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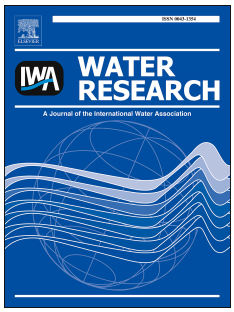

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PII: S0043-1354(15)30289-X

DOI: 10.1016/j.watres.2015.10.024

Reference: WR 11585

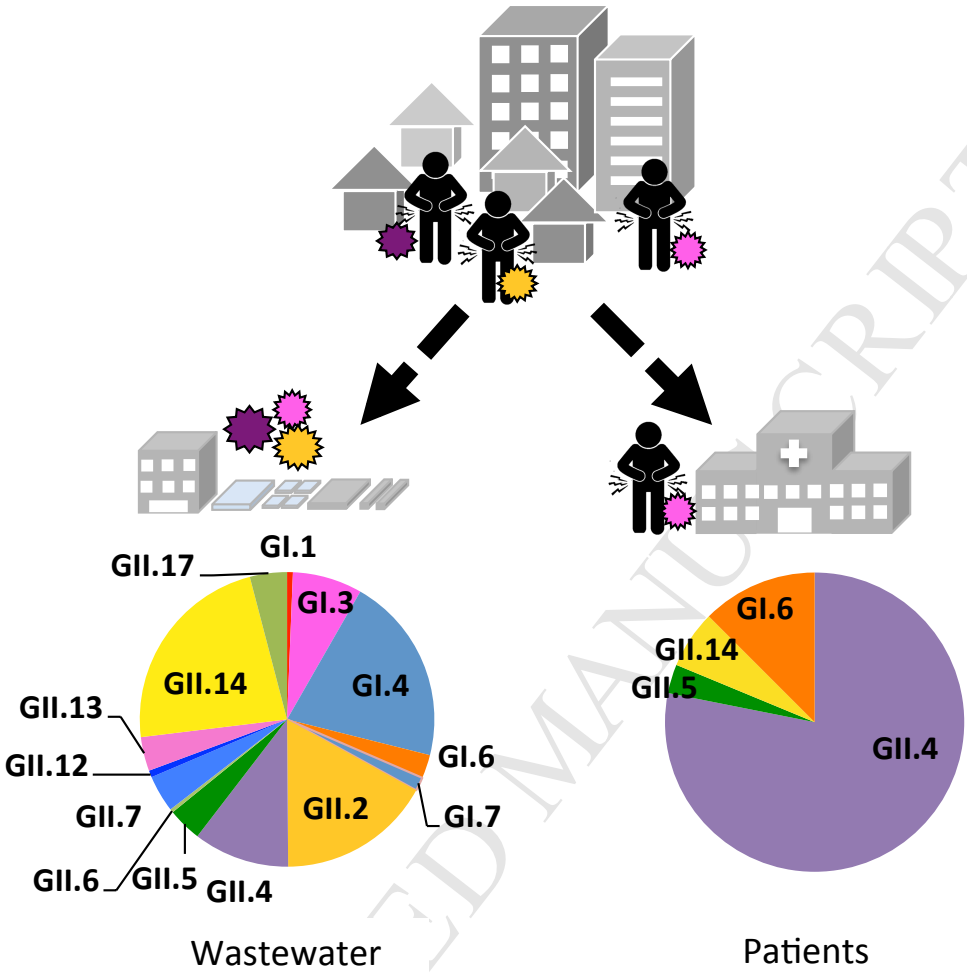
To appear in: *Water Research*

Received Date: 30 January 2015

Revised Date: 14 October 2015

Accepted Date: 17 October 2015

Please cite this article as: Kazama, S., Masago, Y., Tohma, K., Souma, N., Imagawa, T., Suzuki, A., Liu, X., Saito, M., Oshitani, H., Omura, T., Temporal dynamics of norovirus determined through monitoring of municipal wastewater by pyrosequencing and virological surveillance of gastroenteritis cases, *Water Research* (2015), doi: 10.1016/j.watres.2015.10.024.



Temporal dynamics of norovirus determined through monitoring of municipal wastewater by pyrosequencing and virological surveillance of gastroenteritis cases

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ABSTRACT

Norovirus is a leading etiological agent of viral gastroenteritis. Because of relatively mild disease symptoms and frequent asymptomatic infections, information on the ecology of this virus is limited. Our objective was to examine the genetic diversity of norovirus circulating in the human population by means of genotyping the virus in municipal wastewater. We investigated norovirus genogroups I and II (GI and GII) in municipal wastewater in Japan by pyrosequencing and quantitative PCR (qPCR) from November 2012 to March 2013. Virological surveillance for gastroenteritis cases was concurrently conducted in the same area. A total of fourteen distinct genotypes in total (GI.1, 3, 4, 6, 7, GII.2, 4, 5, 6, 7, 12, 13, 14, and 17), with up to eight genotypes detected per sample, were observed in wastewater using pyrosequencing; only four genotypes (GI.6, GII.4, 5, and 14) were obtained from clinical samples. Seventy-eight percent of norovirus-positive stool samples contained GII.4, but this genotype was not dominant in wastewater. The norovirus GII.4 Sydney 2012 variant, which appeared and spread during our study period, was detected in both the wastewater and clinical samples. These results suggest that an environmental approach using pyrosequencing yields a more detailed distribution of norovirus genotypes/variants. Thus, wastewater monitoring by pyrosequencing is expected to provide an effective analysis of the distribution of norovirus genotypes causing symptomatic and asymptomatic infections in human populations.

Keywords

Norovirus; Wastewater; Pyrosequencing; Virological surveillance

1. INTRODUCTION

Noroviruses (family *Caliciviridae*, genus *Norovirus*) are one of the leading causative agents for acute gastroenteritis around the world. Noroviruses, which cause diarrhea and vomiting, affect individuals of all ages and are transmitted primarily through fecal-oral, aerosol-vomitus, or direct-contact routes (Glass et al., 2009). Although the infection can be fatal in vulnerable populations such as infants and the elderly, asymptomatic infections are also common (Glass et al., 2009). Up to 200,000 deaths occur annually among children under the age of five in developing countries (Patel et al., 2008). Outbreaks most frequently occur during cold weather (Ahmed et al., 2013; Lopman et al., 2009; Mounts et al., 2000), and this seasonality is also observed in Japan (Mounts et al., 2000; Siebenga et al., 2009; Yoneda et al., 2014). Noroviruses are classified into five genogroups (GI-GV), which are further subdivided into at least 35 genotypes (Centers for Disease Control and Prevention, 2011; Zheng et al., 2006). Among these, human disease is caused by GI, GII, and GIV (Patel et al., 2009; Zheng et al., 2006). In recent years, most outbreaks have been caused by GII genotype 4 (GII.4) (Glass et al., 2009; Patel et al., 2009; Siebenga et al., 2009). In 2012, a new GII.4 variant called Sydney 2012 was detected in Australia and rapidly spread to many countries (Chan et al., 2014; Eden et al., 2013; Fonager et al., 2013; Giammanco et al., 2013; Leshem et al., 2013b; Mai et al., 2013; Rahman et al., 2013; Vega et al., 2014).

