



HOKKAIDO UNIVERSITY

Title	Temporal variations in genotype distribution of human sapoviruses and Aichi virus 1 in wastewater in Southern Arizona, United States
Author(s)	Kitajima, M.; Rachmadi, A. T.; Iker, B. C. et al.
Citation	Journal of applied microbiology, 124(5), 1324-1332 https://doi.org/10.1111/jam.13712
Issue Date	2018-05
Doc URL	https://hdl.handle.net/2115/73810
Rights	This is the peer reviewed version of the following article: Journal of applied microbiology 124(5) pp.1324-1332 May 2018, which has been published in final form at https://doi.org/10.1111/jam.13712 . This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.
Type	journal article
File Information	Kitajima et al. 2018 JAM (accepted manuscript).pdf



**Temporal Variations in Genotype Distribution of Human Sapoviruses and
Aichi Virus 1 in Wastewater in Southern Arizona, United States**

Masaaki Kitajima^{1,*}, Andri T. Rachmadi¹, Brandon C. Iker^{2,†}, Eiji Haramoto³,
Charles P. Gerba²

¹ *Division of Environmental Engineering, Faculty of Engineering, Hokkaido University,
North 13 West 8, Sapporo, Hokkaido 060-8628, Japan*

² *Department of Soil, Water and Environmental Science, The University of Arizona, 1117 E.
Lowell St., Tucson, Arizona 85721, USA*

³ *Interdisciplinary Center for River Basin Environment, University of Yamanashi, 4-3-11
Takeda, Kofu, Yamanashi 400-8511, Japan*

[†]Present affiliation

Amway, 7575 Fulton Street East, Ada, MI 49355-0001, USA

***Corresponding author:** Masaaki Kitajima

Mailing address: Division of Environmental Engineering, Faculty of Engineering,
Hokkaido University, North 13, West 8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan

Tel: +81(11)-706-7162 **Fax:** +81(11)-706-7162 **E-mail:** mkitajima@eng.hokudai.ac.jp

Abbreviated running headline: Sapovirus and AiV-1 in US wastewater

ABSTRACT

Aims: To investigate the molecular epidemiology, especially temporal variations in genotype distribution, of sapoviruses and Aichi virus 1 (AiV-1) in Arizona, United States, by examining wastewater.

Methods and Results: A total of 26 wastewater samples (13 influent and 13 effluent) were collected monthly from a wastewater treatment plant and viral strains were identified through nested reverse transcription (RT)-PCR followed by cloning and sequencing analysis. Identified sapovirus strains were classified into seven genotypes belonging to three genogroups (GI, GII, and GV): GI.1, GI.2, GI.3, GII.1, GII.2, GII.8, and GV.1, with a clear temporal shift. The majority of AiV-1 strains identified from the wastewater samples were classified into genotype B, and genotype A strains were identified in only two samples.

Conclusions: We identified a number of sapovirus and AiV-1 strains belonging to multiple genotypes in wastewater samples collected over a 13-month period. Our results suggested a temporal shift in prevalent genotypes in the community.

Significance and Impact of Study: This is the first study elucidating the genotype distribution of human sapoviruses and AiV-1 in wastewater in the United States.

Wastewater surveillance is especially useful for understanding molecular epidemiology of viruses that are less commonly tested in clinical diagnosis, including sapoviruses and AiV-1.

Keywords: Sapovirus; Aichi virus 1; Gastroenteritis; Water; PCR; Sequencing; Molecular epidemiology; Genotyping

INTRODUCTION

Sapoviruses and Aichi virus 1 (AiV-1) are both non-enveloped viruses possessing a single-stranded, positive-sense RNA genomes belonging to the families *Caliciviridae* and *Picornaviridae*, respectively (Kitajima and Gerba, 2015; Oka *et al.*, 2015). The prototype strains of these viruses, Sapporo virus and Aichi virus, were discovered in gastroenteritis outbreaks in Japan and named after Sapporo city and Aichi prefecture, respectively, where the first outbreaks of each virus were recognized (Chiba *et al.*, 1979; Yamashita *et al.*, 1991). Sapoviruses and AiV-1 are generally less commonly reported as a causative agent of gastroenteritis than noroviruses and rotaviruses worldwide (Kitajima and Gerba, 2015; Oka *et al.*, 2015). Sapoviruses and AiV-1 were first identified in water in 2007 and 2010, respectively (Hansman *et al.*, 2007; Alcalá *et al.*, 2010). Thereafter, data on the occurrence of these viruses in aquatic environments have been accumulated rapidly within the past decade owing to development and/or improvement of PCR methods for the detection of these viruses in environmental samples (Kitajima and Gerba, 2015; Oka *et al.*, 2015). Recent environmental studies carried out in multiple geographical regions have collectively demonstrated a high prevalence of both sapoviruses and AiV-1 worldwide (Kitajima *et al.*, 2010a, 2011a, 2011b; Sano *et al.*, 2011; Fioretti *et al.*, 2016; Varela *et al.*, 2016; Haramoto and Kitajima, 2017), indicating that these viruses might be more prevalent in the environment than previously appreciated.

Examining municipal wastewater has been considered an effective approach to understand the prevalence of enteric viruses within a population and to obtain genetic information of the circulating strains, because wastewater contains viruses shed from all individuals within a service area regardless of symptoms (Sinclair *et al.*, 2008). In this

regard, wastewater surveillance is especially useful for viruses that are less commonly tested in clinical diagnosis, including sapoviruses and AiV-1. Elucidating their genotype distribution in wastewater is important to understand their molecular epidemiology in a given geographical region as the prevalent genotypes may differ depending on regions.

