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Weekly variations in norovirus genogroup II genotypes in Japanese oysters

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ABSTRACT

Increased levels of norovirus contamination in oysters were reportedly associated with a gastroenteritis epidemic occurring upstream of an oyster farming area. In this study, we monitored the norovirus concentration in oysters weekly between November 2014 and March 2015 and investigated the statistical relationship between norovirus genogroup II (GII) concentrations in oyster and sewage samples and the number of gastroenteritis cases in the area using cross-correlation analysis. A peak correlation coefficient (R = 0.76) at a time lag of +1 week was observed between the number of gastroenteritis cases and norovirus GII concentrations in oysters, indicating that oyster contamination is correlated with the number of gastroenteritis cases with a 1-week delay. Moreover, weekly variations in norovirus GII genotypes in oysters were evaluated using pyrosequencing. Only GII.3 was detected in November and December 2014, whereas GII.17 and GII.4 were present from January to March 2015. GII.17 Kawasaki 2014 strains were detected more frequently than GII.4 Sydney 2012 strains in oyster samples, as previously observed in stool and sewage samples collected during the same study period in Miyagi, Japan. Our observations indicate that there is a time lag between the circulation of norovirus genotypes in the human population and the detection of those genotypes in oysters.

1. Introduction

Norovirus is a leading causative agent of acute gastroenteritis worldwide. Norovirus lineages can be classified into seven genogroups (GI-GVII), which can be further subdivided into at least 38 genotypes (Verhoef et al., 2015; Vinjé, 2015). Viruses belonging to GI, GII, and GIV are responsible for infection in humans, among which GII is the most prevalent genogroup worldwide (Medici et al., 2015; Zheng et al., 2006). A significant number of norovirus outbreaks have occurred since the mid-1990s, with the rapidly evolving GII.4 strain as the major cause. A novel GII variant, GII.17 Kawasaki 2014, emerged during the 2014–2015 norovirus season as a major cause of gastroenteritis outbreaks in Asia, and this variant replaced the previously dominant GII.4 Sydney 2012 variant (Chan et al., 2015; Matsushima et al., 2015). Among the 2133 cases reported in the Japan Infectious Agents Surveillance Report (IASR) from October 2014 to March 2015, GII.4, GII.3, and GII.17 were detected in 17.5%, 6.8%, and 4.7% of cases, respectively. A dramatic increase in the number of cases of GII.17 has been observed, from three in the 2013–2014 season to 100 in the 2014–2015 season in Japan (Matsushima et al., 2015). This lineage has been the predominant genotype since the beginning of the 2014–2015 winter season among both stool and sewage samples collected in Miyagi Prefecture, Japan (Kazama et al., 2017).

Norovirus-associated outbreaks can involve both foodborne and person-to-person transmission. Noroviruses are increasingly becoming the most common cause of gastroenteritis infection associated with the consumption of shellfish (Schaeffer et al., 2013). However, the relative importance of transmission through shellfish as compared with that of person-to-person transmission is unknown. A systematic review of global outbreak surveillance data from 1999 to 2012 indicated that 14% of all norovirus outbreaks were caused by food (Verhoef et al., 2015). From September 2010 to August 2016, the ratio of foodborne norovirus outbreaks was even higher in Japan, which was 20%–34% (27% in average) based on data released by the National

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Epidemiological Surveillance of Infectious Disease (NESID, 2017).

Oysters are one of the most common shellfish eaten raw worldwide. According to the Family Income and Expenditure Survey Report, from 2013 to 2015, the average consumption of oysters per family in Japan was 493 g (Statistics Japan, 2016), which equals approximately 26 oysters assuming that the weight of a single shucked oyster is 19 g. Oysters can bioaccumulate human pathogenic microorganisms when grown in water impacted by sewage. Campos et al. (2017) predicted that an average of 130 sewage spills would lead to 500 copies of norovirus in 1 g of oyster based on a catchment population of 223,008, area of 72,953 ha, sewage spill volume of 1530 m^3/day, and average distance between discharge and sampling point of 4 km. A review of norovirus contamination in shellfish in the UK revealed that among the 74,000 cases of norovirus associated with contaminated food annually, 16% (11,800 cases) were caused by oyster consumption (Hassard et al., 2017). Furthermore, oyster-related gastroenteritis is not always transmitted through locally or domestically harvested oysters. During January and February of 2016 in Denmark, 58 out of 67 persons were infected after consuming oysters harvested off the coast of La Rochelle, France (Rasmussen et al., 2016). Given the numerous reports of norovirus outbreaks in association with oyster consumption, oysters are considered one of the most important pathways for norovirus transmission (Flannery et al., 2012).

In a previous study (Pu et al., 2016), we phylogenetically characterized norovirus isolates from two sewage and four oyster samples. The isolated strains of GII.17 from the sewage and oysters clustered with isolates derived from gastroenteritis cases in the GII.17 Kawasaki 2014 lineage, indicating a strong relationship among the prevalent strains. Thus, in this study, we aimed to improve our understanding of the temporal changes in norovirus genotypes circulating in the human population and those released into the environment in the 2014–2015 norovirus season. Miyagi prefecture, the second largest oyster production area in Japan, with an oyster yield as high as 20,000 tons per year in Matsushima Bay facing the Pacific Ocean, was selected as the target area for this study. We evaluated the correlation between the number of reported gastroenteritis cases and norovirus concentrations in sewage and oyster samples. Furthermore, we investigated variations in norovirus GII genotypes in weekly oyster samples using pyrosequencing.