Virological surveillance is commonly conducted for gastroenteritis cases to detect the occurrence of norovirus. However, in this approach, noroviruses can only be identified in patients who seek medical care; asymptomatic infections are not detected. Moreover, enteric viral identification at medical facilities is not mandatory, even in developed countries. Thus, the number of norovirus infections and the genotypes circulating in the

human populations and water environment are not well understood.

Because human noroviruses (GI, GII and GIV) are host-specific and cannot replicate in other organisms, the presence of the virus in municipal wastewater indicates the presence of infected individuals in the area. Based on this supposition, many studies have been conducted on norovirus occurrence and genotypes in wastewater and other environmental water (Aw and Gin, 2010; Blanco Fernandez et al., 2011; Hernandez-Morga et al., 2009; Katayama et al., 2008; Kitajima et al., 2012; 2010; La Rosa et al., 2010; Lee et al., 2012; Lee et al., 2011; Mans et al., 2013; Pérez-Sautu et al., 2012). Only a few studies have investigated norovirus occurrence and genotypes in both human populations and the water environment at the same time (Kremer et al., 2011; Rajko-Nenow et al., 2013; Ueki et al., 2005). However, because these studies only sequenced a few clones from each sample by direct-sequencing or cloning-sequencing, researchers may have just detected the predominant genotypes. Considering the wide molecular diversity and rapid evolution of the norovirus genome (Bok et al., 2009; Bull et al., 2007; Eden et al., 2014; Zheng et al., 2006), it would be highly advantageous to develop a novel approach capable of detecting multiple genotypes is desired.

Next-generation sequencing (NGS), a high-throughput sequencing technique, has recently been applied to study viral diversity, discover novel viruses, and monitor viral evolution in humans and the environment (Barzon et al., 2011; Cantalupo et al., 2011). This technique allows a large number of nucleotide sequences to be obtained from a single sample. Therefore, we expected that NGS could be used to determine the detailed distribution of norovirus genotypes in wastewater.

The present study aimed to investigate the genomic dynamics of norovirus in an urbanized part of Japan. Specifically, we performed virological monitoring of municipal

93 wastewater using pyrosequencing, an NGS technology. Noroviruses GI and GII in
94 wastewater samples were genotyped using pyrosequencing and also quantified using
95 quantitative PCR (qPCR). In parallel, we conducted virological surveillance for
96 gastroenteritis cases in the catchment area to investigate the number of patients admitted
97 to the hospital with norovirus infection and to determine norovirus genotypes in patient
98 stools. This study was conducted from 2012 to 2013, during an interval when the
99 norovirus GII.4 Sydney 2012 variant was spreading in the study area, permitting us to
100 simultaneously detect the shift in predominant variants in both wastewater and patients.
101 To the best of our knowledge, this is the first report concerning molecular genotyping of
102 noroviruses in wastewater using pyrosequencing.
103
104 **2. MATERIALS AND METHODS**
105 **2.1. Study design**
106 This study was conducted from November 2012 to March 2013 in the town of
107 Matsushima in northeastern Japan's Miyagi Prefecture. As of March 2013, the town had
108 15,141 inhabitants and one municipal wastewater treatment plant, which received
109 wastewater from 67.1% of the population. There are four internal medicine clinics in the
110 town, one of which participated in our virological surveillance for gastroenteritis cases.
111
112 **2.2. Analysis of noroviruses in wastewater samples**
113 2.2.1. Sample collection
114 Primary effluents were collected at the wastewater treatment plant every week from
115 November 14, 2012 (the 46th week of 2012), to March 13, 2013 (the 11th week of 2013),
116 except for the 1st week of 2013; thus a total of 17 weekly samples were collected and

117 analyzed. The grab samples (250 mL each) were collected around 10 a.m., transported
118 to the laboratory on ice, and stored in a deep freezer (−80 °C) until analyzed.
119
120 2.2.2. Virus concentration and nucleic acid extraction
121 To process each weekly specimen, noroviruses were recovered from 40 mL of the
122 primary effluent sample and concentrated using the polyethylene glycol (PEG)
123 precipitation method. Prior to the recovery and concentration process, a known amount
124 (approximately 10^8 genome copies) of the murine norovirus strain S7-PP3 (MNV),
125 provided by Prof. Yukinobu Tohya (Nihon University, Japan), was spiked into each
126 sample as a processing control. Each sample was gently mixed with 3.2 g of PEG 6000
127 (Wako Pure Chemical Industries, Osaka, Japan) and 0.92 g of NaCl (Kanto Kagaku,
128 Tokyo, Japan) using a magnetic stirrer for 12 h at 4 °C, then centrifuged at 9,000×*g* for
129 30 min at 4 °C. Each pellet was resuspended in 1 mL of sterilized, deionized water and
130 mixed using a vortex mixer for 1 min. The resuspended pellets were centrifuged at
131 10,000×*g* for 10 min at 4 °C and the supernatants were collected as virus concentrates.
132 Ribonucleic acid was extracted using QIAamp Viral RNA mini Kit (Qiagen, Hilden,
133 Germany) with QIAcube (Qiagen), and cDNA was synthesized using iScript Advanced
134 cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) with a Veriti 96-well Thermal
135 Cycler (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's
136 instructions.
137
138 2.2.3. Norovirus quantification using qPCR assay
139 Norovirus GI and GII were detected by qPCR using the CFX96 Real-Time PCR
140 Detection System (Bio-Rad). The qPCR was performed using SsoFast Probes Supermix

(Bio-Rad) and the following primers and probes: COG1F, COG1R, RING1(a)-TP, and RING1(b)-TP for GI; and COG2F, COG2R, and RING2AL-TP (5'-FAMTGG GAG GGS GAT CGC RAT CT-TAMRA-3') for GII (Aoki et al., 2010; Ministry of Health , Labour and Welfare, 2007; Kageyama et al., 2003). Each 20-μL reaction mixture contained 5 μL of cDNA, 10 μL of SsoFast Probes Supermix (Bio-Rad), and the primers and probes specified in the references. The PCR cycling conditions were 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C, 60 s at 56 °C, and 30 s at 72 °C.

The number of norovirus genome copies was determined using a standard curve generated from a 10-fold serial dilution of standard DNA (10^8 – 10^1 copies/well). Synthesized oligonucleotide of the target region (Nihon Gene Research Laboratories, Sendai, Japan) was used as the standard DNA. A limit of quantification cycle value (LOQ) was set at 40 to follow the MIQE guidelines (Bustin et al., 2009). Each sample was measured in triplicate. The geometric mean copy number in the sample was converted to concentration in the wastewater sample when all replicated quantification cycle (Cq) values were less than 40. The concentrations of norovirus genome in the wastewater samples were calculated without recovery rates. When one or more replicated Cq value(s) was more than 40, the corresponding concentration was recorded as below LOQ. If all replicates were negative, the corresponding concentration was recorded as "not detected". In order to evaluate overall recovery rates, the MNV spiked into each sample as a processing control was also quantified using qPCR (Hata et al., 2011; Kitajima et al., 2008). The overall recovery rate was calculated by dividing the copy number of the MNV detected in the virus concentrate by the copy number of the MNV spiked into the sample. The recovery rates ranged from 2% to 19% (geometric mean: 8%, n = 17).

