Genetic analysis of noroviruses taken from gastroenteritis patients, river water and oysters

Y. Ueki*,, K. Akiyama*, T. Watanabe** and T. Omura****

* Miyagi Prefectural Institute for Public Health and Environment, Saiwai-cho 4-7-2, Miyagino-ku, Sendai 983-0836, Japan (E-mail: *youeki@ihe.pref.miyagi.jp*)

** Dept of Civil Engineering, Graduate School of Engineering, Tohoku University, Aoba-yama 06, Sendai 980-8579, Japan

Abstract As oysters are eaten raw in Japan, their contamination with the non-bacterial agent of gastroenteritis has become a serious health problem. As it is well known that oysters tend to concentrate noroviruses (NV) in their digestive diverticula, NV may be linked with the acute gastroenteritis. However, since NV cannot be cultivated in cell cultures, and they have genetic diversity, the behaviour of NV in the aquatic environment is little known. In this study, NV samples were taken from gastroenteritis patients; from the river flowing into the oyster-farming area; and from oysters harvested from that river. Genetic identities of NV samples were analysed in capsid and RNA-dependent RNA polymerase (RdRp) regions respectively. In both regions, strains taken from patients were >96% identical with those from river and oyster samples. This proved that oysters were contaminated with NV excreted from patients with gastroenteritis.

Keywords Gastroenteritis; genetic analysis; norovirus; oysters; river water

Introduction

Miyagi Prefecture is located in northeast Japan and is characterised by marine product industries, including oyster farming. As oysters are eaten raw in Japan, contamination of the shellfish with the non-bacterial agent of gastroenteritis has become a serious public health problem. According to a Center for Disease Control and Prevention report (CDCP, 2001), shellfish tend to concentrate noroviruses (NV) in their digestive diverticula. Therefore, NV may be linked with acute gastroenteritis. However, since NV cannot be cultivated in cell cultures, and has genetic diversity, the behaviour of NV in the aquatic environment is little known. Although huge numbers of NV, excreted by patients with gastroenteritis, are discharged into the water environment from the sewer system (Lodder *et al*., 1999), the contamination route of oysters by NV has not yet been proven. The objective of this study was to prove this contamination route through the genetic analysis of NV isolates taken from gastroenteritis patients, river water and oysters.

Materials and methods

Research area and samples

Figure 1 illustrates the research area. According to the Miyagi Prefectural Epidemiological Surveillance of Infectious Agents, from the end of November to the end of December 2001, an outbreak of NV gastroenteritis occurred in cities A and B. River B flows into bay C, where oysters were cultivated. The wastewater treatment plant of city B was located on river B, into which the treated wastewater was discharged. Noroviruses were detected in river B and the oysters cultivated in bay C, from November 2001 to January 2002. In order to conduct the phylogenetic analysis of detected NV, four faecal samples from patients with gastroenteritis in city A, four samples from river B and a sample of oyster from bay C were collected. Cities D and E, located 50 km north of bay C (not in Figure 1), reported outbreaks of NV gastroenteritis in December 2001 and March 2002 respectively; four

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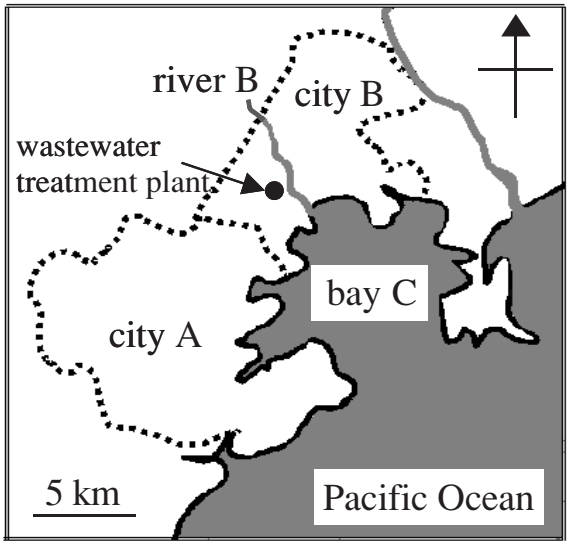

Figure 1 The research area

faecal samples were taken from patients in both cities and used for NV phylogenetic analysis.

