F2, and -R3 primers targeting a 316-bp region of 5' UTR was then performed using Ex *Taq* DNA polymerase (TaKaRa Bio Co., Shiga, Japan), as described previously [1]. The second PCR products were separated by electrophoresis on a 2 % agarose gel and visualized under a UV lamp after ethidium bromide staining. All of the second PCR products with expected size were excised from the gel and cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA). Eight to 15 colonies (clones) per sample were selected, and both strands of direct colony PCR products were sequenced using a BigDye Cycle Sequencing Kit version 3.1 and a 3730*xl* Genetic Analyzer (Applied Biosystems). Nucleotide sequences were assembled using the program Sequencher™ version 5.0.1 (Gene Codes Corporation, Ann Arbor, MI, USA) and aligned with Clustal W version 1.83 (http://clustalw.ddbj.nig.ac.jp/top-e.html). Genetic distances were calculated by Kimura's two-parameter method [13], and a phylogenetic tree from a bootstrap analysis with 1,000 replicates was generated by the neighbor-joining method.

By using the RT-qPCR assay, HCoSV genomes were detected in five (42 %) and six (50 %) influent samples from plant A and B, respectively, and in two (17 %) effluent samples from both plants. Different temporal occurrence between plant A and B was observed based on the RT-qPCR detection; HCoSV genomes were frequently detected during colder months at plant A, whereas samples from plant B showed a high detection rate during warmer months (Table 1). This observation from two plants located in the same region implies the occurrence of sporadic and temporary HCoSV infections within the study area. The influent sample collected from plant B in November 2011 exhibited the highest HCoSV genome concentration of $3.24 \times 10^5$ copies/liter (Table 1). Haramoto and Otagiri reported that HCoSV was detected by the same RT-qPCR assay in 71 % (10/14) of raw wastewater and 29 % (4/14) of treated wastewater samples in Japan, with concentrations of $4.47 \times 10^4$ to $2.80 \times 10^6$ and $3.54 \times 10^3$ to $7.50 \times 10^4$ copies/L, respectively [7]. Detection rates as well as concentrations of HCoSV genomes observed in our wastewater samples in Arizona were generally lower than those in wastewater samples in Japan. One of the possible reasons of this difference in the occurrence of HCoSV in wastewater could be a difference in the incidence of HCoSV infection within the two countries/regions. Use of murine norovirus (MNV S7-PP3 strain; kindly provided by Dr. Y. Tohya, Nihon University, Kanagawa, Japan) as a process control (not used to adjust the viral genome copy numbers) showed no substantial inhibition in the extraction-RT-qPCR process in any of the wastewater samples tested in this study (mean recovery efficiency of more than 75 %; detailed results were reported in our previous study [10]).

By use of the 5' UTR nested RT-PCR assay, seven (15 %) samples tested positive, of which six were positive by the RT-qPCR assay as well, and only one nested-PCR-positive sample was qPCR negative (effluent from plant B in December 2011; Table 1). The overall detection ratio by the nested PCR assay was lower than that of qPCR assay (15/48, 31 %), probably because the qPCR assay is more sensitive than the nested PCR assay when applied to environmental samples.

Based on nucleotide sequencing of a total of 76 selected clones (8 to 15 clones per sample), we identified a total of 35 HCoSV sequences (1 to 8 different sequences per sample), which were classified into species A and D (Table 1, Fig. 1). This result agrees with previous studies

**Table 1** Detection of human cosavirus in wastewater in Arizona, USA

| Year/Month | Plant A | | | | | | Plant B | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Influent | | | Effluent | | | Influent | | | Effluent | | |
| | qPCR[a] | | PCR-sequencing[b] | qPCR | | PCR-sequencing | qPCR | | PCR-sequencing | qPCR | | PCR-sequencing |
| | *Cp* | Copies/L | | *Cp* | Copies/L | | *Cp* | Copies/L | | *Cp* | Copies/L | |
| 2011/Aug | – | – | – | – | – | – | 38.9 | $5.21 \times 10^4$ | Species D | – | – | – |
| Sept | – | – | – | – | – | – | 37.8 | $4.16 \times 10^4$ | Species D | – | – | – |
| Oct | – | – | – | – | – | – | 37.7 | $8.50 \times 10^4$ | – | – | – | – |
| Nov | 38.4 | $2.13 \times 10^4$ | Species D | – | – | – | 36.0 | $3.24 \times 10^5$ | Species A | – | – | – |
| Dec | – | – | – | – | – | – | – | – | – | – | – | Species D |
| 2012/Jan | 37.8 | $5.43 \times 10^4$ | – | – | – | – | 37.1 | $9.69 \times 10^4$ | Species D | – | – | – |
| Feb | 37.1 | $1.03 \times 10^5$ | – | 37.0 | $8.88 \times 10^2$ | – | – | – | – | 36.5 | $8.44 \times 10^2$ | – |
| Mar | 37.0 | $1.09 \times 10^5$ | – | – | – | – | – | – | – | – | – | – |
| Apr | 36.7 | $1.29 \times 10^5$ | Species A | – | – | – | – | – | – | – | – | – |
| May | – | – | – | – | – | – | – | – | – | – | – | – |
| June | – | – | – | 35.9 | $1.54 \times 10^3$ | – | – | – | – | – | – | – |
| July | – | – | – | – | – | – | 37.3 | $9.00 \times 10^4$ | – | 37.9 | $2.40 \times 10^2$ | – |
| % positive | 42 % | | 17 % | 17 % | | 0 % | 50 % | | 33 % | 17 % | | 8 % |

[a] *Cp* results indicate average values from two qPCR tubes. The concentrations of HCoSV genomes were calculated from average cDNA copy numbers of the two qPCR tubes