165

166 2.2.4. Amplification for pyrosequencing

167 Semi-nested PCR was performed using primers as follows. The primers for the first and
168 second PCR were COG1F/G1SKR and G1SKF/G1SKR for GI, and COG2F/G2SKR
169 and G2SKF/G2SKR for GII (Kageyama et al., 2003; Kojima et al., 2002). For the first
170 PCR, the 50-μL reaction mixture contained 15 μL of cDNA, 25 μL of Q5 Hot Start
171 High-Fidelity 2× Master Mix (New England Biolabs, Ipswich, MA, USA), and 25 pmol
172 of both forward and reverse primers. For the second PCR, the 100-μL reaction mixture
173 contained 2 μL of the first PCR product, 50 μL of Q5 Hot Start High-Fidelity 2× Master
174 Mix (New England Biolabs), and 50 pmol of both forward and reverse primers.
175 Amplification by PCR for both the first and second PCR was performed using a Veriti
176 96-well Thermal Cycler (Thermo Fisher Scientific), with reactions initiated by
177 incubation for 30 s at 98 °C, followed by 25 cycles of 10 s at 98 °C, 30 s at 50 °C, and
178 30 s at 72 °C, with a final extension for 30 s at 72 °C. The semi-nested PCR product
179 sizes for GI and GII were approximately 330 bp and 340 bp, respectively.
180 The semi-nested PCR products were visualized using agarose gel electrophoresis, and
181 samples with a band at the expected position were purified and submitted for
182 pyrosequencing. Purification consisted of concentrating 90 μL of the nested-PCR
183 product to 30 μL using the QIAquick PCR Purification Kit (Qiagen) with QIAcube
184 (Qiagen).

185

186 2.2.5. Pyrosequencing

187 In order to perform pyrosequencing on the norovirus amplicons using the GS Junior
188 system (Roche Applied Science, Penzberg, Germany), unique adaptors were ligated to

189 both the 5′ and 3′ ends of the amplicons by fusion PCR. The fusion primers consisted of
190 FLX Titanium Primer A (25-mer sequence used for the sequencing), Multiplex
191 Identifier (10-mer sequence for barcoding each sample), G1SKF or G2SKF primer
192 sequences as forward primers, and FLX Titanium Primer B (25-mer sequence used for
193 the sequencing) and G1SKR or G2SKR primer sequences as reverse primers. The
194 100-μL reaction mixture contained 10 μL of the purified nested-PCR products, 50 μL of
195 Q5 Hot Start High-Fidelity 2× Master Mix (New England Biolabs), and 50 pmol of both
196 forward and reverse primers. The PCR reactions were performed using a Veriti 96-well
197 Thermal Cycler (Thermo Fisher Scientific), and started with 30 s at 98 °C, followed by
198 5 cycles of 10 s at 98 °C, 30 s at 50 °C, and 30 s at 72 °C, with a final extension for 30 s
199 at 72 °C.
200 Ninety microliters of the fusion PCR products were purified and concentrated to 30 μL
201 using QIAquick PCR Purification Kit (Qiagen) with QIAcube (Qiagen). DNA
202 concentrations of the nested PCR products were measured using the Quant-iT
203 PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) with infinite M1000 PRO
204 (TECAN, Männedorf, Switzerland). Three to five samples with identical Multiplex
205 Identifier sequences were mixed for the individual pyrosequencing runs using the GS
206 Junior system (Roche Applied Science) with the Titanium emPCR Kit (Lib-L) and the
207 GS Junior Titanium Sequencing Kit (Roche Applied Science) following the
208 manufacturer's instructions.
209
210 2.2.6. Bioinformatic analysis
211 Although NGS provides large numbers of sequence reads, the sequence data contains
212 non-target sequences such as ambiguous reads with noise and chimeric sequences.

These artifacts are produced during nested-PCR and pyrosequencing, and could result in overestimation of genetic diversity (Quince et al., 2011). Therefore, the bioinformatic analysis was performed as follows using QIIME 1.8.0 software (Caporaso et al., 2010). Quality filtering and primer sequence removal were performed using the software package split_library.py with a minimum quality score parameter of 25. Sequences with incorrect nucleotides produced in the nested-PCR and the pyrosequencing steps were corrected using the denoiser.py package. Chimeric sequences (sequence formed by two or more sequences) were removed using the Perseus software (Quince et al., 2011) after removing reverse primers using the split_library.py package. Sequences were then clustered into operational taxonomic units (OTUs) based on a minimum 97% similarity in nucleotide sequence using the pick_otus.py package, and a representative sequence of each OTU was selected using the pick_rep_set.py package.

Genotypes and variants of the representative sequences were identified using the Norovirus Genotyping Tool Version 1.0 (Kroneman et al., 2011). When not assigned to any genotypes by the tool, sequences were subjected to homology search using BLASTn, and the genotype or variant of top-hit sequences with the highest similarity (exceeding a minimum of 97%) was assigned. If a given sequence could not be assigned using either the Norovirus Genotyping Tool or BLASTN, that sequence was categorized as “not assigned”.

The diversity of the norovirus strains (OTUs) in each wastewater sample was evaluated using a rarefaction curve generated by the Analytic Rarefaction 2.0 software (http://strata.uga.edu/software/).

2.3. Analysis of noroviruses in clinical samples

2.3.1. Sample collection and norovirus screening

Virological surveillance for gastroenteritis cases was conducted at an outpatient Internal Medicine and Pediatrics clinic in Matsushima. Patients with diarrhea who visited the clinic from the 43rd week in 2012 to the 13th week in 2013 (96 patients) were included in this study. Rectal swabs were collected from the patients after obtaining informed consent. The samples were stored at 2-8 °C and transferred to the authors' laboratory weekly.

The samples were tested for norovirus using a real-time PCR assay. Each rectal swab was moistened in 1 mL of phosphate-buffered saline, and ribonucleic acid extraction was performed using the QIAamp Viral RNA Mini Kit (Qiagen) with QIAcube (Qiagen). cDNA was synthesized using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) following the manufacturer's instructions. Noroviruses were detected by real-time PCR (Kageyama et al., 2003) using the TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) and the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific) following the manufacturer's instructions. The PCR cycling conditions were 2 min at 50 °C and 20 s at 95 °C, followed by 45 cycles of 3 s at 95 °C and 35 s at 60 °C. In addition to norovirus, the levels of sapovirus, astrovirus and rotavirus were also tested (Heim et al., 2003; Oka et al., 2006; Pang et al., 2004) for reference.