Extraction of viral RNA from faeces

Stool suspensions (10% in distilled water) were centrifuged (9,200×*g*, 10 min) and guandine thiocyanate (6 M, 200 μL) was added to 200 μL of the supernatant. Glass powder (6 μL, DNA PREP, Dai-Iatoron Co. Ltd, Japan) was added to the mixture, vortexed for 10 min at room temperature and immediately cooled for 10 min on ice. The mixture was centrifuged (9,200×*g*, 1 min) and the supernatant aspirated. Washing buffer (500 μL) (DNA PREP, Dai-Iatoron Co. Ltd, Japan) was added to the pellet, vortexed for a few seconds and centrifuged (3,000×*g*, 3 min), this procedure being repeated. Ethanol (600 μL) was added to the pellet, centrifuged (9,200×*g*, 6 min) and vortexed for a few seconds. The supernatant was aspirated and the pellet dried in a desiccator. After addition of 30 μL distilled water, it was centrifuged (9,200×*g*, 12 min) and the supernatant used for RT-PCR.

Extraction of viral RNA from river water

NV was concentrated from 1 L river water by addition of PEG 6000 (WAKO Pure Chemical Industries Ltd, Japan) and 23 g sodium chloride and stirring overnight at 4°C. After centrifugation (9,200×*g*, 30 min) the supernatant was aspirated and the pellet resuspended in 2 mL distilled water. This was further centrifuged (10,000×*g*, 15 min) and viral RNA extracted using the glass powder method (above).

Extraction of viral RNA from oysters

The digestive diverticula were aseptically removed from the oysters, pooled in batches of five, and frozen for 2 h at –80°C. The tissues were thawed by addition of distilled water at 70°C, centrifuged (9,200×*g*, 10 min) and viral RNA extracted as above.

Amplification of viral RNA

Viral RNA was amplified by the RT-PCR method using primers NV36/35′ (Moe *et al.*, 1994) for the first PCR and NV82, SM82/NV81 (Hayashi *et al.*, unpublished) for the nested-PCR (Table 1). These primers were based upon the sequence of the RNA-dependent

Table 1 Primers used for NV detection

Amplified region	Samples	Primer	
		First PCR	Nested-PCR
RNA-dependent RNA polymerase	Faeces, river water, oysters	NV36/35′	NV82, SM82/NV81
	River water, oysters	COG1F/G1SKR	G1SKF/R
		COG2F/G2SKF/R	G2SKF/R
Capsid	Faeces	G1SKF/R	Not performed
		G2SKF/R	Not performed

RNA poymerase (RdRp) region. Two unpublished primers based on the capsid region were also used. The primer pairs for the first PCR were COG1F/G1SKR and COG2F/G2SKR (Kojima *et al*., 2000; Kageyama *et al*., 2003). The pairs for the nested-PCR were G1SKF/R and G2SKF/R (Kojima *et al*., 2000).

Nested-PCR was performed for samples of river water and oysters, since these samples did not have enough NV to be detected otherwise. The PCR products were analysed by 2% agarose gel electrophoresis and visualised by ethidium bromide (EtBr) staining. Amplicons for RdRp regions were confirmed with Southern hybridisation using the six digoxigenin-labelled probe oligonucleotides consisting of four probe sets (P1-A, P1-B, P2-A and P2-B). The P1-A probe set contained three probes of SR63d, SR65d and SR69d. On the other hand, P1-B, P2-A and P2-B probe sets included single probes of SR67d, SR61d, SR47d respectively. Amplicons for the capsid region were confirmed with Southern hybridisation using four probes (G1-1, 2, 3, 4) in genotype 1 and three probes (G2-1, 2, 3) in genotype 2 (Ishiko *et al*., unpublished).

