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BRIEF COMMUNICATION

Quantification and Genetic Analysis of Salivirus/Klassevirus in Wastewater in Arizona, USA

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Abstract Salivirus/klassevirus sequences were identified in 7 (15 %) wastewater samples collected in Arizona monthly for a year, with the highest concentration of 2.28×10^5 and 2.46×10^4 copies/L in influent and effluent, respectively. This is the first report of quantification and genetic analysis of salivirus/klassevirus in water samples in the United States.

Keywords Salivirus/klassevirus · qPCR · Wastewater treatment plant · Nested PCR · Phylogenetic analysis

Salivirus/klassevirus is a novel member of the family *Picornaviridae* and most closely related to Aichi virus in the genus *Kobuvirus* (Greninger et al. 2009; Holtz et al. 2009; Tapparel et al. 2013). This virus was recently discovered in the feces of children with gastroenteritis in the United States and Australia as well as in a sewage sample collected in Spain (Holtz et al. 2009). Subsequently, it was identified in the fecal samples in African (Li et al. 2009), Asian (Greninger et al. 2010; Han et al. 2010; Li et al. 2009; Shan et al. 2010), European (Calgua et al. 2013; Nielsen et al. 2013), and South American continents as well (Calgua et al. 2013; Nix et al. 2013), suggesting its worldwide distribution. Although it has been suggested that this virus may be a cause of gastroenteritis in infants (Nielsen et al. 2013), its actual prevalence, molecular epidemiology, and role in human enteric diseases still remain unclear.

Since wastewater contains viruses shed from all individuals within a service area regardless of their symptoms, examining wastewater could be an effective approach to determine the circulation of viruses within a population and to obtain sequence information of the circulating strains; especially for emerging viruses or viruses rarely identified in clinical cases, including salivirus/klassevirus. There have been only a limited number of reports describing the occurrence of salivirus/klassevirus in environmental water samples, such as wastewater and river water (Greninger et al. 2009; Han et al. 2014; Haramoto et al. 2013; Haramoto and Otagiri 2013). In the present study, we investigated the prevalence and genetic diversity of salivirus/klassevirus in wastewater in Arizona, the United States, during a 1-year period. Salivirus/klassevirus genomes in wastewater were quantified by quantitative PCR (qPCR), and the virus strains were further characterized based on their partial 5′ untranslated region (UTR) sequences.

Between August 2011 and July 2012, a total of 48 wastewater samples were collected monthly from two WWTPs (Plant A and B, utilizing activated sludge and trickling filter, respectively) located in southern Arizona, which included 12 influent and 12 effluent samples each from two plants. One hundred milliliters of influent and 1,000 mL of effluent wastewater samples were concentrated using an electronegative membrane (Merck Millipore, Tokyo, Japan) and a Centriprep YM-50 device

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Table 1 Detection of salivirus/klassevirus in wastewater in Arizona, USA

Year/ Month	Plant A						Plant B					
	Influent			Effluent			Influent			Effluent		
	qPCR[a]		Nested PCR[b]	qPCR		Nested PCR	qPCR		Nested PCR	qPCR		Nested PCR
	Cp	Copies/L		*Cp*	Copies/L		*Cp*	Copies/L		*Cp*	Copies/L	
2011/Aug	36.4	9.65×10^4	−	38.6	6.95×10^2	−	34.8	1.54×10^5	−	36.3	4.85×10^3	−
Sept	35.7	1.60×10^5	−	36.8	6.05×10^3	−	34.3	2.28×10^5	+	34.6	1.79×10^4	−
Oct	−	−	−	−	−	−	34.6	1.80×10^5	−	−	−	−
Nov	38.0	1.89×10^4	−	−	−	−	35.0	1.49×10^5	−	36.1	7.17×10^3	−
Dec	38.1	1.51×10^4	−	−	−	−	−	−	−	−	−	−
2012/Jan	−	−	−	−	−	−	−	−	−	36.2	2.10×10^3	+
Feb	−	−	−	36.9	4.61×10^3	−	36.5	1.31×10^4	−	35.4	5.47×10^3	−
Mar	−	−	−	−	−	−	−	−	−	−	−	+
Apr	−	−	+	−	−	−	−	−	−	−	−	−
May	−	−	−	−	−	−	38.3	2.32×10^3	−	36.2	2.05×10^3	−
June	−	−	+	35.8	7.42×10^3	−	−	−	−	−	−	−
July	−	−	+	33.6	2.46×10^4	−	37.3	7.82×10^3	+	−	−	−
% positive	33 %		25 %	42 %		0 %	58 %		17 %	50 %		17 %

[a] *Cp* results indicate average values from two qPCR tubes

[b] +, positive; −, negative

(Merck Millipore) to obtain a final volume of 650 µL, as previously described (Kitajima et al. 2014). Viral RNA was extracted and purified using the ZR Viral DNA/RNA Kit (Zymo Research, Irvine, CA), according to manufacturer's instructions. Reverse transcription (RT) was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