2.3.2. Analysis of genotypes and variants

The norovirus-positive stools were assessed using Sanger sequencing. The capsid N/S-encoding domain of the norovirus genome in the norovirus-positive stool samples was amplified by single-round PCR or nested PCR using *Ex Taq* (Takara Bio, Otsu,

Japan) and the primers p290, COG1F, COG2F, G1SKR, and G2SKR, to determine the GI and GII strain genotypes (Jiang et al., 1999; Kageyama et al., 2003; Kojima et al., 2002). The PCR products were purified using QIAquick PCR Purification Kit (Qiagen) with QIAcube (Qiagen), and then subjected to sequencing reactions using the BigDye Terminator v1.1 Sequencing Kit (Thermo Fisher Scientific) following the manufacturer's instructions. The reaction products were purified using BigDye TXTerminator Purification Kit (Thermo Fisher Scientific) followed by sequencing using the Applied Biosystems 3130 Genetic Analyzer or 3730xl DNA Analyzer (Thermo Fisher Scientific) according to the manufacturer's instructions. Multiple alignment and data cleaning were performed using MEGA5 software (Tamura et al., 2011). The genotyping was performed using the Norovirus Typing Tool Version 1.0 (Kroneman et al., 2011).

2.4. Phylogenetic analysis

In order to compare GII.4 sequences obtained from wastewater samples to those obtained from clinical samples, phylogenetic analysis was carried out. The sequences were analyzed using ClustalW; bootstrapped phylogenetic trees were then constructed by the maximum likelihood method with 1,000 bootstrap replications using MEGA5 software (Tamura et al., 2011). The genetic distances were calculated using the Kimura 2-parameter method.

2.5. Nucleotide sequence accession numbers

Nucleotide sequence data from wastewater samples and clinical samples has been deposited in the DDBJ/EMBL/GenBank databases under the accession numbers

285 DRA002609 and LC060872-LC060903, respectively.

286

287

288 **3. RESULTS**

289 **3.1. Noroviruses in wastewater samples by qPCR**

290 The concentrations of noroviruses GI and GII in wastewater samples, as determined by
291 qPCR, are shown in Fig. 1. Norovirus GI was detected in all samples throughout the
292 study period, with the concentrations ranging up to 8.7×10^4 copies/mL. The
293 concentrations were relatively high between December 26, 2012 and January 16, 2013
294 and between February 27, 2013 and March 13, 2013. GII was first detected on
295 December 12, and the concentrations were relatively high (up to 1.3×10^3 copies/mL)
296 from January 9, 2013, to February 20, 2013, except on January 23, 2013.

297

298 **3.2. Noroviruses in wastewater samples by pyrosequencing**

299 In our study, 17 samples were analyzed by pyrosequencing for GI and GII, and a total of
300 999,097 reads (with means of 17,183 reads for GI and 41,587 reads for GII per sample)
301 were obtained. Pyrosequencing produces a high numbers of reads, but these reads
302 typically contain artifacts such as sequences with incorrect nucleotides and chimeric
303 sequences generated during nested-PCR and pyrosequencing. These artifacts must be
304 addressed through correction (denoising) or removal (chimera removal). On average,
305 chimera removal eliminated 0.7% of GI reads and 3.7% of GII reads. Following
306 denoising and chimera removal, the mean numbers of OTUs per sample was 5 for GI
307 and 32 for GII. On the basis of the number of OTUs, GII exhibited a higher diversity
308 than GI in wastewater samples. All rarefaction curves except one reached or almost

309 reached a plateau. The rarefaction curve generated from GI strains in the sample
310 collected on November 14, 2012 (the lowest concentration of GI in wastewater samples)
311 did not reach a plateau. This result indicates that the sequencing was nearly deep
312 enough to understand the diversity of norovirus.
313 The representative sequences of the OTUs were genotyped using the Norovirus Typing
314 Tool and BLASTn search. Figure 2 shows ratios of the number of reads assigned to each
315 genotype/variant divided by the number of reads identified as norovirus in each sample.
316 Because sequences were obtained through nested-PCR and pyrosequencing, genotype
317 distributions (number of reads for each genotype) obtained by pyrosequencing were not
318 expected to be identical with the actual values in the wastewater samples. Therefore,
319 ratios exceeding 10% were denoted as high ratio. Fourteen genotypes in total and up to
320 eight genotypes per wastewater sample (collected on January 9, 2013) were detected.
321 No norovirus sequence was obtained on several dates (November 21, 28, and December
322 12, 2012, for GI; November 21 and 28, 2012, for GII), although noroviruses were
323 detected by qPCR and nested-PCR products were observed at target length in samples
324 from these dates. The numbers of sequences obtained from the samples was very low
325 (less than 100 reads), and these sequences were removed during the quality filtering and
326 chimera removal steps following pyrosequencing. Thus the nested-PCR products were
327 inferred as non-specific products.
328 Norovirus GI was identified in 14 samples by pyrosequencing, including five genotypes
329 (GI.1, 3, 4, 6, and 7). GI.4 was the most frequently detected genotype in 53% (9/17) of
330 the samples and occurred at high ratios (>10%) in eight samples. GI.7 was detected in
331 47% (8/17) of the samples but occurred at high ratios only on December 28, 2012, and
332 March 13, 2013. GI.3 was intermittently detected in 29% (5/17) of the samples in 2013

333 at high ratios, whereas GI.1 was detected in 12% (2/17) of the samples but only
334 observed in 2012. GI.6 was detected in 12% (2/17) of the samples on December 28,
335 2012 and January 9, 2013.
336 Nine GII genotypes (GII.2, 4, 5, 6, 7, 12, 13, 14, and 17) were detected in 14
337 wastewater samples. Single genotypes (GII.2 or GII.14) per sample were detected at the
338 beginning of the monitoring period (November 14, 2012 and December 5, 2012,
339 respectively); multiple genotypes per sample were detected starting from December 12,
340 2012. GII.2 and GII.4 were the most frequently detected genotypes (11/17 samples,
341 65% for both genotypes), followed by GII.14 (10/17 samples, 59%). All of these
342 genotypes appeared at high ratios, except for GII.4 on January 9, 2013. Other genotypes
343 were less frequently detected, namely GII.5 (4/17, 24%), GII.7 (4/17, 24%), GII.17
344 (4/17, 24%), GII.6 (2/17, 12%), GII.12 (1/17, 6%), and GII.13 (1/17, 6%); these
345 genotypes appeared only in 2013.
346 Three GII.4 variants were detected, namely Den Haag 2006b (3/17, 18%), New Orleans
347 2009 (1/17, 5.9%), and Sydney 2012 (10/17, 59%). All three variants were detected
348 until the third week of 2013, but only the Sydney 2012 variant was detected thereafter,
349 implying that Sydney 2012 became the predominant variant from the fourth week of
350 2013.
351