Cloning and sequencing of RT-PCR products

Sequences of PCR products from faecal samples were determined by the direct sequencing method (ABI PRISM 310, Applied Biosystems). Since multiple strains of NV often exist in river water and oysters, PCR products from these samples were cloned in a pPCR-Script Amp SK(+) vector using PCR-Script™ Amp Cloning kit (STRATAGENE). Sequences were determined for at least five randomly selected clones using the BigDye terminator cycle sequence kit and ABI PRISM 310. Sequences of 246 bp of the RdRp region and 243 bp of the capsid region were aligned with Clustal X (Free soft) respectively. On the basis of aligned sequences, phylogenetic analysis was performed with the Neighbor Joining method.

Results and discussion

Strains of NV isolated

From patients. Two NV strains were obtained by amplifying the capsid region among four positive faecal samples taken from gastroenteritis patients in city A. In the same manner, one and two strains were obtained from patients in cities D and E respectively. On the other hand, five strains of NV were obtained from four patients in city A by the amplification of the polymerase region, i.e. two strains were detected from a patient. Two strains were also detected with the primer for the polymerase region from patients in city D.

From river water. Three NV strains were obtained from river B by amplifying a part of the capsid region. Sequences in the amplified region were 100% identical among these strains regardless of different sampling dates.

From oysters. Two strains were detected with the capsid region primer for oyster samples taken on 13 December 2001. On the other hand, a strain of NV was found in the same sam-

