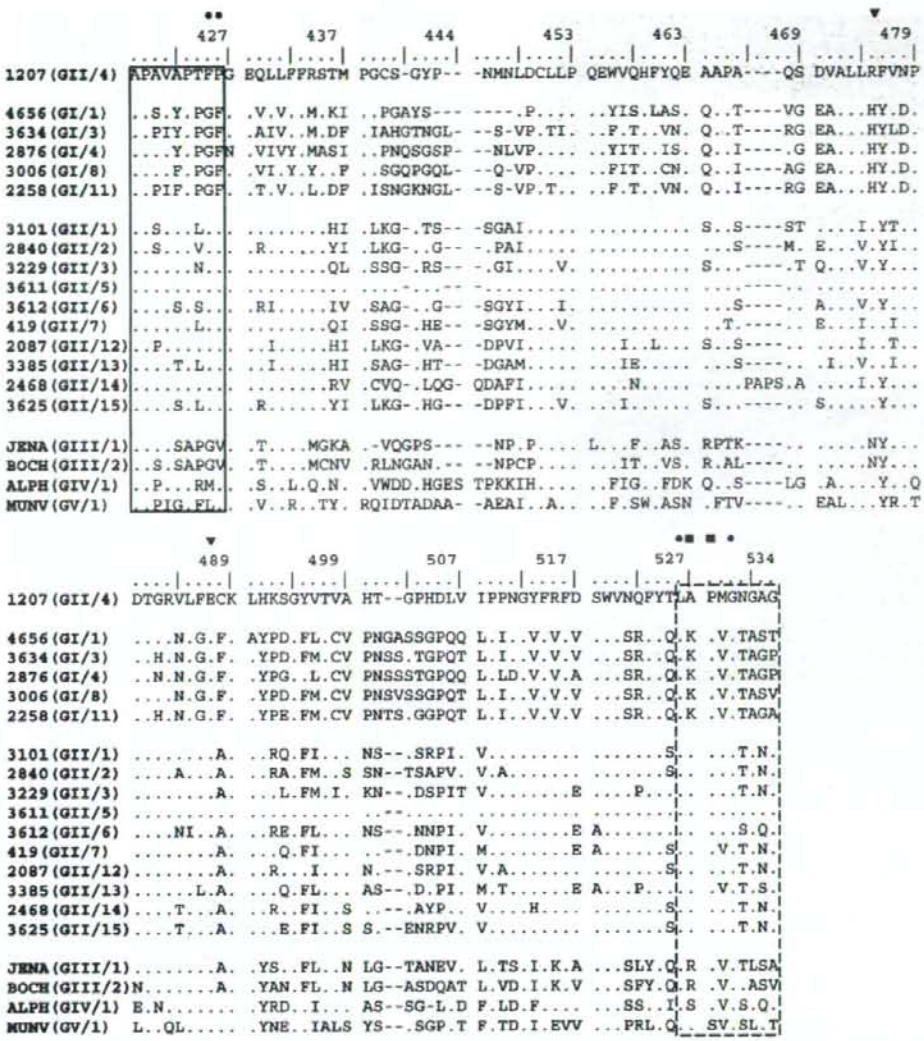

FIG. 2. Alignment of the amino acid sequence of the minimal binding region on noroviruses for MAb14-1. The MAb14-1-specific residues (●), identical components of the previously reported conformational epitope of NV3901 and NV3912 (▼), and the amino acid positions shared between the epitopes of MAb14-1, NV3901, and NV3912 (■) are indicated (37). The solid-line and dashed-line boxes represent the N-terminal antigenic region (A region) and C-terminal antigenic region (B and C region), respectively, on the minimal binding region. Dots indicate identical amino acid residues, and dashes indicate gaps. JENAGIII/1 (Bo/Jena/1980/DE [GenBank accession number AJ011099]), BOCHGIII/2 (Bo/CH126/1998/NL [[GenBank accession number AF320625]), ALPH GIV/1 (Hu/Alphatron/1998/NL [GenBank accession number AF195847]), and MUNV GV/I (Mu/Murine norovirus-1/US [GenBank accession number AY228235]).

fragment analysis, single point mutants, and structure prediction of antigen.