352 **3.3. Norovirus detection and clinical sample characteristics**

353 Figure 3 shows the number of outpatients identified with gastroenteritis and norovirus
354 infection. In total, 96 stool samples were collected from patients with gastroenteritis
355 from the 43rd week in 2012 to the 13th week in 2013. Among these, 32 samples (33%)
356 contained norovirus, including 4 cases of GI (13%) and 28 cases of GII (87%). Other

357 viruses were detected in 21 of 63 norovirus-negative samples, including sapovirus (12
358 samples), astrovirus (7 samples), and rotavirus (2 samples). Although the number of
359 samples was low to show an epidemic curve, the number of norovirus-positive stool
360 samples peaked between the 48th and 50th week of 2012, with the highest number of
361 cases detected during the 50th week (5 cases).
362 Norovirus GI.6 was the only GI genotype detected from patients (n = 4), while three GII
363 genotypes [GII.4 (n = 25), GII.5 (n = 1), and GII.14 (n = 2)] were detected. Norovirus
364 GII.4, the predominant genotype (25 samples, 78%), included three variants: Sydney
365 2012 (18 samples, 72%), Den Haag 2006b (6 samples, 24%), and New Orleans 2009 (1
366 sample, 4%) (Fig. 4). At the beginning of the study period, the Sydney 2012 and Den
367 Haag 2006b variants appeared to be co-circulating; only a single case of New Orleans
368 2009 was detected in this interval. After the 52nd week of 2012, only the Sydney 2012
369 variant was detected (Fig. 3). Although the number of clinical samples was low, our
370 results suggest a shift occurred towards predominance by the GII.4 variant.
371

372 **3.4. Comparison between wastewater samples and clinical samples**

373 Fourteen genotypes were detected in wastewater samples, whereas only four genotypes
374 were detected in stool samples (Figs. 2-5). All genotypes detected from stool samples
375 (GI.6, GII.4, GII.5, and GII.14) also were detected in wastewater. Noroviruses GII.4 and
376 GII.14 were detected in 65% and 59% of the wastewater samples, respectively.
377 Genotype GII.5, which was detected in one stool sample collected during the 8th week
378 of 2013, also was detected in wastewater during an overlapping interval (the 7th, 9th, and
379 10th weeks of 2013). In contrast, GII.2 was detected at high ratios throughout the study
380 period in 65% of wastewater samples but never in stool samples. Furthermore, GI.6 was

exclusively detected in four GI-positive stool samples, whereas GI.6 was detected in only two wastewater samples (12%). The wastewater contained various genotypes that were not observed in the virological surveillance of gastroenteritis cases.

The prevalence of GII.4 variants showed similar temporal trends between wastewater (Fig. 2) and clinical samples (Fig. 3) in our study period. Three variants (Den Haag 2006b, New Orleans 2009, and Sydney 2012) were detected; the Sydney 2012 variant was the most frequently detected in both types of samples. All three variants were detected at the beginning of the study period, both in wastewater and stool samples, but only Sydney 2012 was detected later in the study period. However, there were some notable differences in the timing of this shift. The Sydney 2012 variant was exclusively detected in stool samples during and after the 52nd week of 2012, whereas other variants were detected from wastewater until the 3rd week of 2013.

Fig. 5 shows a phylogenetic tree derived using GII.4 sequences obtained from the wastewater and stool samples. Given that the wastewater samples yielded large numbers of OTUs, representative sequences of OTUs containing more than 1000 reads were used for the phylogenetic analysis. All the sequences identified as the same variant clustered on distinct branches. These results indicated that genotypes/variants detected in wastewater using pyrosequencing indeed reflected gastroenteritis cases in human populations.

4. DISCUSSION

In this study, we used pyrosequencing and qPCR to assess the genomic diversity of noroviruses using virological monitoring of both wastewater and clinical samples, including analysis of genotypes and concentration in wastewater. Wastewater

405 monitoring allowed us to identify in detail the diversity of norovirus genotypes. This
406 incuded a total of 14 different genotypes, as well as on samples, which by itself
407 contained eight genotypes (January 9, 2013). Some previous attempts have been made
408 to show the relationship between noroviruses in feces and their presence in the water
409 environment (Kremer et al., 2011; Rajko-Nenow et al., 2013). However, because those
410 previous studies used direct-sequencing or cloning-sequencing methods to genotype
411 noroviruses from environmental samples, the respective laboratories detected only one
412 or two genotypes in a given sample. Thus, detection may have been limited to
413 predominant genotypes only. Although it is possible to detect multiple genotypes using
414 cloning-sequencing, this requires large amounts of cloning and considerable cost and
415 effort.
416 We additionally note that the use of NGS, as employed in the present work, could also
417 be used to evaluate wastewater treatment processing and water quality. A recent study
418 reported that distinct norovirus genotypes may exhibit different tolerances in wastewater
419 treatment processing and different accumulation rates in oysters (Rajko-Nenow et al.,
420 2013). Detection of multiple genotypes using NGS would enhance investigations such
421 as genotype-dependent tolerance in wastewater treatment processes and oyster
422 environments.
423 Norovirus GII.4 was detected most frequently in stool samples (78%), but this variant
424 did not dominate in wastewater samples. One possible explanation for this seemingly
425 contradictory finding is that GII.4 is more likely to cause symptomatic infection as
426 compared with other genotypes (Barreira et al., 2010; Bucardo et al., 2010; Okabayashi
427 et al., 2008). Because the clinical samples were obtained from symptomatic patients
428 who sought medical care, GII.4 is expected to be detected more frequently. In contrast,

429 all genotypes causing both symptomatic and asymptomatic infection are deposited in
430 wastewater. Similarly, previous studies reported that the GI concentration in wastewater
431 was comparable to that of GII in wastewater (Katayama et al., 2008; Kitajima et al.,
432 2012; Nordgren et al., 2009), but GII genotypes were most frequently detected in stool
433 samples (Chan et al., 2006; Fukuda et al., 2009; Ozawa et al., 007). Consistent with
434 these results, other studies have reported that several genotypes that were not detected in
435 patient stool samples were observed in wastewater or polluted river water (Aw et al.,
436 2009; Rajko-Nenow et al., 2013).
437 Due to the low number of samples, the epidemic trend of GII.4 variants may not have
438 been well represented by our study. Nonetheless, we noted that three GII.4 variants
439 (Den Haag 2006b, New Orleans 2009, and Sydney 2012) were detected early in our
440 study period, and the Sydney 2012 variant was detected in the late study period in both
441 environmental and clinical samples. The Sydney 2012 variant is known to differ
442 antigenically from other GII.4 variants (Debbink et al., 2013) and to cause more severe
443 clinical symptoms compared to other genotypes and variants (Leshem et al., 2013b; Mai
444 et al., 2013). This property has resulted in its emergence as the predominant strain in
445 Japan and other countries (Fioretti et al., 2014; Fonager et al., 2013; Giammanco et al.,
446 2013; Leshem et al., 2013b; Rahman et al., 2013).
447 Norovirus-positive patients were detected from the 45th week of 2012, but GII
448 noroviruses were first detected in wastewater from the 50th week of 2012. This
449 difference may, in part, reflect the fact that virological surveillance is based on symptom
450 onset, whereas wastewater concentration reflects the number of people shedding
451 norovirus at the time that the samples were collected. Therefore, the detection of
452 norovirus GII in wastewater may have been delayed in relation to its incidence in

