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BRIEF COMMUNICATION

# Pyrosequencing Analysis of Norovirus Genogroup II Distribution in Sewage and Oysters: First Detection of GII.17 Kawasaki 2014 in Oysters

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**Abstract** Norovirus GII.3, GII.4, and GII.17 were detected using pyrosequencing in sewage and oysters in January and February 2015, in Japan. The strains in sewage and oyster samples were genetically identical or similar, predominant strains belonging to GII.17 Kawasaki 2014 lineage. This is the first report of GII.17 Kawasaki 2014 in oysters.

**Keywords** Norovirus · Oyster · Sewage · Pyrosequencing

A significant number of norovirus outbreaks have occurred worldwide since the mid-1990s, with the genogroup II genotype 4 (GII.4) as the major cause. A new variant, GII.17 Kawasaki 2014, emerged in the 2014–2015 norovirus season and replaced the previously prevalent GII.4 Sydney 2012 variant (Matsushima et al. 2015; Chan et al. 2015). Oysters can accumulate norovirus when grown in contaminated marine environment and are considered one of the most important pathways of norovirus transmission (Flannery et al. 2012). In this study, we investigated the presence of norovirus GII in sewage and oysters using pyrosequencing techniques to determine norovirus diversity detected in this important pathway.

Sewage samples were collected from a wastewater treatment plant in Miyagi Prefecture, Japan on January 7 and February 4, 2015. Oyster samples (>12 individuals/batch) were collected from the same geographic location on January 14, 22, 28, and February 4, 2015. Sewage samples were processed by the polyethylene glycol precipitation method to concentrate viruses present in the samples (Kazama et al. 2016). Digestive tissues were excised from the oyster samples and mixed with an enzyme solution containing amylase (6.24 mg/mL), proteinase-K (0.25 mg/mL), and lipase (6.24 g/mL) for virus extraction. In brief, the virus extraction was performed with two stainless beads on Micro Smash (TOMY, Tokyo, Japan) at 4200 rpm for 60 s and then incubated with the enzyme solution for 60 min at 37 °C followed by 15 min at 60 °C. Viral RNA was extracted using the NucliSENS miniMAG Kit following the manufacturer's instructions. Complementary DNA was reverse transcribed and used for a nested PCR assay using the COG2F/G2SKR and G2SKF/G2SKR primer sets to amplify the RdRp and capsid N/S-encoding domain (RdRp-N/S) region (Kazama et al. 2016). The nested PCR products were separated by agarose gel electrophoresis and products with the expected length (344 bp) were excised from the gel and purified using the Qiagen Gel Extraction Kit (Qiagen). The purified products were applied in a fusion PCR assay, and subjected to pyrosequencing using the GS Junior system (Roche Applied Science) and bioinformatics analysis was performed as described by Kazama et al. (2016).

Genotype distribution of norovirus strains present in sewage and oyster samples in January and February 2015

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**Table 1** Norovirus genotypes detected in sewage and oysters[a]

| Genotype | Sewage | | Oysters | | | |
|---|---|---|---|---|---|---|
| | Jan 7 | Feb 4 | Jan 14 | Jan 22 | Jan 28 | Feb 4 |
| GII.2 | – | 7.2 | – | – | – | – |
| GII.3 | – | 24 | 19 | 25 | 79 | 4.8 |
| GII.4 (Den Haag 2006b) | – | 0.43 | – | 12 | – | – |
| GII.4 (Sydney 2012) | 16 | 0.0082 | 6.4 | 0.53 | 5.0 | 2.2 |
| GII.4 (not assigned) | 0.58 | 0.14 | – | 0.025 | 0.19 | – |
| GII.6 | 13 | – | – | – | – | – |
| GII.13 | – | 2.8 | – | – | – | – |
| GII.14 | 3.6 | – | – | – | – | – |
| GII.17 | 67 | 65 | 74 | 62 | 16 | 93 |

[a] The numbers represent the rate of sequence reads (%) calculated as the number of reads assigned to each genotype/variant divided by the total number of reads in each sample

was obtained by pyrosequencing (Table 1). An average of 12,409 reads of norovirus GII was obtained for each sewage or oyster sample, with a rate of chimera reads ranging from 0.2 to 1.2 %. All rarefaction curves reached a plateau, indicating a deep coverage of norovirus GII diversity. We found four to five genotypes involving GII.2, GII.3, GII.4, GII.6, GII.13, GII.14, and GII.17 in each sewage sample and three genotypes (GII.3, GII.4, and GII.17) in each oyster sample. Although GII.3 and GII.4 have previously been detected in oyster samples (Rajko-Nenow et al. 2013), GII.17, a genotype previously undetected in oysters, was identified in our oyster samples for the first time. Furthermore, GII.17 was the predominant genotype, followed by the GII.3 or GII.4 genotypes, in almost all the sewage and oyster samples, except in the oyster samples collected on January 28. In a study performed in Ireland in 2010, although not the dominant genotype, GII.17 was detected in influent wastewater, but not in effluent wastewater and oyster samples (Rajko-Nenow et al. 2013).

