Genetic Analysis of Norwalk Virus（NV）Detected in the River Water and the Oysters

Yo UEKI[1,5] Tomikazu ARITA[1] Ikuo GOTO[1]
Chizuko SATO[1] Yoko OKIMURA[1] Hiroyuki SHIRAISHI[1]
Kazuo AKIYAMA[2] Osamu HASHIMOTO[3] Hiroaki ISHIKO[3]
Naokazu TAKEDA[4] Toru WATANABE[5] Tatsuo OMURA[5]

Key words：Norwalk Virus, River Water, Oysters

INTRODUCTION

Miyagi prefecture is located on northeast of Japan and characterized by marine products industries. Such as the oyster-farming is one of marine products industries in Miyagi prefecture. As oysters are often eaten raw in Japan, the contamination of oysters with the non-bacterial agent concerned with the gastroenteritis has become a serious social problem. According to the report by Center for Disease Control and Prevention, USA(2001), the shellfish tends to concentrate Norwalk virus(NV)in their digestive diverticula[1]. Therefore, NV may be concerned with the acute gastroenteritis. However, owing to the inability to cultivate NV in cell lines and the genetic diversity of NV, the behavior of NV in the aquatic environment has been hardly known. The objective of this study is to investigate the distribution of NV in the river and the oysters cultivated at Sendai Bay, Miyagi and the homogeneity of NV by the genetic analysis.

MATERIALS AND METHODS

The detection of NV was performed for 89 samples taken from 11 rivers that flow into three oyster-farming areas and 44 samples of oysters cultivated at the areas（Figure 1）. These samples were collected by Miyagi prefectural government from September 1999 to March 2000 and from October 2000 to March 2001.

NV was isolated from the sample of river water by the following procedures. Sample of river water（1 L）was concentrated with polyethyleneglycol and centrifuged at 10,000 x g for 30 min. The supernatant was removed with aspirator. The pellet was suspended with 2 ml of distilled water. Viral RNA was purified with glass powder method. For the detection of NV from the sample of oyster, digestive diverticula was separated from the oyster and frozen at －80 ℃ for 2 hours. Then it was melted by adding the distilled water at 70 ℃ and centrifuged at 9,200 x g for 15 min. Viral RNA was isolated from the supernatant with DNA PREP（ASAHI GLASS COMPANY）.

RNA of NV was detected with RT-PCR method using primers designed as amplified polymerase regions（Tables 1 and 2）. The RT profile was run with 37 ℃ for 1 hour and at 98 ℃ for 5 min. PCR profiles for NV series[2] and Yuri series[3][4] of primers were run with procedures in Table 3. The PCR products of 330bp for NV series and 373bp for Yuri series were analyzed by 2.0% agarose gel electrophoresis, revealed by ethidium bromide（EtBr）staining, and Southern transferred to positively charged nylon membranes（Roche Molecular Biochemicals, Indianapolis, Ind.）for oligohy-bridization. The membranes were prehybridized for 3 hours at 58 ℃ then hybridized with digoxigenin-labelled probes at the same temperature.

Genotype of NV detected by RT-PCR method was determined by using the primer designed as amplified capsid regions（Mitsubishi Kagaku Bio-Clinical Laboratory INC.）. Some of PCR products（161bp）were cloned into plasmids with PCR-ScriptTM Amp Cloning Kit (STRATAGENE) and sequenced with the BigDye Terminator Cycle Sequencing FS Ready Kit（Applied Biosystems）and ABI PRISM 310（Applied Biosystems）. The cloned NV gene was analyzed with Neighbor Joining method. The dendrogram of predicted phylogenetic relationship among strains of NV was created by GENETYX-MAC（SOFTWARE DEVELOPMENT CO., LTD.）.

Table 1. Primers employed for the detection of NV by RT-PCR methods.

RT	NV series	Yuri series
RT	35'	MR 4
1 st. PCR	36	MR 3
2 nd. PCR	NV82 and SM82	Yuri22F and 22R

１）保健環境センター 微生物部
２）仙台保健福祉事務所塩釜総合支所
３）㈱三菱化学ビーシーエル研究開発部
４）国立感染症研究所ウイルス第２部
５）東北大学大学院工学研究科

Table 2. Sequences of primers.

NV series	35'	5'-CTTGTTGGTTTGAGGCCATA-3'
	36	5'-ATAAAAGTTGGCATGAACA-3'
	NV81	5'-ACAATCTCATCATCACCATA-3'
	NV82	5'-TCATTTTGATGCAGATTA-3'
	SM82	5'-CCACTATGATGCAGATTA-3'
Yuri series	MR 3	5'-CCGTCAGAGTGGGTATGAA-3'
	MR 4	5'-AGTGGGTTTGAGGCCGTA-3'
	Yuri22F	5'-ATGAATGAGGATGGACCCAT-3'
	Yuri22R	5'-CATCATCCCCGTAGAAAGAT-3'

Table 3. Procedures for PCR with primers of NV and Yuri serieses.

(a) NV series

1st. PCR	1 cycle of 94℃ for 3 min. 40 cycles of 94℃ for 1 min, 50℃ for 1min. and 72℃ for 1 min. 1 cycle of 72℃ for 15min.
2st. PCR	Same as 1st. PCR

(b) Yuri series

1st. PCR	5 cycle of 94℃ for 1 min, 51℃ for 2 min. and 60℃ for 4min. 30 cycles of 94℃ for 1 min, 51℃ for 80sec. and 72℃ for 1 min.
2st. PCR	5 cycle of 94℃ for 1 min, 45℃ for 2 min. and 60℃ for 4min. 30 cycles of 94℃ for 1 min, 45℃ for 80sec. and 72℃ for 1 min.