The presence of salivirus/klassevirus genomes was first examined with a recently developed TaqMan MGB-based qPCR (Haramoto et al. 2013), using a LightCycler® 480 Real-Time PCR Instrument II (Roche Diagnostics, Mannheim, Germany). A 10-fold serial dilution of the standard plasmid DNA was used to make a standard curve, enabling us to obtain quantitative data on salivirus/klassevirus genomes in the wastewater samples. The qPCR reactions were performed in duplicate (i.e., two PCR tubes per sample) and considered positive only when both tubes fluoresced with sufficient intensity and the average crossing point (*Cp*) value was not more than 40, as recommended by the guidelines described elsewhere (Bustin et al. 2009). By use of the RT-qPCR assay, salivirus/klassevirus genomes were detected in 4 (33 %) and 7 (58 %) influent and 5 (42 %) and 6 (50 %) effluent samples from plant A and B, respectively. The influent sample collected from plant B in September 2011 exhibited the highest salivirus/klassevirus genome concentration of 2.28×10^5 copies/L (Table 1). Haramoto et al. (2013) reported that salivirus/klassevirus was detected by the RT-qPCR assay in 93 % (13/14) of raw wastewater and 29 % (4/14) of treated wastewater in Japan with concentration of 3.7×10^5 to 9.7×10^6 and 1.7×10^4 to 2.7×10^5 copies/L, respectively. Both positive rate and concentration of salivirus/klassevirus observed in our wastewater samples in Arizona were lower than those of Japanese wastewater, probably because of the differences in incidence of infection within the different counties and/or the differences in the amount and type (i.e., industrial and domestic) of water use per capita. Use of murine norovirus (S7-PP3 strain) as a process control showed no substantial inhibition in the extraction-RT-qPCR process in any of the wastewater samples tested in this study (recovery efficiency of as low as 52 %).

The nested PCR assay using SAL-L1, -R1, -L2, and -R2 primers was then performed to generate 414-bp products of 5′ UTR using Ex *Taq* DNA polymerase (TaKaRa Bio Co., Shiga, Japan), as previously described (Shan et al. 2010). The PCR products were separated by electrophoresis on a 2 % agarose gel and visualized by staining with ethidium bromide. Seven (15 %) samples were positive by the 5′ UTR nested PCR assay, of which 3 samples were positive by the qPCR assay as well, and the other 4 nested PCR-positive samples were qPCR-negative; conversely, 19 qPCR-positive samples were tested negative by nested PCR. This discrepancy between the results of detection by qPCR and nested PCR may be due to the difference of detection sensitivity between the two assays—i.e., the qPCR assay being slightly more sensitive than the nested PCR assay, as described in Haramoto et al. (2013)—as well