Upon phylogenetic analysis, we found that the nucleotide sequences of samples isolated from sewage and oysters formed a distinct cluster that can be called sub-cluster Kawasaki308 2015 in the GII.17 Kawasaki 2014 lineage, as shown in Fig. 1 (Dinu et al. 2015). In February 2015, norovirus GII.17 strains were isolated from three individuals infected in an oyster-related outbreak in Miyagi

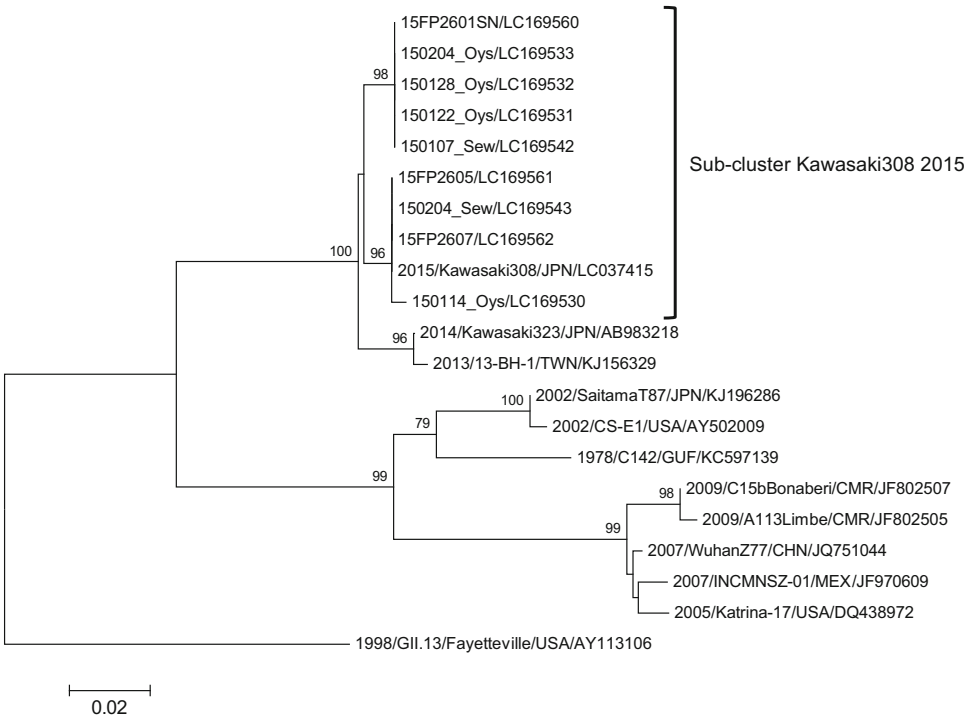


**Fig. 1** Phylogenetic tree of the RdRp-N/S region of GII.17 strains obtained from sewage and oyster samples. The obtained sequences are designated with names starting sampling date (yymmdd) followed by "Sew" (sewage) or "Oys" (oyster), and accession number. The reference sequences are designated with names starting year followed by the name of strain, country, and accession number. Numbers at nodes represent the rates of 1000 bootstrap replicates

Prefecture. Notably, the three strains (15FP2601SN, 15FP2605, 15FP2607) clustered in the same sub-cluster with the sewage- and oyster-derived strains (Fig. 1). This result clearly indicated that there is strong correlation among the prevalent norovirus strains detected in sewage, oysters, and in the gastroenteritis cases.

The GII.17 genotype can be traced back to as early as 1978, and from 1978 to 2013, GII.17 strains have been reported in Africa, Asia, Europe, North America, and South America (de Graaf et al. 2015). In the 2014–2015 norovirus season, the GII.17 Kawasaki 2014 variant was detected more frequently than the GII.4 Sydney 2012 variant in gastroenteritis cases in our study area, Miyagi Prefecture (data not shown), as reported in other areas in Japan (Matsushima et al. 2015). The simultaneous existence and dominance of the GII.17 strains in sewage, oysters, and gastroenteritis cases highlights the importance of studying diverse norovirus genotypes circulating in the human population and those released in the environment. Future studies should investigate the long-term temporal variation of norovirus genotypes in sewage, oysters, and gastroenteritis cases.

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