We previously investigated the seasonal occurrence and reduction of sapoviruses and AiV-1 as well as other viruses at wastewater treatment plants, which served as the first study showing quantitative data on the occurrence of sapoviruses and AiV-1 in an urban wastewater system in the United States (Kitajima *et al.*, 2014b). This previous study demonstrated a high prevalence of both sapoviruses and AiV-1 in wastewater, which prompted us to carry out further investigations to elucidate their molecular epidemiology by characterizing the virus strains. In the present study, we investigated the temporal variations in genotype distribution of sapoviruses and AiV-1 in wastewater in Arizona, the United States, over a 13-month period. The viral genomes in wastewater were detected with reverse transcription-quantitative PCR (RT-qPCR) and nested RT-PCR assays, and the sapovirus and AiV-1 strains were further characterized based on partial capsid gene and 3CD sequences, respectively.

MATERIALS AND METHODS

Collection of wastewater samples

Between July 2011 and July 2012, a total of 26 wastewater grab samples (13 influent and 13 effluent, 1 l each) were collected monthly from a wastewater treatment plant (WWTP) utilizing a conventional activated sludge process located in southern Arizona. This plant used chlorination for disinfection. The characteristics of this plant are described in our

previous report (Kitajima *et al.*, 2014a). All samples were collected in sterile plastic bottles, stored on ice, and transported to the laboratory, where they were processed within 12 h of collection.

Concentration of viruses in wastewater samples

The wastewater samples were concentrated using an electronegative filter method as described previously (Katayama *et al.*, 2002) with slight modification. Briefly, 2.5 mol l⁻¹ MgCl₂ was added to the wastewater samples to obtain a final concentration of 25 mmol l⁻¹. The samples (100 ml influent and 1000 ml effluent) were subsequently passed through the electronegative filter (cat. no. HAWP-090-00; Merck Millipore, Billerica, MA, USA) attached to a glass filter holder (Advantec, Tokyo, Japan). Magnesium ions were removed by passing 200 ml of 0.5 mmol l⁻¹ H₂SO₄ (pH 3.0) through the filter, and the viruses were eluted with 10 ml of 1.0 mmol l⁻¹ NaOH (pH 10.8). The eluate was recovered in a tube containing 50 µl of 100 mmol l⁻¹ H₂SO₄ (pH 1.0) and 100 µl of 100× Tris–EDTA buffer (pH 8.0) for neutralization, followed by further centrifugal concentration using a Centriprep YM-50 (Merck Millipore) to obtain a final volume of approximately 650 µl. The concentrates were stored at –80 °C until further analysis.

Viral RNA extraction and RT

Viral RNA was extracted from the concentrated wastewater sample spiked with murine norovirus (MNV, S7-PP3 strain; kindly provided by Prof. Y. Tohya, Nihon University, Kanagawa, Japan) as a process control using the ZR Viral DNA/RNA Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's protocol. The RT reaction was performed using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 10 µl of extracted RNA was added to 10 µl of RT

mixture containing 2 μ l of 10 $\times$ reverse transcription buffer, 0.8 μ l of 25 $\times$ deoxynucleoside triphosphates (dNTPs), 2 μ l of 10 $\times$ random hexamers, 50 units of MultiScribeTM reverse transcriptase, and 20 units of RNase inhibitor. The RT reaction mixture was incubated at 25°C for 10 min, followed by 37°C for 120 min, and finally 85°C for 5 min to inactivate the enzyme.

AiV-1 genotype-specific qPCR

AiV-1 genotype-specific qPCR was performed with a LightCycler® 480 Real-Time PCR Instrument II (Roche Diagnostics, Mannheim, Germany), according to the protocol described previously (Kitajima *et al.*, 2013). Reaction mixture (25 μ l) consisted of 12.5 μ l of LightCycler® 480 Probes Master (Roche Diagnostics), forward and reverse primers (AiV-AB-F and AiV-AB-R), probes (AiV-A-TP and AiV-B-TP), and 2.5 μ l of (c) DNA template. The reaction mixture was subjected to thermal cycling, and fluorescence readings were collected and analyzed with LightCycler® 480 Software version 1.5 (Roche Diagnostics). The genome copy numbers of each genotype were determined based on the standard curve prepared with 10-fold serial dilutions of standard plasmid DNA, at a concentration of 10⁷ to 10⁰ copies per reaction based on the plasmid DNA concentration determined by measuring the optical density at 260 nm. For each sample, the qPCR reaction was duplicated and an averaged genome copy number from the duplicate was reported. Negative controls were included to avoid false-positive results and no false-positive qPCR signal was observed.

Use of MNV as a process control, which was quantified with an MNV-specific RT-qPCR assay (Kitajima *et al.*, 2010b), showed no substantial inhibition in the extraction-RT-qPCR process in any of the wastewater samples tested in this study (mean

recovery efficiency of greater than 83 %). Specific recovery efficiency data for the influent and effluent samples were reported in our previous study (Kitajima *et al.*, 2014b). This set of wastewater samples was also examined previously with RT-qPCR assays targeting sapoviruses (Oka *et al.*, 2006) and AiV-1 genotypes A and B (AiV-universal assay) (Kitajima *et al.*, 2013), as reported the previous study (Kitajima *et al.*, 2014b). These previous qPCR results were compared with the results from the present study.

Nested PCR for sapoviruses and AiV-1

Nested PCR for sapoviruses was performed using SaV124F/1F/5F and SV-R13/R14 primer set and 1245Rfwd and SV-R2 primer set for first- and second-round PCR, respectively, to amplify the 430-bp partial capsid region (Kitajima *et al.*, 2010a). Nested PCR for AiV-1 was performed using 6261 and 6779 primer set and C94b and 246k primer set for first- and second-round PCR, respectively, to amplify the 266-bp 3CD junction region (Yamashita *et al.*, 2000). The PCR reactions were performed in 50 μ l of reaction volume containing DNA template (5 μ l of cDNA for the first PCR, and 2 μ l of the first PCR product for the second PCR), 2.5 units of Ex *Taq* Hot Start DNA polymerase (TaKaRa Bio Co., Shiga, Japan), and 20 pmol of each primer. Negative controls were included to avoid false-positive results due to cross-contamination, and no false-positive nested PCR product was observed. The same set of the wastewater samples was examined previously with an RT-qPCR assay targeting both genotypes A and B of AiV-1 (AiV-universal assay).

Cloning and sequencing

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 (Thermo Fisher Scientific). The plasmid constructs were then transformed into *Escherichia coli* TOP10 competent cells (Thermo Fisher Scientific) and the transformants were incubated at 37°C on a Luria broth agar plate containing 50 µg ml⁻¹ of ampicillin and 50 µg ml⁻¹ of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). At least eight colonies (clones) per sample were selected, insertion sizes were checked by direct colony PCR amplifications with an M13 forward (5'-GTAAAACGACGGCCAG-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primer set. Both strands of direct colony PCR products were sequenced using a BigDye Cycle Sequencing Kit version 3.1 and a 3730xl Genetic Analyzer (Thermo Fisher Scientific). Nucleotide sequences were assembled using the program SequencherTM version 5.0.1 (Gene Codes Corporation, Ann Arbor, MI, USA) and aligned with Clustal W version 2.1 (<http://clustalw.ddbj.nig.ac.jp/top-e.html>). The distances were calculated by Kimura's two-parameter method (Kimura, 1980), and the phylogenetic tree from a bootstrap analysis with 1,000 replicates was generated by the neighbor-joining method.

Nucleotide sequence accession numbers

The nucleotide sequences determined in the present study have been deposited in GenBank under accession numbers LC324793–LC324898.

RESULTS

Detection of sapoviruses and AiV-1

Table 1 summarizes the results of detection of sapovirus and AiV-1 genomes in 13 influent and 13 effluent wastewater samples. In our previous study (Kitajima *et al.*, 2014b), the wastewater samples were examined for the presence of sapovirus and AiV-1 genomes

using RT-qPCR assays (Oka *et al.*, 2006; Kitajima *et al.*, 2013); sapoviruses were detected in 12 (92%) influent and 7 (54%) effluent samples, and all samples tested positive with AiV-1 universal assay (Table 1). As an additional analysis of AiV-1 in the wastewater samples, an AiV-1 genotype-specific duplex RT-qPCR assay (Kitajima *et al.*, 2013) was performed in the present study. Five (38%) influent and 7 (54%) effluent samples were positive for both genotypes A and B, and one influent sample collected in June 2012 was negative for both genotype-specific assays; other samples tested positive for either genotype A or B (Table 1).

By use of a nested RT-PCR assay followed by cloning-sequencing, sapovirus sequences were obtained from eight (62%) influent and one (8%) effluent samples, all of which were positive by qPCR as well (Table 1). AiV-1 sequences were also obtained from seven (54%) influent and seven (54%) effluent samples (Table 1).

Sapovirus genotype distribution

We identified a total of 45 sapovirus sequences (1 to 10 different sequences per sample) based on nucleotide sequencing of a total of 100 selected clones (8 to 15 clones per sample) of the sapovirus nested PCR products. The sapovirus strains identified in the present study were classified into seven genotypes belonging to three genogroups (GI, GII, and GV): GI.1, GI.2, GI.3, GII.1, GII.2, GII.8, and GV.1 (Fig. 1). No GIV strain was identified. Strains identified from the same sample tended to form a tight genetic cluster within a genotype and coexistence of multiple genotypes was observed only in two influent samples (March and June 2012), demonstrating a limited genetic variation among the strains identified in each sample. By contrast, substantial genetic variations were observed between the strains identified from different samples, which indicates dynamic genotype/genogroup changes in

the restricted geographical area. In late 2011, only GII genotypes (GII.1 and GII.8) were identified; only GI genotypes (GI.1, GI.2, and GI.3) were found in early 2012; GI.2, GII.2 as well as GV.1 were detected in mid 2012 (Fig. 1).

AiV-1 genotype distribution

Based on nucleotide sequencing of a total of 146 selected clones (8 to 16 clones per sample) of the AiV-1 nested PCR products, we identified a total of 61 AiV-1 sequences (1 to 8 different sequences per sample). Genotype B strains were abundant in the wastewater samples, whereas genotype A strains were identified in only two samples (July 2011 influent and May 2012 effluent) (Fig. 2). All the genotype B strains were clustered together with the Chshc5 strain (FJ890516) isolated in China (Yang *et al.*, 2009). Four clones obtained from the effluent sample in May 2012 formed a unique genetic cluster (“untyped”) that are distinct from any of the existing AiV-1 genotypes, exhibiting highest nucleotide identity of 96.4% to 2011-RW01 (LC200524) identified from sewage in Nepal (Haramoto and Kitajima, 2017). The genotyping results were generally consistent with the results of multiplex genotype-specific qPCR assays (Table 1). For the samples that were confirmed for the presence of certain genotype by sequencing, genotype-specific qPCR assay of corresponding genotype showed higher concentrations than the other genotype (except for 2011 November sample).

DISCUSSION

The purpose of this study was to elucidate the molecular epidemiology of sapoviruses and AiV-1, especially their temporal variations in genotype distribution, in a given geographical region by characterizing the virus strains identified in wastewater. Sapoviruses

and AiV-1 are excreted in feces of infected individuals and have been identified in environmental samples, including wastewater and surface water (Kitajima and Gerba, 2015; Oka *et al.*, 2015). However, there have been only limited studies reporting genotype distribution of these viruses in water samples (Kitajima *et al.*, 2010a, 2011a, 2011b; Sano *et al.*, 2011; Haramoto and Kitajima, 2017). We conceived the present study because usefulness of virus monitoring in wastewater for understanding viral epidemiology has been demonstrated for other waterborne pathogenic viruses, such as noroviruses and hepatitis A virus (Hellmér *et al.*, 2014; Kazama *et al.*, 2017).