453 clinical samples. Indeed, norovirus shedding is known to continue for 9-56 days after
454 infection (Aoki et al., 2010; Atmar et al., 2008; Tu et al., 2008).
455 In 2013, GII.17 was detected in wastewater samples on January 16, 23, 30, and
456 February 6, but this variant was not detected in stool samples or in the national
457 surveillance system for gastroenteritis in Japan during our study period. Representative
458 nucleotide sequences of the largest GII.17 OTUs (including >99% of GII.17 reads in
459 each sample) detected in three wastewater samples (January 23, 30, and February 6,
460 2013) were 100% identical. This sequence was 99% identical (100% coverage) to the
461 strain detected in Okinawa, Japan, in 2012 (Accession No.: AB901276) and 99%-100%
462 identical (68%-94% coverage) to strains detected in asymptomatic food handlers
463 (Accession Nos. KF773972-3. KF773977, KF773989, and KF774001) and groundwater
464 samples in the Republic of Korea in 2012 (Accession Nos. KC413399-403; Cho et al.,
465 2014). However, there were no other sequences in the DDBJ/EMBL/GenBank database
466 that were more than a 97% match with our sequences. These results suggest that the
467 GII.17 strain caused local outbreaks across East Asia during 2012.
468 Moreover, GI.6 was the only GI genotype detected in four of the stool samples.
469 Emergence of this genotype, which peaks during the summer, was reported in 2010 in
470 the United States (Leshem et al., 2013a). Although GI.6 also was detected in two
471 wastewater samples, other GI genotypes (GI.3, 4) were detected more frequently,
472 perhaps because our study, which was conducted from November to March, did not
473 include summer months.
474 Even after pre-processing, many OTUs were produced, and up to eight genotypes were
475 detected in one wastewater sample, demonstrating a high genetic diversity of
476 noroviruses in wastewater. In contrast, only four genotypes were detected in the stool

477 samples. This discrepancy likely reflects the fact that only one outpatient clinic
478 participated in our surveillance efforts. Moreover, a previous report indicated that 90%
479 of patients with norovirus infections do not seek medical care (Hall et al., 2013). These
480 factors limited the number of samples that could be analyzed (n = 32). According to a
481 nationwide Japanese database, 19 genotypes were reported in Japan from September
482 2012 to August 2013 (National Institute of Infectious Deseases, 2014). Thus, more stool
483 samples would have been needed for a detailed analysis of infectious gastroenteritis
484 cases. However, considering the cost and effort involved in clinical surveillance,
485 wastewater monitoring could more easily provide a detailed distribution of genotypes
486 circulating in the population. Another possible explanation for the discrepancy is that
487 pyrosequencing was used for environmental samples but not for the clinical samples.
488 Since wastewater recieves noroviruses from all patients in the served area, multiple
489 genotypes were expected. This is why NGS is beneficial for detection of genotypes in
490 wastewater samples. In contrast, the aim of virological surveillance of gastroenteritis
491 cases is to identify a genotype/variant causing clinical symptom of each patient.
492 Because infection by multiple genotypes/variants is rare (Saito et al., 2014;
493 Thongprachum et al., 2014; Chan et al., 2015), the conventional Sanger sequencing
494 method is preferable both in terms of cost and experimental simplicity including
495 bioinformatics analysis. We have applied both pyrosequencing and Sanger sequencing
496 to three of the stool samples, and the results were identical: two samples contained a
497 single genotype/variant and the other contained two genotypes/variants, possibly caused
498 by co-infection (data not shown). To identify co-infection, we always examined the
499 chromatograms obtained by Sanger sequencing, and confirmed that there were no
500 samples with ambiguous nucleotides (e.g. peaks of multiple bases in the same position).

5. CONCLUSIONS

Our study showed that norovirus monitoring in municipal wastewater by pyrosequencing could provide similar or better information regarding temporal and genomic dynamics of norovirus than the virological surveillance of gastroenteritis cases. We demonstrated that ten more genotypes were detected in wastewater samples as compared with stool samples. Because novel genotypes or variants caused by genome recombination are frequently reported, monitoring of multiple genotypes is important, even if the genotypes are not dominant. This approach would also be useful in regions where nationwide clinical investigation is difficult and gastroenteritis surveillance systems do not exist. Applying this approach in such regions would enhance understanding of global movement of noroviruses. Further investigation, including screening for other pathogens, is necessary to permit virological surveillance based on municipal wastewater monitoring using NGS.

ACKNOWLEDGMENTS

We thank Mr. Yoshimitsu Konta for technical assistance on wastewater analysis and Dr. Hitoshi Onodera for his support with virological surveillance of gastroenteritis cases. This study was supported by CREST from the Japan Science and Technology Agency.

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777

778 **FIGURE LEGENDS**

779

780 **Fig. 1** Concentrations (as determined by qPCR) of norovirus genotypes I and II (GI and
781 GII) in wastewater samples collected at the indicated intervals.
782 < LOQ, below the limit of quantification; ND, not detected.

783

784 **Fig. 2** Genotypes and GII.4 variants identified from wastewater samples.
785 The numbers show ratios calculated as the number of reads assigned to each
786 genotype/variant divided by the number of reads identified as norovirus in each sample.
787 Heat map (color coding) is as indicated.

788

789 **Fig. 3** Number of infectious gastroenteritis cases and norovirus-positive/negative stool
790 samples collected each week.
791 For a given interval, the number of infectious gastroenteritis cases in the clinic is plotted
792 as solid line (asterisk (*)); the numbers and variants of norovirus are plotted as a bar
793 graph with color coding as indicated. Discordance between the number of the stool
794 samples and number of the infectious gastroenteritis cases in a given week reflects the
795 number of stool samples that could not be obtained for analysis.

796

797 **Fig. 4** Genotypes and GII.4 variants detected in stool samples.

798

799 **Fig. 5** Phylogenetic tree of GII.4 sequences obtained from wastewater samples and
800 clinical samples. The number on each branch shows the bootstrap value. Because many
801 OTUs were obtained from the wastewater samples, representative sequences of OTUs

802 containing more than 1000 reads were used for the analysis. The sequences obtained
803 from the wastewater samples are designated with names starting with “W”, followed by
804 date, variant (2006b, Den Haag 2006b; 2009, New Orleans 2009; 2012, Sydney 2012),
805 and identification number of OTU (e.g., W_Dec28_2012_OTU1). The sequences
806 obtained from the clinical samples are designated with names starting with “P”,
807 followed by variant and date of onset (e.g., P_2006b_Nov29). The reference sequences
808 are designated with names starting with “II.4” followed by accession number (e.g.,
809 II.4|2012|JX459908).