2. Materials and methods

2.1. Oyster sampling and viral RNA extraction

Nine individual oyster samples were collected each Wednesday from November 5, 2014 to March 26, 2015 from a farming area in Miyagi Prefecture, Japan. The oyster samples were sent to the laboratory on ice within 24 h, and digestive tissue (DT) was excised immediately after arrival. Highest accumulations of noroviruses have been found in DT compared to other tissues in oysters, and thus it is often used for the detection of norovirus in oysters (Le Guyader et al., 2006; Tian et al., 2006; Wang et al., 2008). The DTs of the oyster samples were weighed (ranging from 2.1 to 4.9 g), and then each of the nine samples was placed in a 5-mL tube with two stainless steel beads (3.2 mm in diameter) and 1 mL enzyme solution containing 6.3 mg/mL α-amylase (A-3176 Type VI-B; Sigma, St. Louis, MO, USA), 0.25 mg/mL proteinase-K (#03115801001, PCR grade; Roche, Indianapolis, IN, USA), and 6.3 g/mL lipase (L1754 Type VII; Sigma). Each tube was processed on a Micro Smash-100 (TOMY, Tokyo, Japan) at 4200 rpm for 60 s, and samples were then incubated for 60 min at 37 °C, followed by 15 min at 60 °C. The mixture was then centrifuged at 9100 × *g* for 12 min. The supernatants (approximately 4 mL) from three individual samples were mixed to form one composite sample. The three oyster composite samples produced each week were stored at −80 °C until subsequent analysis.

For RNA extraction, 500 μL of 400 mM citrate buffer (pH 2.5) was added to 500 μL of each composite sample, and the samples were mixed by vortexing for 10 s and centrifuged at 9100 × *g* for 12 min. Approximately 50 μL of RNA was extracted from the supernatant using NucliSENS miniMAG reagents (BioMérieux, Marcy-l'Étoile, France) following the manufacturer's instructions. The RNA extracts were stored at −80 °C until further analysis.

2.2. Reverse transcription (RT) and quantitative real-time PCR (qPCR)

RNA was reverse-transcribed into complementary DNA (cDNA) using an iScript Advanced cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) with a T100 thermal cycler (Bio-Rad) following the manufacturer's instructions. The RT reaction was conducted with the following protocol: 42 °C for 30 min for RT reaction, followed by 85 °C for 5 min for inactivation of reverse transcriptase. Oyster sample cDNA was stored at −80 °C for further analysis.

For qPCR, the cDNAs were analyzed with a CFX96 Touch Real-Time PCR detection system (Bio-Rad) for the quantification of norovirus GII. qPCR was performed with 5 μL of cDNA and 15 μL of reaction mix containing Sso Advanced Universal Probes Supermix (Bio-Rad), together with primers and probes [COG2F, COG2R, and RING2AL-TP (5′-FAMTGG GAG GGS GAT CGC RAT CT-TAMRA-3′)] as previously described (Kageyama et al., 2003; Kazama et al., 2016; Nakamura et al., 2010; Pu et al., 2016). PCR amplification was performed under the same conditions as those described by Kazama et al. (2016).

A ten-fold dilution was prepared for each cDNA sample, and both undiluted and diluted samples were analyzed by qPCR. Tenfold serial dilutions (10^5–10^1 copies) of plasmid containing the target region were prepared for each standard curve (Kageyama et al., 2003). qPCR was performed in duplicate for both samples and standards. The DT of each oyster was spiked with murine norovirus (MNV, approximately 10^7 genome copies) during viral extraction, as a process control (Hata et al., 2011). MNV was also quantified by qPCR with the primers and probe described by Hata et al. (2011). The recovery rate for each sample was obtained by dividing the MNV amount measured by qPCR by the amount added to each oyster sample.

2.3. Quality controls for qPCR

For qPCR, samples with MNV recovery rates higher than 1% were considered validated for norovirus quantification (ISO 15216-1, 2017). Positive results were obtained from amplifications with fewer than 40 quantification cycles (C_q value < 40), in compliance with the MIQE guidelines (Bustin et al., 2009). The limit of detection (LOD) was approximately 1.1 log copies/g DT, based on the average oyster sample DT weight (calculated from three composite oyster samples each week for 18 weeks). Tubes used in all procedures, if not specifically mentioned, were sterilized polyethylene low-binding tubes.

2.4. Nested PCR

Considering the unquantifiable but possibly positive sample, one composite sample was selected from each of the eighteen weeks, with a Cq value below 45 according to qPCR. Sample cDNAs were used for nested PCR. The COG2F/G2SKR (CARGARBCNATGTTYAGRTGGATGAG/CCRCCNGCATRHCCRTTRTACAT) and G2SKF/G2SKR (CNTGGG AGGGCGATCGCAA/CCRCCNGCATRHCCRTTRTACAT) primer sets were used to amplify the capsid N/S-encoding domain (N/S) region using NebNext High Fidelity 2× PCR MasterMix (New England Biolabs, Ipswich, MA, USA), as described by 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 a Qiagen Gel Extraction Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions.