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

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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} $MgCl_2$ 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} H_2SO_4 (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} H_2SO_4 (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 μl of 10 × reverse transcription buffer, 0.8 μl of 25 × deoxynucleoside triphosphates (dNTPs), 2 μl of 10× random hexamers, 50 units of MultiScribe™ 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 μl) consisted of 12.5 μl 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 μl 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 10^7 to 10^0 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

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

143 **Nested PCR for sapoviruses and AiV-1**

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

156 **Cloning and sequencing**

157 The second PCR products were separated by electrophoresis on a 2 % agarose gel and
158 visualized under a UV lamp after ethidium bromide staining. All of the second PCR
159 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 3730*xl* Genetic Analyzer (Thermo Fisher Scientific). 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 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

183 using RT-qPCR assays (Oka *et al.*, 2006; Kitajima *et al.*, 2013); sapoviruses were detected
184 in 12 (92%) influent and 7 (54%) effluent samples, and all samples tested positive with
185 AiV-1 universal assay (Table 1). As an additional analysis of AiV-1 in the wastewater
186 samples, an AiV-1 genotype-specific duplex RT-qPCR assay (Kitajima et al., 2013) was
187 performed in the present study. Five (38%) influent and 7 (54%) effluent samples were
188 positive for both genotypes A and B, and one influent sample collected in June 2012 was
189 negative for both genotype-specific assays; other samples tested positive for either genotype
190 A or B (Table 1).
191 By use of a nested RT-PCR assay followed by cloning-sequencing, sapovirus sequences
192 were obtained from eight (62%) influent and one (8%) effluent samples, all of which were
193 positive by qPCR as well (Table 1). AiV-1 sequences were also obtained from seven (54%)
194 influent and seven (54%) effluent samples (Table 1).
195 **Sapovirus genotype distribution**
196 We identified a total of 45 sapovirus sequences (1 to 10 different sequences per sample)
197 based on nucleotide sequencing of a total of 100 selected clones (8 to 15 clones per sample)
198 of the sapovirus nested PCR products. The sapovirus strains identified in the present study
199 were classified into seven genotypes belonging to three genogroups (GI, GII, and GV): GI.1,
200 GI.2, GI.3, GII.1, GII.2, GII.8, and GV.1 (Fig. 1). No GIV strain was identified. Strains
201 identified from the same sample tended to form a tight genetic cluster within a genotype and
202 coexistence of multiple genotypes was observed only in two influent samples (March and
203 June 2012), demonstrating a limited genetic variation among the strains identified in each
204 sample. By contrast, substantial genetic variations were observed between the strains
205 identified from different samples, which indicates dynamic genotype/genogroup changes in

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

209 **AiV-1 genotype distribution**

210 Based on nucleotide sequencing of a total of 146 selected clones (8 to 16 clones per
211 sample) of the AiV-1 nested PCR products, we identified a total of 61 AiV-1 sequences (1
212 to 8 different sequences per sample). Genotype B strains were abundant in the wastewater
213 samples, whereas genotype A strains were identified in only two samples (July 2011
214 influent and May 2012 effluent) (Fig. 2). All the genotype B strains were clustered together
215 with the Chshc5 strain (FJ890516) isolated in China (Yang *et al.*, 2009). Four clones
216 obtained from the effluent sample in May 2012 formed a unique genetic cluster ("untyped")
217 that are distinct from any of the existing AiV-1 genotypes, exhibiting highest nucleotide
218 identity of 96.4% to 2011-RW01 (LC200524) identified from sewage in Nepal (Haramoto
219 and Kitajima, 2017). The genotyping results were generally consistent with the results of
220 multiplex genotype-specific qPCR assays (Table 1). For the samples that were confirmed
221 for the presence of certain genotype by sequencing, genotype-specific qPCR assay of
222 corresponding genotype showed higher concentrations than the other genotype (except for
223 2011 November sample).

224

225 **DISCUSSION**

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

229 and AiV-1 are excreted in feces of infected individuals and have been identified in
230 environmental samples, including wastewater and surface water (Kitajima and Gerba, 2015;
231 Oka *et al.*, 2015). However, there have been only limited studies reporting genotype
232 distribution of these viruses in water samples (Kitajima *et al.*, 2010a, 2011a, 2011b; Sano *et*
233 *al.*, 2011; Haramoto and Kitajima, 2017). We conceived the present study because
234 usefulness of virus monitoring in wastewater for understanding viral epidemiology has been
235 demonstrated for other waterborne pathogenic viruses, such as noroviruses and hepatitis A
236 virus (Hellmér *et al.*, 2014; Kazama *et al.*, 2017).
237 In our previous study, we investigated the occurrence of sapoviruses and AiV-1 in
238 untreated and treated wastewater using RT-qPCR (Kitajima *et al.*, 2014b). The RT-qPCR
239 assays for sapoviruses and AiV-1 used in this previous study react broadly with
240 genogroups/genotypes infecting humans; specifically, the sapovirus assay targets GI, GII,
241 GIV, and GV (Oka *et al.*, 2006), and the AiV-1 universal assay detects both genotypes A
242 and B (Kitajima *et al.*, 2013). In the present study, we carried out genotype-specific duplex
243 RT-qPCR assay (Kitajima *et al.*, 2013) as an additional analysis of AiV-1 genome
244 quantification. The AiV-1 universal and genotype-specific RT-qPCR results exhibited good
245 agreement, because 25 (96%) out of 26 AiV-1 universal assay-positive samples tested
246 positive for genotype A and/or B by the genotype-specific assay. This serves as additional
247 evidence on the applicability of this RT-qPCR system (Kitajima *et al.*, 2013) for
248 quantification of genotyping of AiV-1 in environmental water samples.
249 We also performed nested RT-PCR to detect and amplify sapovirus and AiV-1 genomes
250 (Yamashita *et al.*, 2000; Kitajima *et al.*, 2010a). The overall detection ratio by the nested
251 PCR assays was lower than that of qPCR assay (Table 1). This is inconsistent with previous

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

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

272 AiV-1 has been identified in wastewater and reclaimed water through viral
273 metagenomics in the United States (Rosario *et al.*, 2009; Cantalupo *et al.*, 2011; Ng *et al.*,
274 2012); however, these studies did not carry out genotyping analysis. In the present study,

275 we investigated genotype distribution of AiV-1 in wastewater using two approaches,
276 namely, genotype-specific quantification of viral genomes with qPCR and phylogenetic
277 analysis based on sequencing of cloned PCR products. Although these two approaches
278 provided reasonably consistent results in terms of the identified genotypes in each sample,
279 the results were not fully congruent. For instance, there were several cases where both
280 genotypes were detected by qPCR but only genotype B was identified by
281 cloning-sequencing from a given sample (Table 1). This could be due in part to the limited
282 number of clones selected for sequencing (8 to 15 clones per sample), which is one of the
283 technical limitations of the cloning-sequencing method. To obtain the genotyping data that
284 represent the actual genotype distribution more accurately, the number of sequencing reads
285 per sample should be increased by sequencing more numbers of clones, or more realistically,
286 applying a next-generation sequencing technology for dramatically greater sequencing
287 depth. Despite this technical limitation, our results contribute to enhance our understanding
288 of the global distribution of AiV-1 genotypes. Recent studies have suggested that genotype
289 B is more prevalent in South America, Europe, and Southeast Asia (Alcalá *et al.*, 2010; Di
290 Martino *et al.*, 2013; Lodder *et al.*, 2013; Burutarán *et al.*, 2016; Haramoto and Kitajima,
291 2017), whereas genotype A has been reported to be more prevalent in East Asia and North
292 Africa (Sdiri-Loulizi *et al.*, 2010; Kitajima *et al.*, 2011b). The present study demonstrated
293 that genotype B was dominant in wastewater in southern Arizona, which serves as the first
294 report on genotype distribution of AiV-1 in North America.
295 Human sapoviruses and AiV-1 have been increasingly recognized as causative agents of
296 gastroenteritis and waterborne pathogenic viruses (Kitajima and Gerba, 2015; Oka *et al.*,
297 2015). However, knowledge of the temporal, geographical, and genotype distributions of

298 these viruses in the environment is limited compared with that of other major gastroenteritis
299 viruses, such as noroviruses and rotaviruses (Lopman *et al.*, 2012; Kiulia *et al.*, 2015). We
300 therefore designed the present study to elucidate the molecular epidemiology, especially
301 temporal variations in genotype distribution, of human sapoviruses and AiV-1 in a given
302 geographical region by examining wastewater. Genotype distribution of human sapoviruses
303 and AiV-1 in wastewater in North America has not been reported. To our knowledge, this is
304 the first study elucidating the genotype distribution of human sapoviruses and AiV-1 in
305 wastewater throughout a year in the United States. The present study also suggests the
306 usefulness of combination of RT-qPCR and nested RT-PCR assays for identifying and
307 characterizing human sapoviruses and AiV-1 in environmental samples. Further
308 environmental studies on the occurrence and genotype distribution of these viruses utilizing
309 these molecular tools, even in combination with more high-throughput next-generation
310 sequencing approach, are required worldwide for a better understanding of their molecular
311 epidemiology, seasonal and geographical distributions, and potential health risks to humans.
312

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CONFLICT OF INTEREST

No conflict of interest declared.

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435

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] (copies/L)	Genotype(s)	qPCR[a] (copies/L)	Genotype(s)	qPCR[b] (copies/L)			Genotype(s)	qPCR[b] (copies/L)			Genotype(s)
					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% (12/13)	62% (8/13)	54% (7/13)	8% (1/13)	100% (13/13)	69% (9/13)	62% (8/13)	54% (7/13)	100% (13/13)	62% (8/13)	92% (12/13)	54% (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.