The results of the fragment analysis for VP1 showed that the epitope for MAb14-1 exists on the C-terminal P1 domain, which is more conserved than the N-terminal P1 and P2 domains based on sequence identity among noroviruses. This location of the epitope may be the reason for the same reactivity under different pH conditions because particle (P domain on the surface of the particle) or a single capsid protein did not relate to the accessibility of the epitope on the P domain for MAb14-1.

The fragment analysis for the C-terminal P1 domain and structural analysis showed that almost the whole C-terminal P1 domain generated the conformation of the minimal binding region. Both terminal antigenic regions (amino acid positions 418 to 426 and 526 to 534) on the minimal binding region approached each other via the insertion region (positions 427

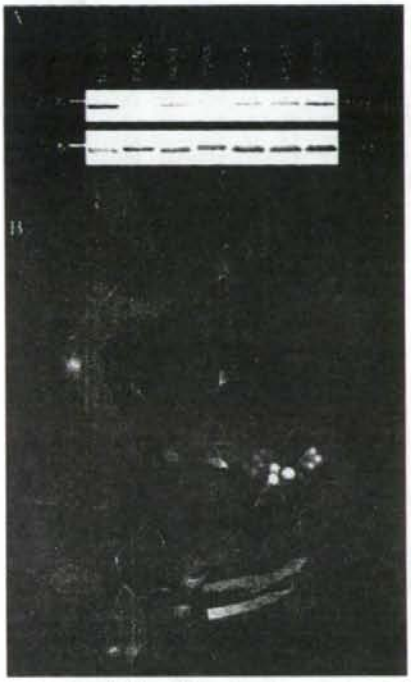

FIG. 3. (A) Reactivities of the six point mutations by Western blotting analysis. 20 K, 20,000; a-His, anti-His_5 antibody. (B) Position of each point mutation on the r1207 prediction structure. The phenylalanine at position 426 (blue), proline at position 427 (red), leucine at position 526 (yellow), alanine at position 527 (green), proline at position 528 (orange), and glycine at position 530 (magenta) are shown.

to 525). This motif forms the conformational epitope and may explain the broad reactivity, because MAb14-1 was generated by immunization of GII/4, which is the most sophisticated strain for immune response, implying a potential evolutionary selection.

The components of the epitope for MAb14-1 were determined by mutational analysis. It was found that the components comprise six amino acids and are classified into four major groups, groups 1 to 4, by the following reactivities.

(i) Not only was L526 conserved in all rVLP sequences but also the same conserved residues in other genogroups of norovirus were observed. More interestingly, L526 was even conserved among other caliciviruses, suggesting that this leucine residue might be influence the calicivirus-specific reactivity of MAb14-1 (7).

(ii) There were two interpretations of the role of F425, which is conserved among GII. One of them was the generation of high-titer GII-specific antigenicity for MAb14-1. A previous study by Chakravarty et al. also supported this observation (5). The other interpretation was the generation of genus-specific antigenicity, due to the existence of a GI-specific phenylalanine close to GII-specific phenylalanine. For confirmation of this interpretation, site-directed mutagenesis on the GI capsid needs to be performed in the future.

(iii) Three residues, P426, A527, and P528, gave the same result in inducing a significant reduction in reactivity, but their roles were probably different from each other. P426 possibly constructed the epitope directly. Parker et al. previously reported that K527 (GI) directly interacted with E487, generating the GI-specific structure (35). As a result, K527 (GI) may induce a low titer of MAb14-1 for GI. Our results also supported the previous observations reported by Parker et al. (35) and Chakavraty et al. (5), in that the difference between K and A induced a difference of antigenicity between GI and GII. P528, which is conserved among all noroviruses, except for

TABLE 4. Cross-reactivities of representative previously reported broadly reactive monoclonal antibodies with various norovirus capsids by Western blotting and/or ELISA

Monoclonal antibody	Isotype	Reactivity[a] of rVLP or recombinant capsid protein with:																				Recognition domain	Minimal binding region	Reference(s)
		Genogroup I genotype[b]							Genogroup II genotype[b]															
		1	2	3	4	6	8	11	1	2	3	4	5	6	7	8	12	13	14	15	17			
MAb14-1	IgG1	+	+	+/−[c]			+	+	+	+	+	+	+	+	+	+	+	+				C-terminal P1	418–534	This study
NV23	IgG1	+	+		+					+		−		+			+		+					20
NS14	IgG1	−	−		−					+		−		+			+		+			C-terminal P1	473–494[d]	20, 34
NV3901 and NV3912	IgG1	+	+		+					−		−		−			−		−			C-terminal P1	454–520[e]	14, 20, 35
1B4	IgG1	+	+	+	−	+		+	+	+	+		+	+		+	+				+	S	31–70[f]	46, 47
1F6	IgG2	+/−[g]	−	+/−[h]	−	+		−	+	+	+		+	+		+	+				+	S	31–70[f]	46, 47
MAb5		+	+		+					+	+	+		+					+					39