[b] Nested PCR followed by cloning and nucleotide sequencing. HCoSV species identified by this method are indicated. –, negative

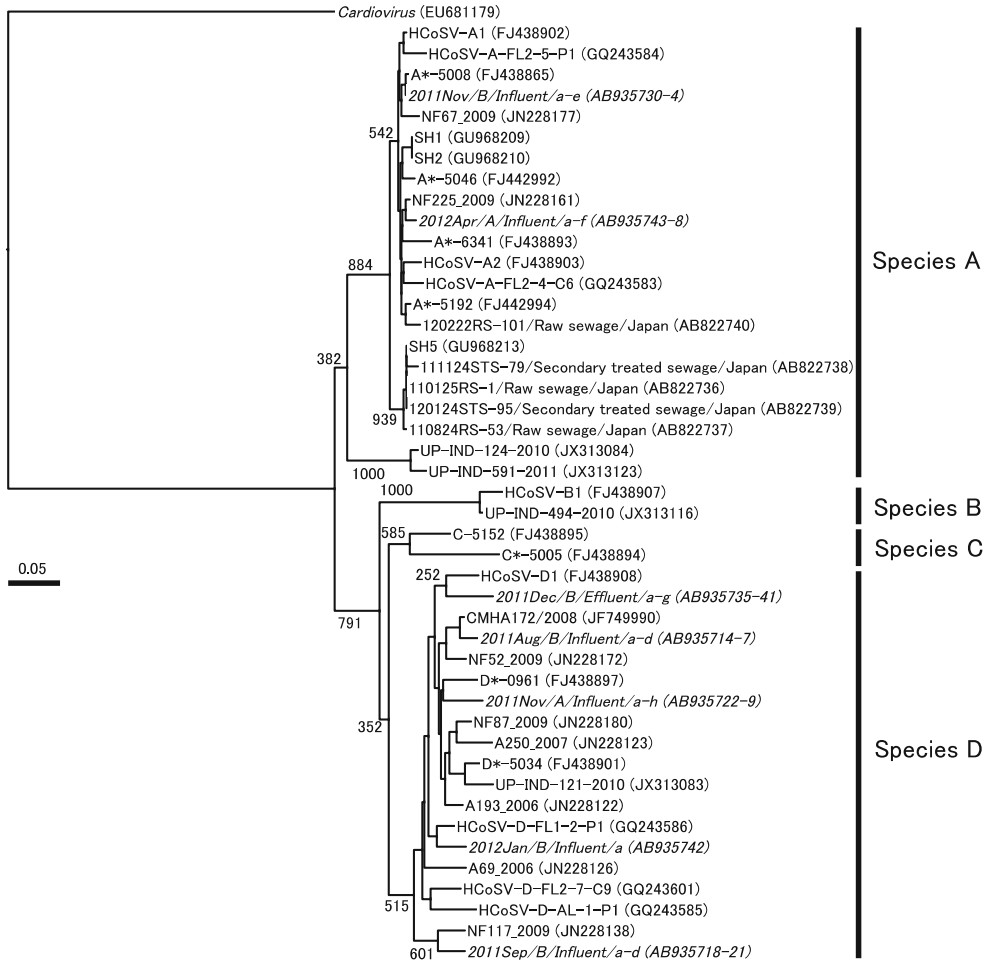


**Fig. 1** Phylogenetic tree for HCoSV strains using 276 nucleotides of the 5' UTR gene sequences. The tree was generated by the neighbor-joining method with representative strains among slightly different sequences derived from the same wastewater sample as well as reference strains. The numbers on each branch indicate the bootstrap values obtained from a bootstrap analysis with 1,000 replicates, and the scale represents nucleotide substitutions per site. Strains shown in *italic* are representative HCoSV strains identified in the present study, representing the year and month of sample collection

reporting the predominance of species A and D in clinical samples [1, 2]. The HCoSV strains identified in the same wastewater sample belonged to the same genetic cluster (only representative strains among the slightly different sequences derived from the same sample are shown in Figure 1), which exhibits low genetic diversity among strains in a single wastewater sample. However, the strains identified in different months and treatment plants were genetically distinct from each other (Fig. 1), demonstrating the heterogeneity of HCoSV strains causing infections and a temporal shift in the predominant species/strains in the study area.

Viral metagenomic analysis has been a powerful tool for discovering new viral pathogens and determining the diversity of viruses in clinical and environmental samples. In fact, this allowed the discovery of HCoSV in human stool samples [1] and in subsequent identification in sewage and biosolids [14, 15]. However, clinical and environmental surveillance of viral pathogens of particular interest/importance using a targeted detection approach, such as qPCR quantification and target-specific PCR amplicon sequencing, are still necessary and complementary to the metagenomic strategy once new viruses are discovered and their genetic information becomes available. In addition, a recent study reported the *in vitro* propagation and infectivity assay for HCoSV using a cell culture system [16]. This may allow development of a strategy to assess the infectivity of HCoSV in environmental samples using cell culture assays and/or integrated cell culture PCR (ICC-PCR), which will contribute to a better understanding of environmental persistence and public health risks of HCoSV, if any, in the future.

Until now, only two studies have reported molecular detection of HCoSV in environmental water samples in the

United States and Japan [6, 7]. The present study describes additional evidence of the prevalence and genetic diversity of HCoSV in wastewater based on a year-long study in southern Arizona, United States. Our results suggested that HCoSV of species A and D was circulating among humans and aquatic environments with a clear temporal shift in the predominant strains in the study area. Further environmental studies, even in combination with clinical studies, are required worldwide to understand the molecular epidemiology and geographical distribution of this emerging virus with a currently unknown etiological role in human enteric diseases.

## Nucleotide sequence accession numbers

The nucleotide sequences determined in the present study have been deposited in GenBank under accession numbers AB935714–AB935748.

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