RESULTS AND DISCUSSIONS

NV were detected from 8 of 35 samples of river water and 12 of 35 samples of oyster in September 1999 to March 2000, and 4 of 54 samples of river water and 11 of 44 samples of oysters in October 2000 to March 2001, respectively (Figure 1).

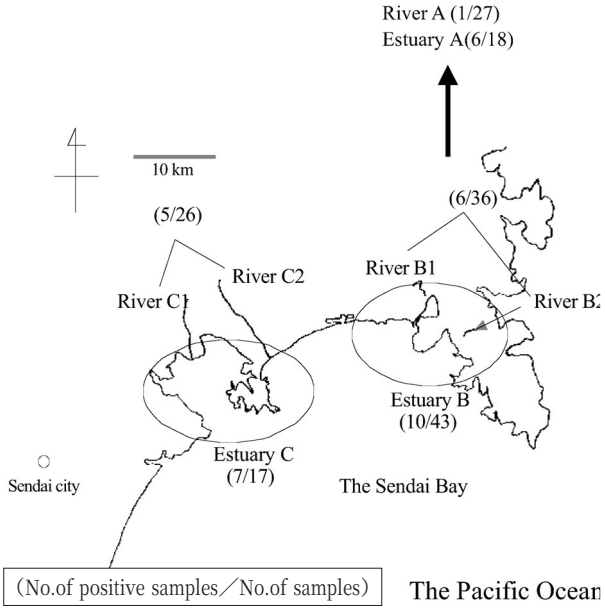

Figure 1. The research area and the results of detection of NV in the river and the oysters cultivated in the estuary. Estuary A and river A are located on 80km north from estuaries B and C. NV were detected from 1 of 27 samples in river A and 6 of 18 samples of oysters in estuary A.

These results indicated that rivers and oysters in Sendai Bay were contaminated broadly. In Japan, outbreaks of gastroenteritis by NV through eating oysters occur mainly in winter. ThereforeSo, the rate of positive samples rises in winter related to these outbreaks.

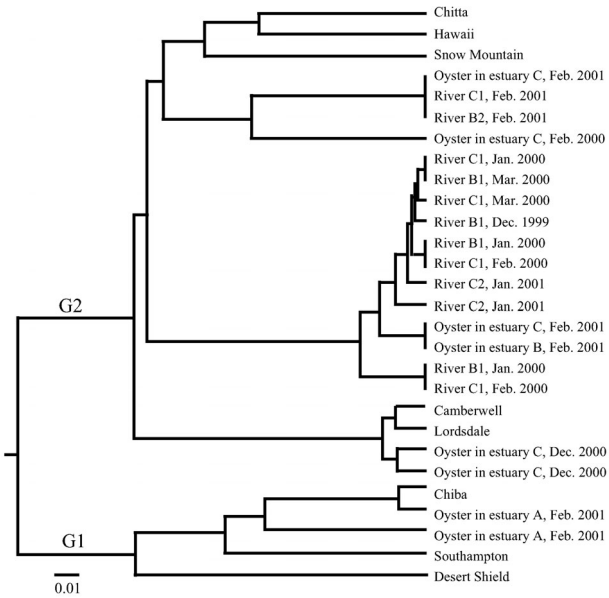

Figure 2. Dendrogram of predicted phylogenetic relationship among 28 strain of NV. The length of the abscissa to the connecting node is proportional to the genetic distance between sequences.

Twenty strains were obtained from 14 samples(９ samples of river water and ５ samples of oysters). Figure ２ shows the dendrogram of predicted phylogenetic relationship among 28 strains of NV. On the basis of sequences of nucleic acid and amino acids in the RNA dependent RNA polymerase (RdRp) regions and capsid regions, NV was categorized into two groups (G１ and G２). Strains in G２ group are often detect-ed from outbreak of gastroenteritis by NV in Japan. Both groups of G１ and G２ had about 70 percent of homogeneity, respectively. The homogeneity between G１ and G２ groups was about 50 percents. Sequences of nucleic acid in the capsid regions of NV in rivers C１ and C2, and oysters cultivated in estuary C were similar to those in rivers B１ and B2, and oysters cultivated in estuary B regardless of the sampling season. Only oysters cultivated in estuary A were categorized into G１ group. Since estuary A is located on 80 km north from estuaries B and C, the difference may be related to this distance.

Sequence of nucleic acid in the capsid regions of NV in the oyster taken in estuary C in February, 2001 is identical to that of NV in river C１ at the same time. This result showed the possibility that NV transported from the river to the estuary was concentrated in the oyster. Through investigations into

the distribution of NV in the rivers and the oysters at the Sendai Bay, and the homogeneity of NV by genetic analysis, it shows that NV discharged from infected persons contributed through the river to the contamination of oysters in the estuary.

REFERENCES

1) Center for Disease Control and Prevention (2001). Norwalk-Like Viruses, Recommendations and Reports, 50(RR- 9), 1-18, June 1
2) Moe C.l. et al. : J Clin Microbiol 32 ; 642 (1994)
3) Lew J.F. et al. : J Virol 68 ; 3391 (1994)
4) Saito H. et al. : Microbiol Immunol 42 ; 439 (1998)