[a] Symbols: +, reactive; −, not reactive. Blanks mean the reactivity was unconfirmed. Homologous reactivities are shown in boldface type, especially NS14 against mixture of recombinant norovirus and recombinant Snow Mountain agent (SMA).

[b] Genetic classification based on the method described by Kageyama et al. (17).

[c] MAb14-1 has weak reactivity for GI/3.

[d] Amino acid numbers correspond to the sequence of Norwalk virus (GenBank accession number M87661).

[e] Amino acid numbers correspond to the sequence of Houston virus related to Lordsdale virus (GenBank accession number X86557).

[f] Amino acid numbers correspond to the sequence of the immunogen used to generate the specific antibodies: recombinant norovirus 36 for 1B4 and 1F6 (GenBank accession number AB028244).

[g] +/− indicates positive for KU4hGI (GenBank accession number AB067549) but negative for Gifu'96 (GenBank accession number AB0456039).

[h] +/− indicates positive for Desrt Shield (GenBank accession number U04469) but negative for Stav'95/Nor (GenBank accession number AF145709).

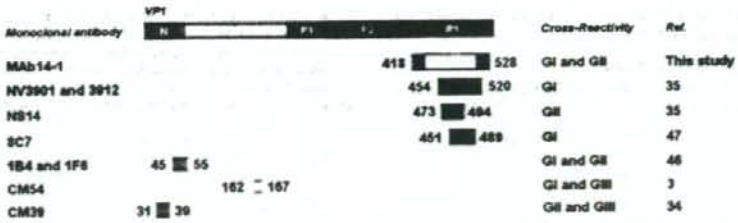

FIG. 4. Location map of the norovirus cross-reactive monoclonal antibody binding sites (being or containing an epitope) on VP1. The blank (amino acids positions 427 to 525) on the binding site for MAb14-1 means that it is not the region for a binding site but is necessary for generating a binding site structurally. Amino acid numbers correspond to the sequences of the immunogens used to generate the specific antibodies: Southampton virus for CM54 (GenBank accession number L07418) Jena virus for CM39 (GenBank accession number AJ011099) Norwalk virus for NV3901 and NV3912 (GenBank accession number M87661), SMA for NS14 (GenBank accession number U70059), recombinant Norwalk virus capsid protein (NV 96-908) for 8C7 (GenBank accession number AB028247), and recombinant genogroup II virus capsid protein (NV 36) for 1B4 and 1F6 (GenBank accession number AB028244). Ref., reference.

murine norovirus, is the component of the epitope and induces GI and GII cross-reactivity of MAb14-1.

(iv) G530 is the critical component of the epitope. Functional change of protein was usually ignored in the change from G to A, because there is not much difference in character between G and A (35). Nevertheless, our results showed that the change was important. It is suggested that a slight difference from G to A generates moderate effect on reactivity when glycine is the main component of the epitope.

These mutational analyses elucidated the character of the epitope residues, explaining GI and GII cross-reactivity of the epitope and difference in titer among GI and GII. High conservation of the six amino acids among GII explains high GII-specific titer of MAb14-1. Genus-specific residues generate tolerant reactivity for GI. GI-specific residues induce low reactivity of MAb14-1 for GI. These results imply that the epitope for MAb14-1 is the genus-specific epitope. To investigate this possibility, the reactivity of MAb14-1 for GIII-V rVLPs needs to be elucidated further.

Our results could not explain the low affinity to GI/3 for MAb14-1 because we could not find appropriate GI/3-specific mutations in the minimal binding region. Two possible explanations for this were proposed. First, the epitope on GI/3 may be inhibited by a conformational change derived from the remote amino acid residue(s) in the minimal binding region. Second, other domains, such as N, S, N-terminal P1, and P2 domains, may shield or mask the epitope, as in previous reports about human immunodeficiency virus or picornavirus (4, 8, 23, 26, 36, 44). To confirm these hypotheses, we need to conduct further investigation including crystallography studies.