In our previous study, we investigated the occurrence of sapoviruses and AiV-1 in untreated and treated wastewater using RT-qPCR (Kitajima *et al.*, 2014b). The RT-qPCR assays for sapoviruses and AiV-1 used in this previous study react broadly with genogroups/genotypes infecting humans; specifically, the sapovirus assay targets GI, GII, GIV, and GV (Oka *et al.*, 2006), and the AiV-1 universal assay detects both genotypes A and B (Kitajima *et al.*, 2013). In the present study, we carried out genotype-specific duplex RT-qPCR assay (Kitajima *et al.*, 2013) as an additional analysis of AiV-1 genome quantification. The AiV-1 universal and genotype-specific RT-qPCR results exhibited good agreement, because 25 (96%) out of 26 AiV-1 universal assay-positive samples tested positive for genotype A and/or B by the genotype-specific assay. This serves as additional evidence on the applicability of this RT-qPCR system (Kitajima *et al.*, 2013) for quantification of genotyping of AiV-1 in environmental water samples.

We also performed nested RT-PCR to detect and amplify sapovirus and AiV-1 genomes (Yamashita *et al.*, 2000; Kitajima *et al.*, 2010a). The overall detection ratio by the nested PCR assays was lower than that of qPCR assay (Table 1). This is inconsistent with previous

studies using the same set of nested PCR and qPCR for the detection of sapoviruses and AiV-1 in wastewater samples, which reported similar positive rate between nested PCR and qPCR (Kitajima *et al.*, 2011a, 2013). The reason of this inconsistency remains unknown, but the present study provides additional data on the comparison of RT-qPCR and nested RT-PCR assays for the detection of human sapoviruses and AiV-1 in environmental water samples.

Characterization of the detected virus strains based on nucleotide sequencing analysis is important to understand the molecular epidemiology of human viruses in the population. Based on sequencing analysis, we identified genetically diverse sapovirus strains in wastewater, which were classified into a total of seven different genotypes in three genogroups (Figure 1). One of the most interesting observations of the present study is a dynamic change of the detected sapovirus genogroups. GII strains were detected in November and December 2011, only GI strains were detected in the subsequent five months (January to May 2012), and three genogroups (GI, GII, and GV) were detected in June 2012 (Fig. 1). A few previous clinical surveillance carried out in Japan also reported dynamic changes of sapovirus genogroups and genotypes identified from gastroenteritis patients in a restricted geographical region (Harada *et al.*, 2009, 2012, 2013). Additional epidemiological studies, from both clinical and environmental aspects, need to be conducted to confirm these observations on a dynamic change in prevailing sapovirus genogroup in a certain region.

AiV-1 has been identified in wastewater and reclaimed water through viral metagenomics in the United States (Rosario *et al.*, 2009; Cantalupo *et al.*, 2011; Ng *et al.*, 2012); however, these studies did not carry out genotyping analysis. In the present study,

we investigated genotype distribution of AiV-1 in wastewater using two approaches, namely, genotype-specific quantification of viral genomes with qPCR and phylogenetic analysis based on sequencing of cloned PCR products. Although these two approaches provided reasonably consistent results in terms of the identified genotypes in each sample, the results were not fully congruent. For instance, there were several cases where both genotypes were detected by qPCR but only genotype B was identified by cloning-sequencing from a given sample (Table 1). This could be due in part to the limited number of clones selected for sequencing (8 to 15 clones per sample), which is one of the technical limitations of the cloning-sequencing method. To obtain the genotyping data that represent the actual genotype distribution more accurately, the number of sequencing reads per sample should be increased by sequencing more numbers of clones, or more realistically, applying a next-generation sequencing technology for dramatically greater sequencing depth. Despite this technical limitation, our results contribute to enhance our understanding of the global distribution of AiV-1 genotypes. Recent studies have suggested that genotype B is more prevalent in South America, Europe, and Southeast Asia (Alcalá *et al.*, 2010; Di Martino *et al.*, 2013; Lodder *et al.*, 2013; Burutarán *et al.*, 2016; Haramoto and Kitajima, 2017), whereas genotype A has been reported to be more prevalent in East Asia and North Africa (Sdiri-Loulizi *et al.*, 2010; Kitajima *et al.*, 2011b). The present study demonstrated that genotype B was dominant in wastewater in southern Arizona, which serves as the first report on genotype distribution of AiV-1 in North America.

Human sapoviruses and AiV-1 have been increasingly recognized as causative agents of gastroenteritis and waterborne pathogenic viruses (Kitajima and Gerba, 2015; Oka *et al.*, 2015). However, knowledge of the temporal, geographical, and genotype distributions of

these viruses in the environment is limited compared with that of other major gastroenteritis viruses, such as noroviruses and rotaviruses (Lopman *et al.*, 2012; Kiulia *et al.*, 2015). We therefore designed the present study to elucidate the molecular epidemiology, especially temporal variations in genotype distribution, of human sapoviruses and AiV-1 in a given geographical region by examining wastewater. Genotype distribution of human sapoviruses and AiV-1 in wastewater in North America has not been reported. To our knowledge, this is the first study elucidating the genotype distribution of human sapoviruses and AiV-1 in wastewater throughout a year in the United States. The present study also suggests the usefulness of combination of RT-qPCR and nested RT-PCR assays for identifying and characterizing human sapoviruses and AiV-1 in environmental samples. Further environmental studies on the occurrence and genotype distribution of these viruses utilizing these molecular tools, even in combination with more high-throughput next-generation sequencing approach, are required worldwide for a better understanding of their molecular epidemiology, seasonal and geographical distributions, and potential health risks to humans.