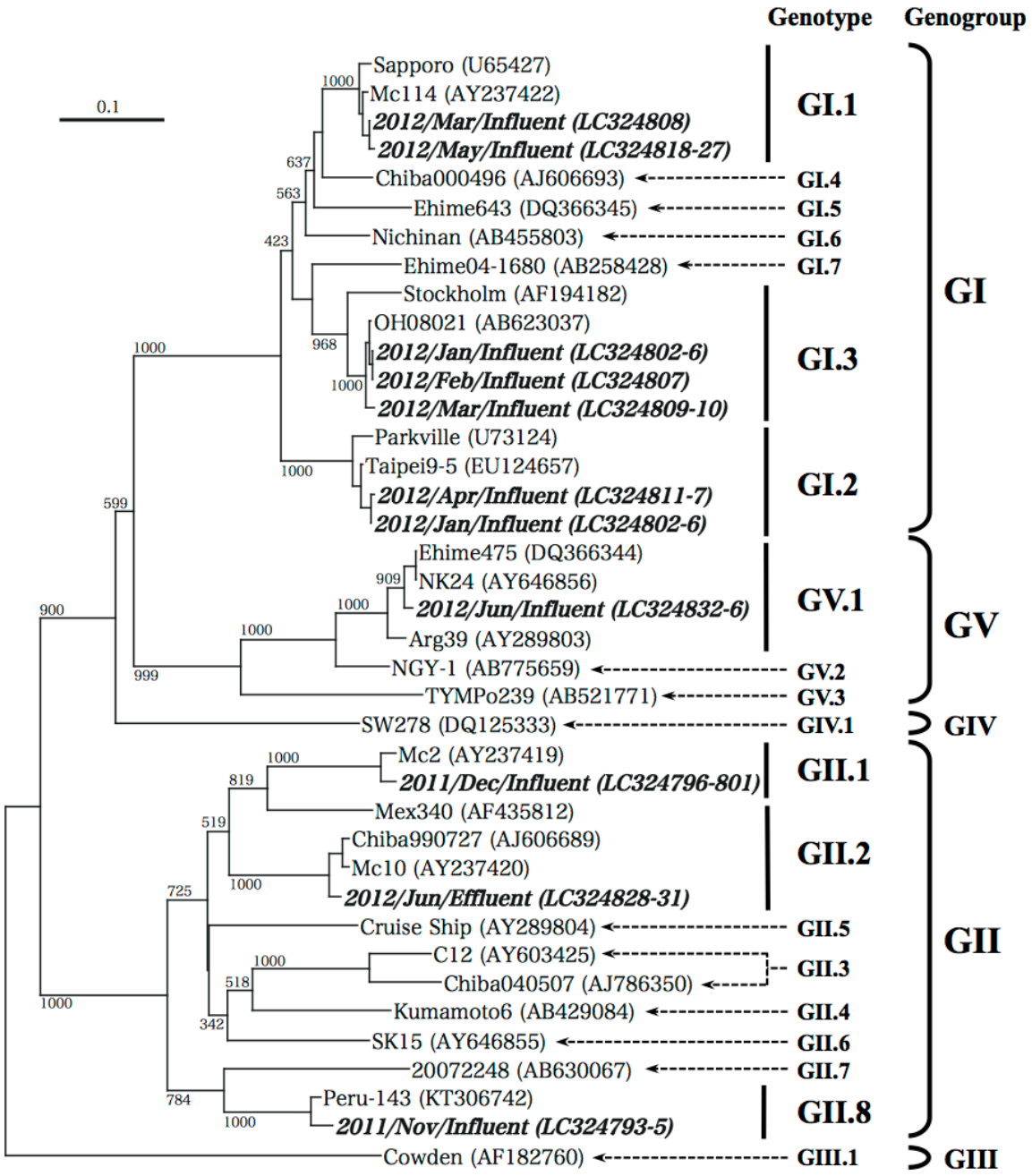
Genotype
Genogroup
0.1
Sapporo (U65427)
Mc114 (AY237422)
2012/Mar/Influent (LC324808)
2012/May/Influent (LC324818-27)
GI.1
Chiba000496 (AJ606693)
GI.4
Ehime643 (DQ366345)
GI.5
Nichinan (AB455803)
GI.6
Ehime04-1680 (AB258428)
GI.7
Stockholm (AF194182)
OH08021 (AB623037)
2012/Jan/Influent (LC324802-6)
2012/Feb/Influent (LC324807)
2012/Mar/Influent (LC324809-10)
GI.3
Parkville (U73124)
Taipei9-5 (EU124657)
2012/Apr/Influent (LC324811-7)
2012/Jan/Influent (LC324802-6)
GI.2
GI
Ehime475 (DQ366344)
NK24 (AY646856)
2012/Jun/Influent (LC324832-6)
Arg39 (AY289803)
GV.1
NGY-1 (AB775659)
GV.2
TYMPo239 (AB521771)
GV.3
GV
SW278 (DQ125333)
GIV.1
GIV
Mc2 (AY237419)
2011/Dec/Influent (LC324796-801)
GII.1
Mex340 (AF435812)
Chiba990727 (AJ606689)
Mc10 (AY237420)
2012/Jun/Effluent (LC324828-31)
GII.2
Cruise Ship (AY289804)
GII.5
C12 (AY603425)
Chiba040507 (AJ786350)
GII.3
Kumamoto6 (AB429084)
GII.4
SK15 (AY646855)
GII.6
20072248 (AB630067)
GII.7
Peru-143 (KT306742)
2011/Nov/Influent (LC324793-5)
GII.8
GII
Cowden (AF182760)
GIII.1
GIII

Figure 1

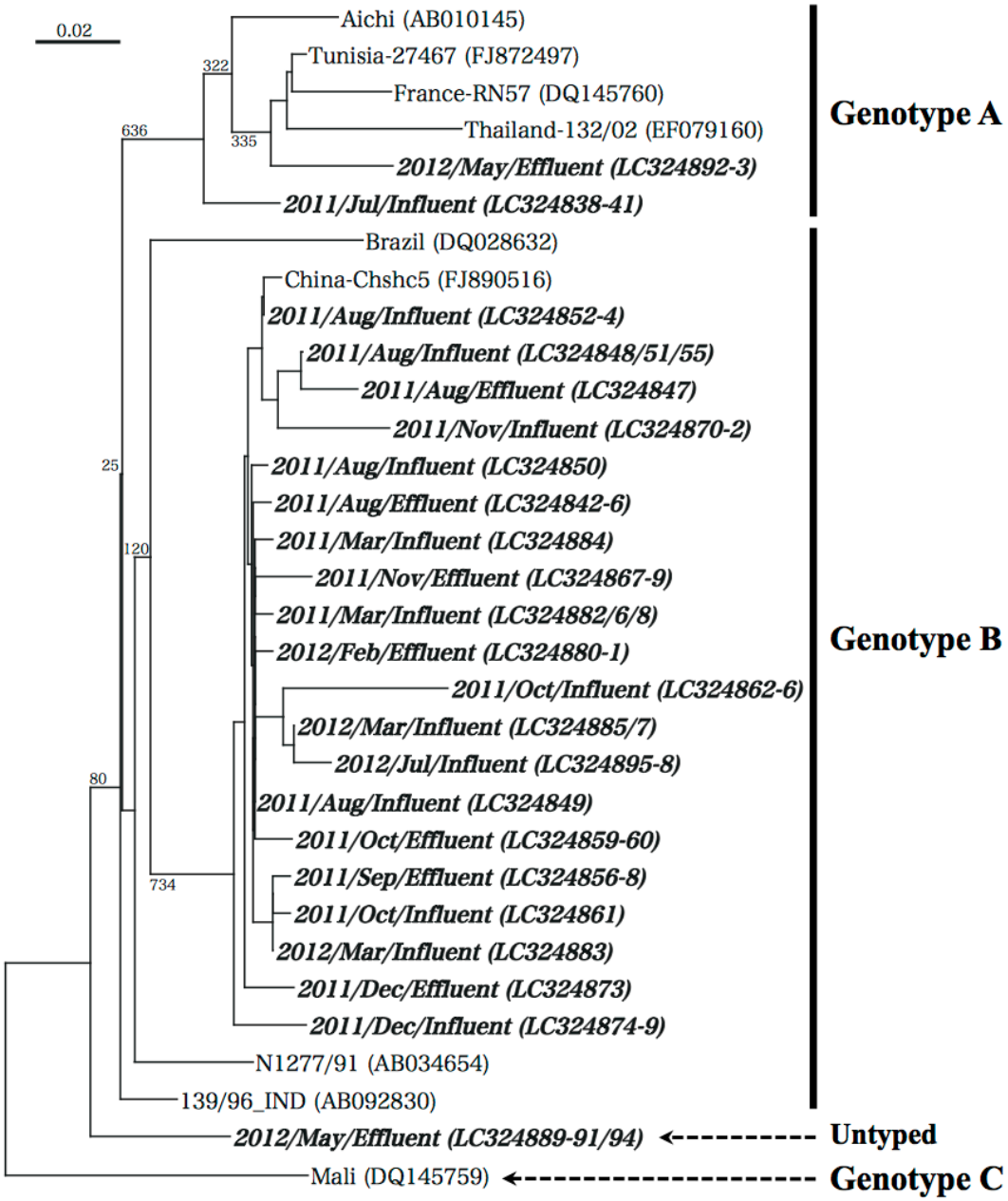
0.02
Aichi (AB010145)
Tunisia-27467 (FJ872497)
France-RN57 (DQ145760)
Thailand-132/02 (EF079160)
2012/May/Effluent (LC324892-3)
2011/Jul/Influent (LC324838-41)
Genotype A
Brazil (DQ028632)
China-Chshc5 (FJ890516)
2011/Aug/Influent (LC324852-4)
2011/Aug/Influent (LC324848/51/55)
2011/Aug/Effluent (LC324847)
2011/Nov/Influent (LC324870-2)
2011/Aug/Influent (LC324850)
2011/Aug/Effluent (LC324842-6)
2011/Mar/Influent (LC324884)
2011/Nov/Effluent (LC324867-9)
2011/Mar/Influent (LC324882/6/8)
2012/Feb/Effluent (LC324880-1)
2011/Oct/Influent (LC324862-6)
2012/Mar/Influent (LC324885/7)
2012/Jul/Influent (LC324895-8)
2011/Aug/Influent (LC324849)
2011/Oct/Effluent (LC324859-60)
2011/Sep/Effluent (LC324856-8)
2011/Oct/Influent (LC324861)
2012/Mar/Influent (LC324883)
2011/Dec/Effluent (LC324873)
2011/Dec/Influent (LC324874-9)
N1277/91 (AB034654)
139/96_IND (AB092830)
Genotype B
2012/May/Effluent (LC324889-91/94)
Untyped
Mali (DQ145759)
Genotype C
322
636
335
25
120
80
734

Figure 2