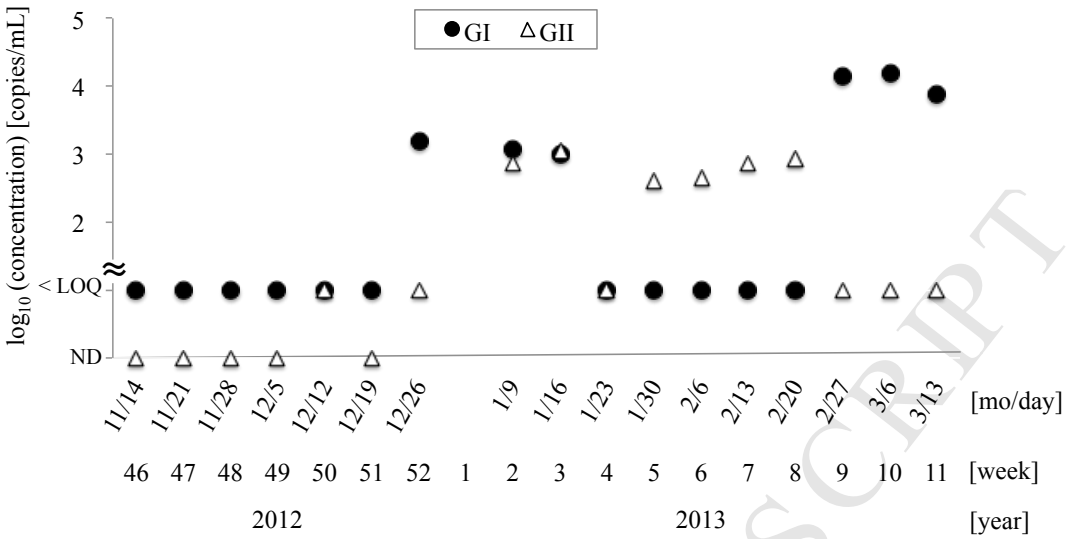

Fig. 1 Concentrations (as determined by qPCR) of norovirus genotypes I and II (GI and GII) in wastewater samples collected at the indicated intervals.

< LOQ, below the limit of quantification; ND, not detected.

	year	2012							2013									
	week	46	47	48	49	50	51	52	1	2	3	4	5	6	7	8	9	10	11
	mo/day	11/14	11/21	11/28	12/5	12/12	12/19	12/28		1/9	1/16	1/23	1/30	2/6	2/13	2/20	2/27	3/6	3/13
GI	GI.1	100.0						13.3											
	GI.3									19.0	99.7		98.9			96.9		100.0	
	GI.4				100.0		100.0	10.0		69.5		100.0	0.0	99.9	99.7		99.6		
	GI.6							47.7		11.5									
	GI.7							29.0			0.3		1.1	0.1	0.3	3.1	0.4		100.0
GII	GII.2	100.0				31.3		31.2		21.0	21.7	45.6	23.5	19.5	32.4	23.6	18.2		
	GII.4					22.1		29.9		6.4	19.9		13.1	15.5	27.8	23.7	18.5	20.1	39.8
	GII.5														39.8		18.2	14.3	
	GII.6													1.6				4.4	
	GII.7									3.1							27.1		60.2
	GII.12									32.7									
	GII.13																	43.5	
	GII.14				100.0	46.3		38.9		36.8	58.0		32.5	26.5		52.7	17.1	16.9	
	GII.17										0.3	54.4	30.2	36.9					
	GII (not assigned)					0.2							0.7	0.1			0.9	0.8	
GII.4 variants	Den Haag 2006b					92.3		23.7			30.0								
	New Orleans 2009							9.1											
	Sydney 2012							62.6		76.5	61.5		95.2	84.8	96.6	94.2	87.7	90.5	98.6
	GII.4 (not assgined)					7.7		4.6		23.5	8.6		4.8	15.2	3.4	5.8	12.3	9.5	1.4

Fig. 2 Genotypes and GII.4 variants identified from wastewater samples.

The numbers show ratios calculated as the number of reads assigned to each genotype/variant divided by the number of reads identified as norovirus in each sample. Heat map (color coding) is as indicated.

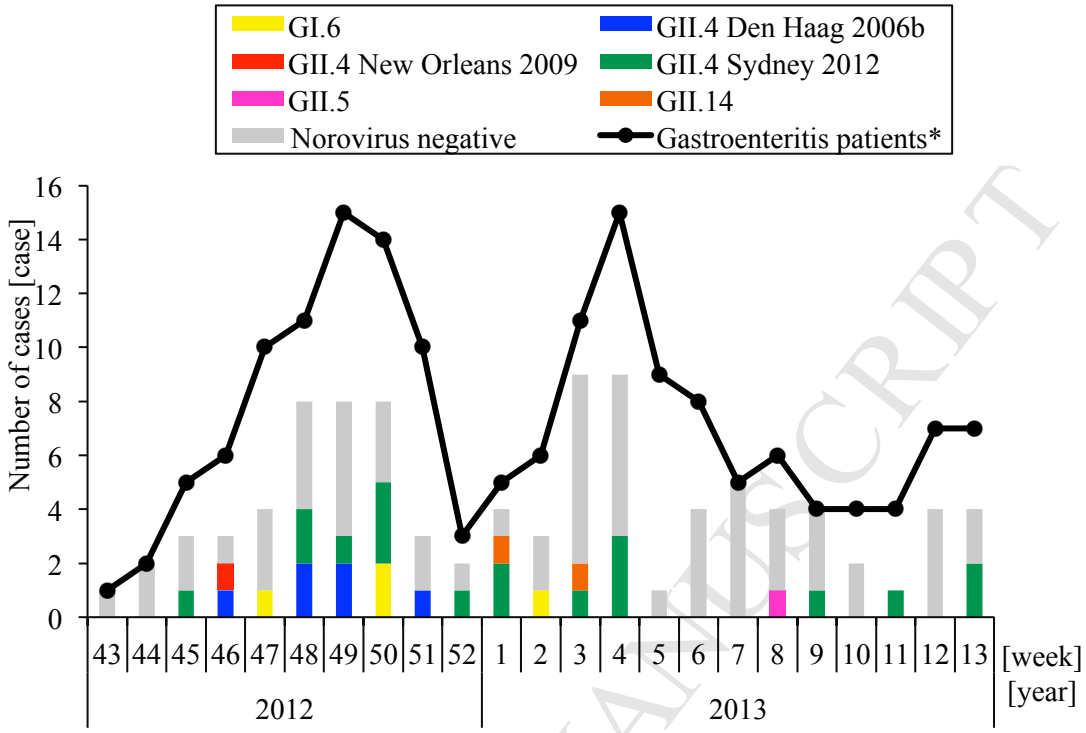

Fig. 3 Number of infectious gastroenteritis cases and norovirus-positive/negative stool samples collected each week.