ACKNOWLEDGMENTS

The authors would like to thank Kelly Reynolds at the University of Arizona Mel and Enid Zuckerman College of Public Health for her laboratory contributions and two anonymous wastewater treatment plants in southern Arizona for providing wastewater samples.

This study was partly supported by National Science Foundation (NSF) Water and Environmental Technology (WET) Center and Water Resources Research Center (WRRC), the University of Arizona.

We also wish to acknowledge the support of the Japan Society for the Promotion of Science (JSPS) to Masaaki Kitajima, under JSPS Postdoctoral Fellowships for Research Abroad. Andri T. Rachmadi was a recipient of 2012 Fulbright Master of Science and Technology Scholarship.

CONFLICT OF INTEREST

No conflict of interest declared.

REFERENCES

- Alcalá, A., Vizzi, E., Rodríguez-Díaz, J., Zambrano, J.L., Betancourt, W., and Liprandi, F. (2010) Molecular detection and characterization of Aichi viruses in sewage-polluted waters of Venezuela. *Appl. Environ. Microbiol.* **76**: 4113–4115.
- Burutarán, L., Lizasoain, A., García, M., Tort, L.F.L., Colina, R., and Victoria, M. (2016) Detection and molecular characterization of Aichivirus 1 in wastewater samples from Uruguay. *Food Environ. Virol.* **8**: 13–17.
- Cantalupo, P.G., Calgua, B., and Zhao, G. (2011) Raw sewage harbors diverse viral populations. *MBio* **2**: 1–11.
- Chiba, S., Sakuma, Y., Kogasaka, R., Akihara, M., Horino, K., Nakao, T., and Fukui, S. (1979) An outbreak of gastroenteritis associated with calicivirus in an infant home. *J. Med. Virol.* **4**: 249–254.
- Fioretti, J.M., Rocha, M.S., Fumian, T.M., Ginuino, A., da Silva, T.P., de Assis, M.R., et al. (2016) Occurrence of human sapoviruses in wastewater and stool samples in Rio De Janeiro, Brazil. *J. Appl. Microbiol.* **121**: 855–862.
- Hansman, G.S., Sano, D., Ueki, Y., Imai, T., Oka, T., Katayama, K., et al. (2007) Sapovirus in water, Japan. *Emerg. Infect. Dis.* **13**: 133–135.
- Harada, S., Oka, T., Tokuoka, E., Kiyota, N., Nishimura, K., Shimada, Y., et al. (2012) A confirmation of sapovirus re-infection gastroenteritis cases with different genogroups and genetic shifts in the evolving sapovirus genotypes, 2002-2011. *Arch. Virol.* **157**: 1999–2003.
- Harada, S., Okada, M., Yahiro, S., Nishimura, K., Matsuo, S., Miyasaka, J., et al. (2009) Surveillance of pathogens in outpatients with gastroenteritis and characterization of sapovirus strains between 2002 and 2007 in Kumamoto prefecture, Japan. *J. Med. Virol.* **81**: 1117–1127.
- Harada, S., Tokuoka, E., Kiyota, N., Katayama, K., and Oka, T. (2013) Phylogenetic analysis of the nonstructural and structural protein encoding region sequences, indicating successive appearance of genomically diverse sapovirus strains from gastroenteritis patients. *Jpn. J. Infect. Dis.* **66**: 454–457.
- Haramoto, E. and Kitajima, M. (2017) Quantification and genotyping of Aichi virus 1 in water samples in the Kathmandu Valley, Nepal. *Food Environ. Virol.* 1–4.
- Hellmér, M., Paxéus, N., Magnius, L., Enache, L., Arnholm, B., Johansson, A., et al. (2014) Detection of pathogenic viruses in sewage provided early warnings of hepatitis A virus and norovirus outbreaks. *Appl. Environ. Microbiol.* **80**: 6771–6781.

365 Katayama, H., Shimasaki, A., and Ohgaki, S. (2002) Development of a virus concentration
 366 method and its application to detection of enterovirus and Norwalk virus from coastal
 367 seawater. *Appl. Environ. Microbiol.* **68**: 1033–1039.

368 Kazama, S., Miura, T., Masago, Y., Konta, Y., Tohma, K., Manaka, T., et al. (2017)
 369 Environmental surveillance of norovirus genogroups I and II for sensitive detection of
 370 epidemic variants. *Appl. Environ. Microbiol.* **83**: e03406-16.

371 Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions
 372 through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**: 111–120.

373 Kitajima, M. and Gerba, C. (2015) Aichi virus 1: Environmental occurrence and behavior.
 374 *Pathogens* **4**: 256–268.

375 Kitajima, M., Haramoto, E., Iker, B.C., and Gerba, C.P. (2014b) Occurrence of
 376 *Cryptosporidium*, *Giardia*, and *Cyclospora* in influent and effluent water at wastewater
 377 treatment plants in Arizona. *Sci. Total Environ.* **484**: 129–136.

378 Kitajima, M., Haramoto, E., Phanuwat, C., and Katayama, H. (2011a) Genotype distribution
 379 of human sapoviruses in wastewater in Japan. *Appl. Environ. Microbiol.* **77**: 4226–4229.

380 Kitajima, M., Haramoto, E., Phanuwat, C., and Katayama, H. (2011b) Prevalence and genetic
 381 diversity of Aichi viruses in wastewater and river water in Japan. *Appl. Environ.*
 382 *Microbiol.* **77**: 2184–2187.

383 Kitajima, M., Hata, A., Yamashita, T., Haramoto, E., Minagawa, H., and Katayama, H.
 384 (2013) Development of a reverse transcription-quantitative PCR system for detection
 385 and genotyping of Aichi viruses in clinical and environmental samples. *Appl. Environ.*
 386 *Microbiol.* **79**: 3952–3958.