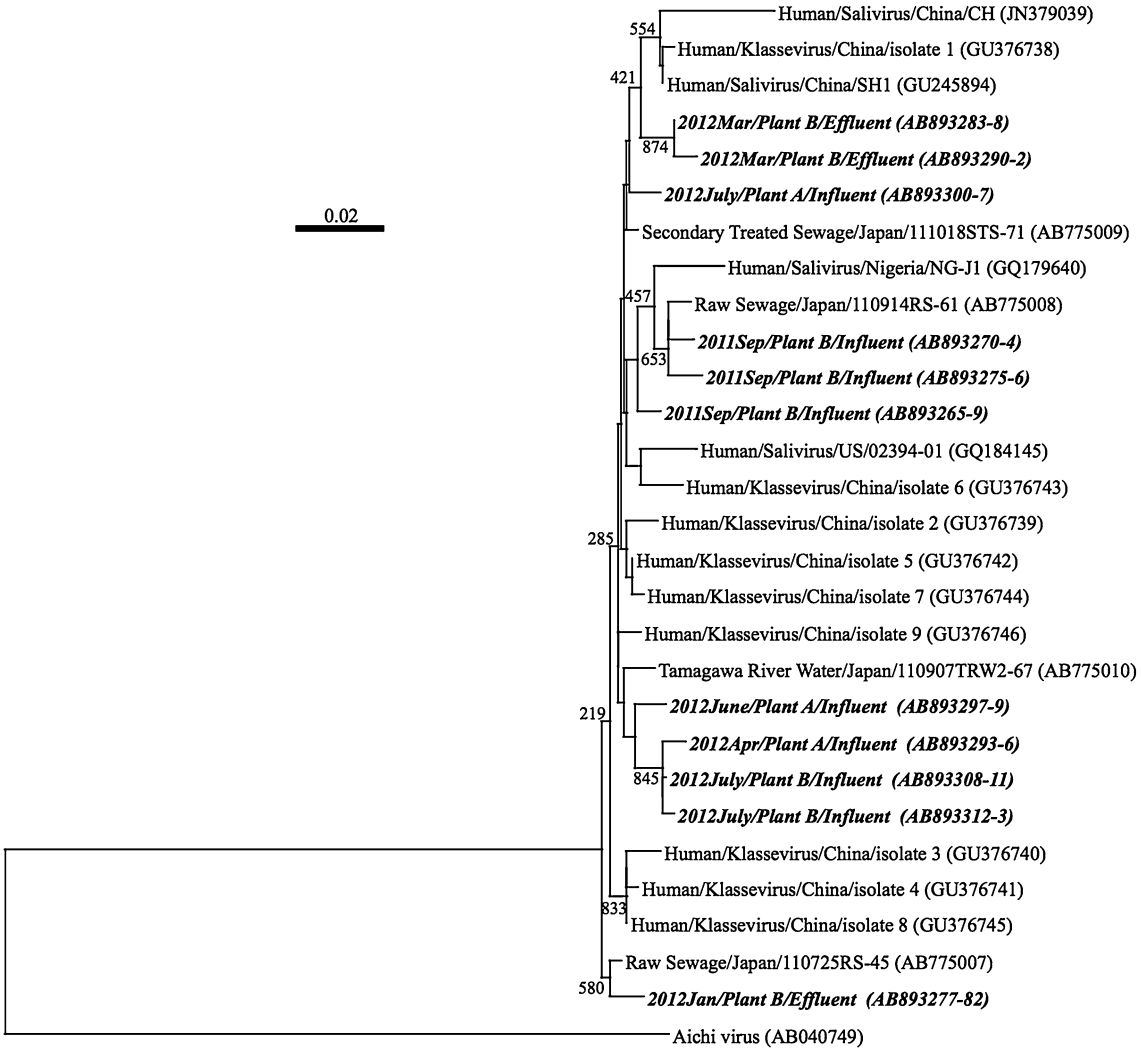

Fig. 1 Phylogenetic tree for salivirus/klassevirus strains using 374 nucleotides of the 5′ UTR gene sequences. The tree was generated by the neighbor-joining method with representative strains derived from wastewater and reference strains. The *numbers on each branch* indicate the bootstrap values obtained from a bootstrap analysis with 1,000 replicates, and the *scale* represents nucleotide substitutions per site. Strains shown in *italic bold* are representative salivirus/klassevirus strains identified in the present study, representing the year and month of sample collection, WWTP (plant A or B), sample type (influent or effluent), and GenBank accession number.

as the variability of target genome copy numbers/presence in a PCR tube especially in a low concentration range (at the edge of lower detection limit).

All of the positive second PCR products were cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA). A total of 88 clones (11 to 15 clones per sample) were selected, and both strands were sequenced with the BigDye Cycle Sequencing Kit version 3.1 and 3730*xl* Genetic Analyzer (Applied Biosystems). Nucleotide sequences were assembled using the program Sequencher (TM) version 5.0.1 (Gene Codes Corporation, Ann Arbor, MI, USA) and aligned using Clustal W version 1.83 (http://clustalw.ddbj.nig.ac.jp/top-e.html), and distances were calculated by Kimura's two-parameter method (Kimura 1980). The phylogenetic tree from a bootstrap analysis with 1,000 replicates was generated by the neighbor-joining method.

Based on the sequencing analysis, we identified a total of 49 salivirus/klassevirus sequences (3 to 12 different sequences per sample), which were closely related to known environmental and clinical strains (Fig. 1). The salivirus/klassevirus strains identified in the same wastewater sample tended to form the same genetic cluster with sequence similarity of 98 % or higher, but the strains identified in different months were, in general, genetically distinct from each other (as low as 96 % sequence similarity). This result demonstrates the diversity of salivirus/klassevirus strains infecting to humans and temporal shift of the prevalent strains in the study area.

This study describes novel findings on the prevalence and genetic diversity of salivirus/klassevirus in wastewater in the United States. A few studies have reported molecular detection of this virus in wastewater samples in Japan and

Korea (Han et al. 2014; Haramoto et al. 2013; Haramoto and Otagiri 2013); however, since these studies performed direct sequencing without molecular cloning, genetic diversity of the virus may have been underestimated. To improve upon these previous studies, we cloned the PCR products before sequence analysis so that we could detect multiple salivirus/klassevirus strains in one sample, not just the predominant strain. As a result, we were able to better assess the variations of salivirus/klassevirus strains in the wastewater samples. The present study highlights the importance of further studies on salivirus/klassevirus toward a better understanding of its molecular epidemiology, geographical distribution, and etiological role, if any, in human enteric diseases.

Nucleotide Sequence Accession Numbers

The nucleotide sequences determined in the present study have been deposited in GenBank under accession numbers AB893265–AB893313.

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