For a given interval, the number of infectious gastroenteritis cases in the clinic is plotted as solid line (asterisk (*)); the numbers and variants of norovirus are plotted as a bar graph with color coding as indicated. Discordance between the number of the stool samples and number of the infectious gastroenteritis cases in a given week reflects the number of stool samples that could not be obtained for analysis.

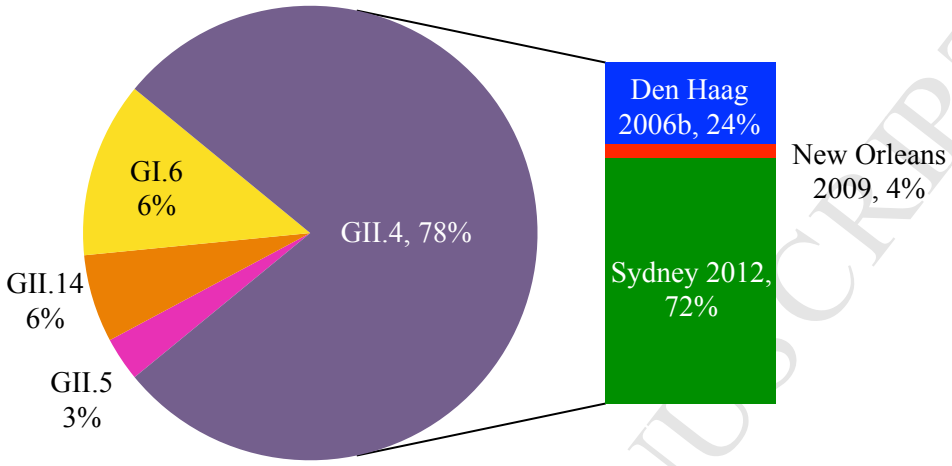

FIG 4 Genotypes and GII.4 variants detected in stool samples.

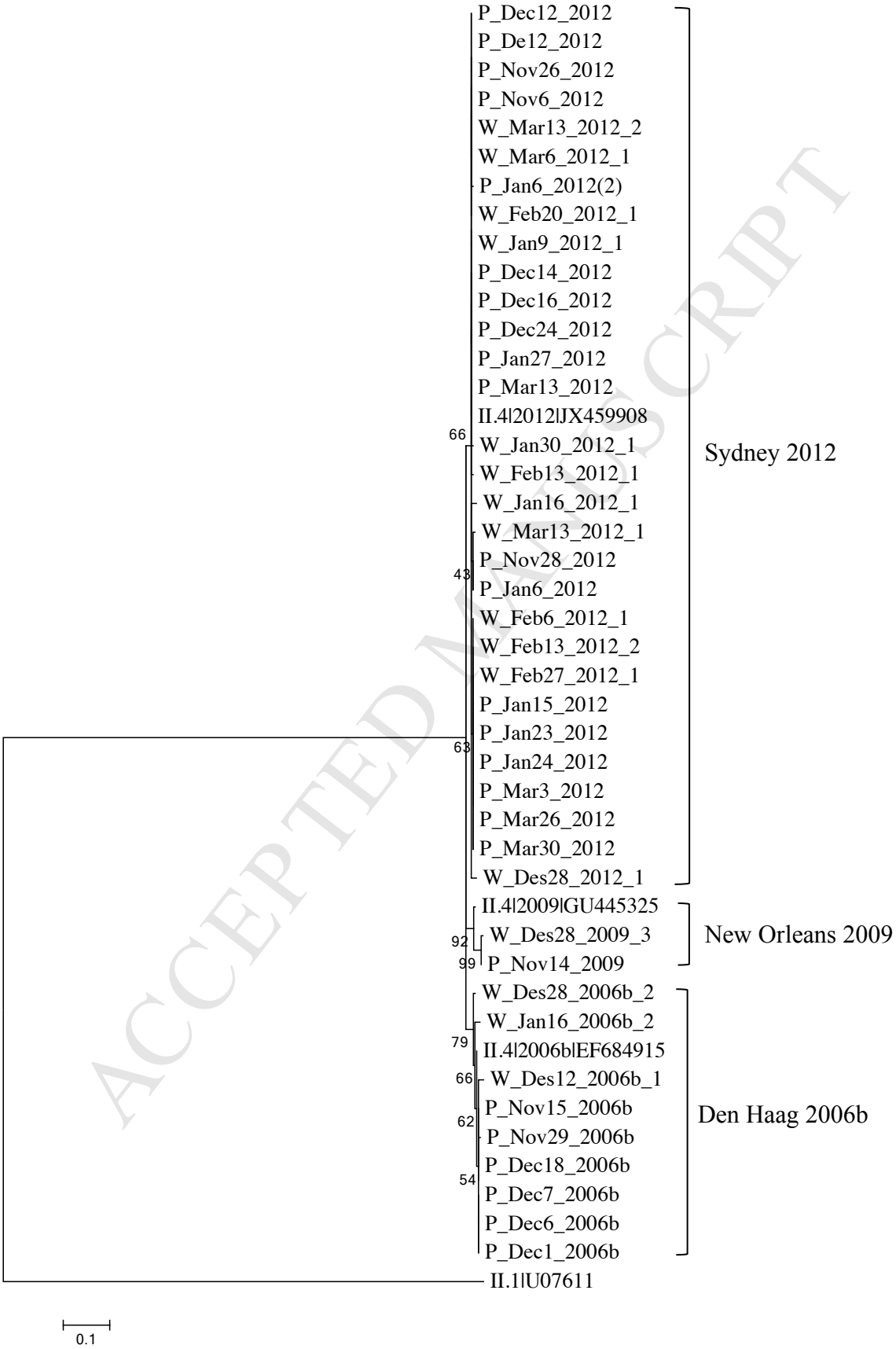

Fig. 5 Phylogenetic tree of GII.4 sequences obtained from wastewater samples and clinical samples. The number on each branch shows the bootstrap value. Because many OTUs were obtained from the wastewater samples, representative sequences of OTUs containing more than 1000 reads were used for the analysis. The sequences obtained from the wastewater samples are designated with names starting with "W", followed by date, variant (2006b, Den Haag 2006b; 2009, New Orleans 2009; 2012, Sydney 2012), and identification number of OTU (e.g., W_Dec28_2012_OTU1). The sequences obtained from the clinical samples are designated with names starting with "P", followed by variant and date of onset (e.g., P_2006b_Nov29). The reference sequences are designated with names starting with "II.4" followed by accession number (e.g., II.4|2012|JX459908).

Highlights

1. Pyrosequencing revealed diverse *Norovirus* genotypes in wastewater.
2. Ten more genotypes were detected in wastewater (14) than in patients' stools (4).
3. Spread of *Norovirus* GII.4 Sydney variant was observed in both sample types.
4. A rare strain of *Norovirus* GII.17 was detected in wastewater.
5. The developed method is applicable to any types of environmental samples.