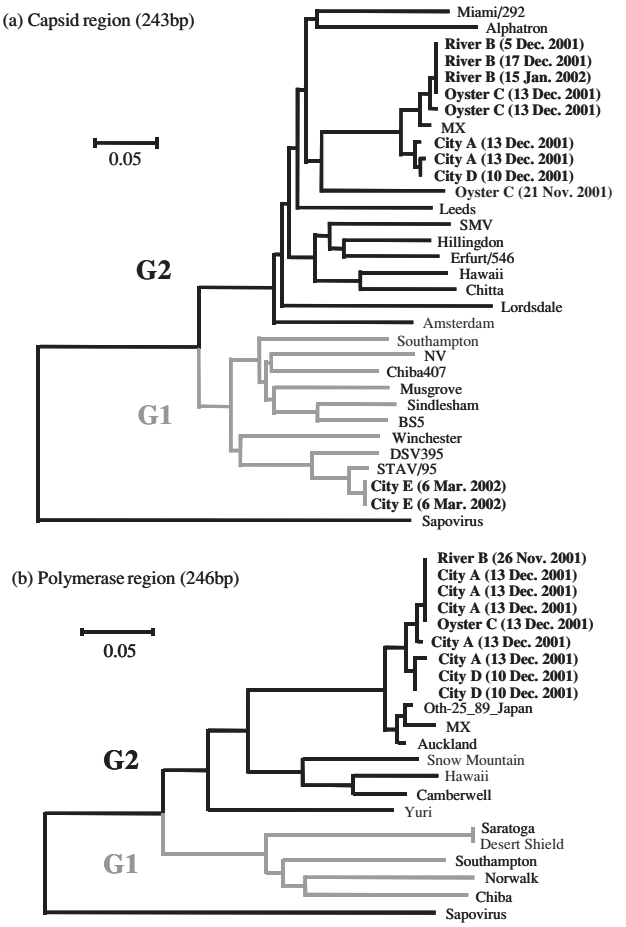

Figure 2 Dendrograms of capsid and polymerase regions in NV genes detected from gastroenteritis patients in cities A, D and E, the river B and oysters cultivated in the bay C. (The GenBank accession numbers of the reference strains are as follows: Miami/292, AF414410; Alphatron, AF195847; MX, U22498; Leeds, AJ277608; SMV, U70059; Hillingdon, AJ277607; Erfurt/546, AF427118; Hawaii, U07611; Chitta, AB032758; Lordsdale, X86557; Amsterdam, AF195848; Southampton, L07418; NV, M87661; Chiba407, AB022679; Musgrove, AJ277614; Sindlesham, AJ277615; BS5, AF09397; Winchester, AJ277609; DSV395, U04469; STAV/95, AF145709; Oth-25 89 Japan, L23830; Auckland, U34381; Camberwell, AF145896; Yuri, AB009876; Saratoga, U07614; Sapovirus, U65427. The length of the abscissa to the connecting node is proportional to the genetic distance between sequences.)

ples with the polymerase region primer. The 21 November 2001 oyster samples gave one strain using the polymerase region primer.

Contamination route of oysters with NV

On the capsid region. Figure 2a illustrates the results of phylogenetic analysis for capsid belonging to genogroup 2 (G2). A strain of NV taken from oysters on 13 December 2001 had the same sequence as those from river B on 5 and 17 December 2001 and 15 January 2002. Three strains found in patient faeces in city A on 13 December 2001 were similar to this group (96% nucleotide identity). Since the strain isolated from oyster on 21 November 2001 was not included in the above group, this strain was not the same as those isolated from oysters on 13 December 2001.

On the polymerase region. Figure 2b illustrates the results of the phylogenetic analysis for the polymerase region in NV genes. A strain from river B (26 November 2001), three strains from patients (city A, 13 December 2001) and a strain from oysters cultivated in bay C (13 December 2001) were 100% identical in the polymerase region. According to the phylogenetic analyses for both the capsid and polymerase regions in NV genes, the same strain (>96% identity) was isolated from gastroenteritis patients in city A, river B and oysters cultivated in bay C between 26 November 2001 and 15 January 2002.

Although faecal samples were not collected from gastroenteritis patients in city B, there was a high possibility that the outbreak here was caused by the same NV strain as in city A, as both outbreaks occurred at the same time. This was supported by the same NV strain that occurred in city A being detected in river B following discharge of wastewater from city B. On the basis of the phylogenetic analyses, the contamination route of oysters with NV could be explained as follows. Firstly, river B was contaminated with NV excreted from gastroenteritis patients in city B. Then NV in the river was transported into bay C through the sewer system and concentrated in oysters.

Relationship between outbreaks of gastroenteritis

As shown in Figure 2, strains of NV isolated from patients in city A (13 December 2001) were the same as those from patients in city D (10 December 2001). This indicated that the two outbreaks were caused by the same NV strain. The city D outbreak might have been related to NV detected from bay C oysters, although city D was located 50 km north of the bay. On the other hand, NV taken from patients in city E (6 March 2002) was categorised as G1 by phylogenetic analysis for the capsid region. Therefore, the outbreak of gastroenteritis in city E was not related to oysters cultivated in bay C.

Prevention of contamination of oysters with NV

In order to prevent NV contamination of oysters, viruses must be removed somewhere along the contamination route shown in this study. Various methods to remove or inactivate NV in oysters are presently being investigated. However, effective removal technologies have not been developed as yet, because the NV accumulation mechanism in oysters is not well known. In general, virus removal from water is easier than from oysters. Therefore, virus removal at the wastewater treatment plants should significantly contribute to the decrease in the risk of infection from eating oysters.

Conclusions

In order to investigate the contamination route of oysters with NV, phylogenetic analyses were performed for NV strains isolated from gastroenteritis patients, river water and oysters. Sequences in both capsid and polymerase regions were >96% identical among these strains. Therefore, the contamination route of oysters with NV could be explained as river B being contaminated with NV excreted from gastroenteritis patients in city B, followed by transportation into bay C and concentration by oysters. On the basis of this route, virus removal at wastewater treatment plants would significantly contribute to the decrease of infectious risk associated with the eating of oysters.

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