387 Kitajima, M., Iker, B.C., Pepper, I.L., and Gerba, C.P. (2014a) Relative abundance and
 388 treatment reduction of viruses during wastewater treatment processes — Identification of
 389 potential viral indicators. *Sci. Total Environ.* **488–489**: 290–296.

390 Kitajima, M., Oka, T., Haramoto, E., Katayama, H., Takeda, N., Katayama, K., and Ohgaki, S.
 391 (2010a) Detection and genetic analysis of human sapoviruses in river water in Japan.
 392 *Appl. Environ. Microbiol.* **76**: 2461–2467.

393 Kitajima, M., Oka, T., Takagi, H., Tohya, Y., Katayama, H., Takeda, N., and Katayama, K.
 394 (2010b) Development and application of a broadly reactive real-time reverse
 395 transcription-PCR assay for detection of murine noroviruses. *J. Virol. Methods* **169**:
 396 269–273.

397 Kiulia, N., Hofstra, N., Vermeulen, L., Obara, M., Medema, G., and Rose, J. (2015) Global
 398 occurrence and emission of rotaviruses to surface waters. *Pathogens* **4**: 229–255.

399 Lodder, W.J., Rutjes, S.A., Takumi, K., and de Roda Husman, A.M. (2013) Aichi virus in
 400 sewage and surface water, the Netherlands. *Emerg. Infect. Dis.* **19**: 1222–1230.

- Lopman, B., Gastañaduy, P., Park, G.W., Hall, A.J., Parashar, U.D., and Vinjé, J. (2012) Environmental transmission of norovirus gastroenteritis. *Curr. Opin. Virol.* **2**: 96–102.
- Di Martino, B., Di Profio, F., Ceci, C., Di Felice, E., and Marsilio, F. (2013) Molecular detection of Aichi virus in raw sewage in Italy. *Arch. Virol.* **158**: 2001–2005.
- Ng, T.F.F., Marine, R., Wang, C., Simmonds, P., Kapusinszky, B., Bodhidatta, L., et al. (2012) High variety of known and new RNA and DNA viruses of diverse origins in untreated sewage. *J. Virol.* **86**: 12161–12175.
- Oka, T., Katayama, K., Hansman, G.S., Kageyama, T., Ogawa, S., Wu, F.-T., et al. (2006) Detection of human sapovirus by real-time reverse transcription-polymerase chain reaction. *J. Med. Virol.* **78**: 1347–1353.
- Oka, T., Wang, Q., Katayama, K., and Saif, L.J. (2015) Comprehensive review of human sapoviruses. *Clin. Microbiol. Rev.* **28**: 32–53.
- Rosario, K., Nilsson, C., Lim, Y.W., Ruan, Y., and Breitbart, M. (2009) Metagenomic analysis of viruses in reclaimed water. *Environ. Microbiol.* **11**: 2806–2820.
- Sano, D., Pérez-Sautu, U., Guix, S., Pintó, R.M., Miura, T., Okabe, S., and Bosch, A. (2011) Quantification and genotyping of human sapoviruses in the Llobregat river catchment, Spain. *Appl. Environ. Microbiol.* **77**: 1111–1114.
- Sdiri-Loulizi, K., Hassine, M., Aouni, Z., Gharbi-Khelifi, H., Sakly, N., Chouchane, S., et al. (2010) First molecular detection of Aichi virus in sewage and shellfish samples in the Monastir region of Tunisia. *Arch. Virol.* **155**: 1509–1513.
- Sinclair, R.G., Choi, C.Y., Riley, M.R., and Gerba, C.P. (2008) Pathogen surveillance through monitoring of sewer systems. *Adv. Appl. Microbiol.* **65**: 249–269.
- Varela, M.F., Polo, D., and Romalde, J.L. (2016) Prevalence and genetic diversity of human sapoviruses in shellfish from commercial production areas in Galicia, Spain. *Appl. Environ. Microbiol.* **82**: 1167–1172.
- Yamashita, T., Kobayashi, S., Sakac, K., Nakata, S., Chiba, S., and Ishihara, Y. (1991) Isolation of cytopathic small round viruses with BS-C-1 cells from patients with gastroenteritis. *J. Infect. Dis.* **7**: 954–957.
- Yamashita, T., Sugiyama, M., Tsuzuki, H., Sakae, K., Suzuki, Y., and Miyazaki, Y. (2000) Application of a reverse transcription-PCR for identification and differentiation of Aichi virus, a new member of the Picornavirus family associated with gastroenteritis in humans. *J. Clin. Microbiol.* **38**: 2955–2961.
- Yang, S., Zhang, W., Shen, Q., Yang, Z., Zhu, J., Cui, L., and Hua, X. (2009) Aichi virus strains in children with gastroenteritis, China. *Emerg. Infect. Dis.* **15**: 1703–1705.

TABLE

Table 1. Detection of sapoviruses and Aichi virus 1 in wastewater in Arizona, USA.

Year/Month	Sapovirus				AiV-1							
	Influent		Effluent		Influent				Effluent			
	qPCR ^a	Genotype(s)	qPCR ^a	Genotype(s)	qPCR ^b (copies/L)			Genotype(s)	qPCR ^b (copies/L)			Genotype(s)
	(copies/L)		(copies/L)		AB ^a	A	B		AB ^a	A	B	
2011/July	4.6×10 ⁴	— ^c	—	—	7.88×10 ⁴	3.65×10 ⁵	—	A	3.55×10 ³	3.32×10 ³	—	—
Aug	5.8×10 ⁴	—	—	—	1.35×10 ⁵	4.02×10 ⁴	1.98×10 ⁵	B	9.95×10 ³	4.79×10 ³	2.04×10 ⁴	B
Sept	—	—	—	—	9.63×10 ⁴	3.61×10 ⁴	—	—	2.12×10 ⁴	4.18×10 ³	1.65×10 ⁴	B
Oct	9.2×10 ⁴	—	—	—	1.42×10 ⁵	4.21×10 ⁴	1.04×10 ⁵	B	8.96×10 ³	—	2.19×10 ⁴	B
Nov	1.5×10 ⁴	GII.8	—	—	3.68×10 ⁵	3.28×10 ⁴	—	B	2.18×10 ⁴	—	2.62×10 ⁴	B
Dec	9.4×10 ⁴	GII.1	2.83×10 ⁴	—	6.36×10 ⁴	—	4.12×10 ⁴	B	5.66×10 ³	3.94×10 ³	7.49×10 ³	B
2012/Jan	2.8×10 ⁵	GI.3	—	—	1.68×10 ⁴	—	1.41×10 ⁴	—	2.47×10 ³	—	3.90×10 ³	—
Feb	1.9×10 ⁵	GI.3	5.62×10 ²	—	4.70×10 ⁴	3.01×10 ⁴	3.48×10 ⁴	—	2.16×10 ⁴	8.86×10 ³	2.32×10 ⁴	B
Mar	3.2×10 ⁶	GI.1, GI.3	3.54×10 ³	—	2.34×10 ⁵	2.11×10 ⁴	2.16×10 ⁵	B	1.77×10 ⁴	—	2.38×10 ⁴	—
Apr	6.6×10 ⁵	GI.2	1.06×10 ⁵	—	1.44×10 ⁵	1.10×10 ⁵	1.51×10 ⁵	—	8.39×10 ³	6.96×10 ³	8.41×10 ³	—
May	1.0×10 ⁶	GI.1	5.91×10 ⁴	—	7.13×10 ³	2.09×10 ⁴	—	—	3.91×10 ³	4.80×10 ³	4.56×10 ³	A, B, untyped
June	1.8×10 ⁵	GI.2, GV.1	6.92×10 ³	GII.2	6.40×10 ⁴	—	—	—	2.18×10 ⁴	7.79×10 ³	9.69×10 ³	—
July	1.4×10 ⁴	—	7.10×10 ³	—	1.49×10 ⁵	—	2.95×10 ⁴	B	2.54×10 ⁴	—	3.92×10 ³	—
% positive	92%	62%	54%	8%	100%	69%	62%	54%	100%	62%	92%	54%
	(12/13)	(8/13)	(7/13)	(1/13)	(13/13)	(9/13)	(8/13)	(7/13)	(13/13)	(8/13)	(12/13)	(7/13)

^a qPCR data reported previously (Kitajima, Iker, *et al.*, 2014).^b AB, AiV-1 universal assay; A, genotype A-specific assay; B, genotype B-specific assay (Kitajima *et al.*, 2013).^c —, negative.

FIGURE LEGEND

Figure 1. Phylogenetic tree for sapovirus strains based on about 400 nucleotides (nt) of the partial capsid gene sequences. The tree was generated by the neighbor-joining method with representative strains derived from wastewater and reference strains. The scale represents nucleotide substitutions per site. Strains shown in ***italic bold*** are representative sapovirus strains identified in the present study, representing the year and month of sample collection, sample type (influent or effluent), and GenBank accession number. Genotype numbers were assigned according to a recently updated sapovirus strain classification (Oka *et al.*, 2015).

Figure 2. Phylogenetic tree for AiV-1 strains based on 224 nucleotides (nt) of the 3CD junction region sequences. The tree was generated by the neighbor-joining method with representative strains derived from wastewater and reference strains. The scale represents nucleotide substitutions per site. Strains shown in ***italic bold*** are representative AiV-1 strains identified in the present study, representing the year and month of sample collection, sample type (influent or effluent), and GenBank accession number.

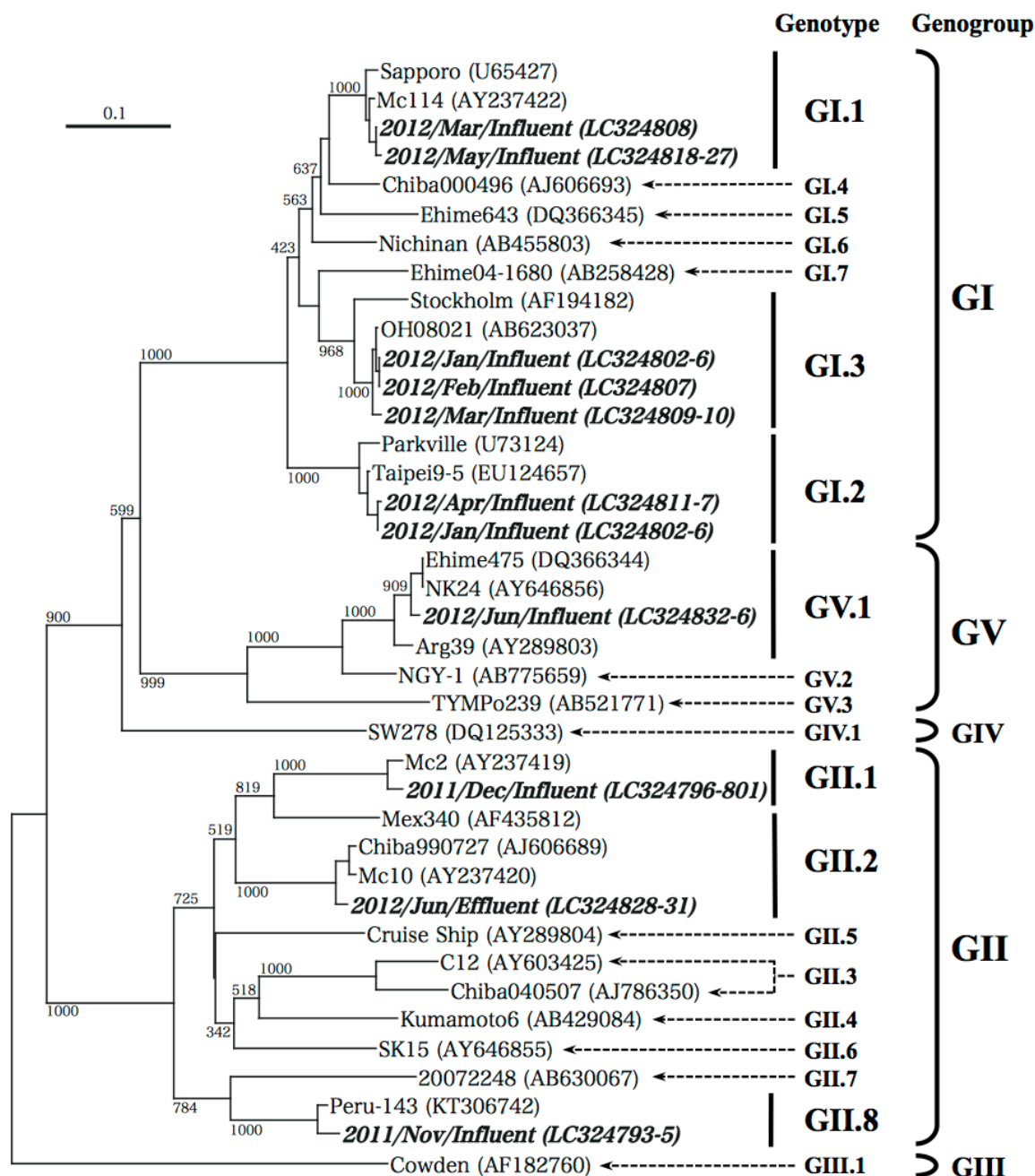


Figure 1

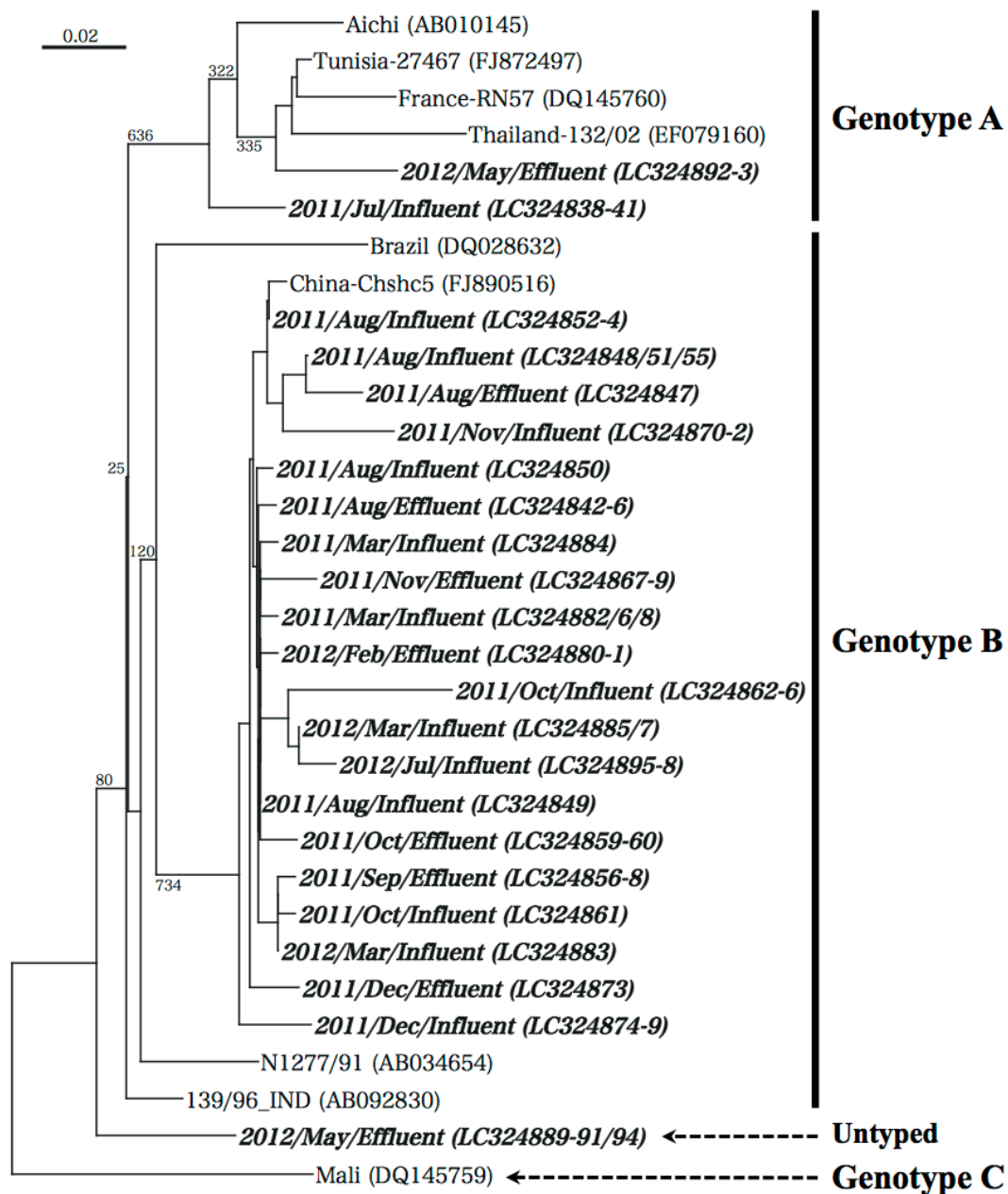


Figure 2