The fragment, sequence, structural, and mutational analyses identified the epitope formed by the six amino acids and excluded any other amino acids composing the epitope. The structural sequence of these six amino acids generates a linear region; therefore, we can consider this epitope to have potential as a linear epitope with the binding property for the monoclonal antibody. Moreover, in a previous finding on the linear epitope, five amino acid residues were essential for antibody binding, which supports our supposition (10).

In comparison with the location of a previously reported cross-reactive epitope on VP1 (3, 34, 35, 46, 47), our identified epitope is obviously a novel conformational one (Fig. 4). However, a previously reported GI cross-reactive conformational epitope for monoclonal antibodies, NV3901 and NV3912, and the novel identified epitope in this study shared two amino acid positions, 527 and 528, but the MAb14-1 showed both GI and GII cross-reactivity (Fig. 2) (35). In addition, previous studies reported that broadly reactive monoclonal antibodies, GI and GII cross-reactive antibodies, NS14, 1B4 and 1F6, have linear epitopes (35, 46, 47). Therefore, MAb14-1 had more advantages than previously reported broadly reactive monoclonal antibodies did. In other words, we were the first to identify the GI and GII cross-reactive monoclonal antibody, which recognizes the novel conformational epitope.

With the absence of an appropriate cultivation system, we are not able to use neutralization methods to determine the neutralizing antibody for norovirus. The potentially neutralizing monoclonal antibodies were indirectly determined by Vance's study using histo-blood group antigens assumed to be a receptor for norovirus infection and the putative neutralizing antibodies detecting P2 epitopes (15, 25). Recently, Batten et al. (3) and Oliver et al. (34) reported that anti-human norovirus monoclonal antibody could detect the bovine norovirus, while the opposite was also true. Until now, several neutralizing epitopes have been reported for caliciviruses, except for norovirus (9, 27, 41–43). These previous findings suggest that there are genus-specific neutralizing epitopes on caliciviruses. If this suggestion were true, a broadly reactive monoclonal antibody, such as MAb14-1, which has potential for detecting other caliciviruses, may neutralize calicivirus infection. The use of MAb14-1 may be contribute towards antiviral and vaccine development.

In conclusion, to our knowledge, we were the first group to determine the conformational epitope on the norovirus capsid for GI (GI/1, 4, 8, and 11) and GII (GII/1 to 7 and 12 to 15) cross-reactive novel monoclonal antibody, which showed a weak affinity to GI/3. From these data, MAb14-1 could be applied further for the development of the immunochromatography test.

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Genotyping and Quantitation of Noroviruses in Oysters from Two Distinct Sea Areas in Japan

Tomoko Nishida*[1,5], Osamu Nishio[2], Masahiko Kato[3], Takehisa Chuma[4], Hirotomo Kato[5], Hiroyuki Iwata[5], and Hirokazu Kimura[2]

[1]Yamaguchi Prefectural Research Institute of Public Health, Yamaguchi, Yamaguchi 753–0821, Japan, [2]Infectious Diseases Surveillance Center, National Institute of Infectious Diseases, Musashimurayama, Tokyo 201–0011, Japan, [3]Gunma Prefectural Institute of Public Health and Environmental Sciences, Maebashi, Gunma 371–0052, Japan, [4]Department of Veterinary Hygiene, Kagoshima University, Kagoshima, Kagoshima 890–0065, Japan, and [5]Department of Veterinary Hygiene, Yamaguchi University, Yamaguchi, Yamaguchi 753–8511, Japan

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Abstract: **Norovirus (NV) is a causative agent of acute gastroenteritis in humans, and shellfishes including oysters act as major vehicles of the virus. To investigate the genetic characteristics of NVs, we collected 1,512 oysters for raw consumption between October 2002 and March 2005 from two distinct areas (area A: the Sanriku Sea area; area B: the Setouchi Sea area). We detected the capsid gene and subjected it to phylogenetic analysis. By further quantification of the copy number of the genome by using real-time PCR, the NV capcid gene was detected in approximately 5% of the oysters, and they showed wide diversity. Two percent of the oysters from area B showed relatively large number of NVs, i.e., over 100 copies of capsid gene/oyster, whereas this was not observed in area A. Most of the detected NVs from oysters and humans were genetically related when the capsid region was compared. These results suggested that NVs obtained from humans and those obtained from oysters showed a potential relationship to each other and that some populations of Japanese oysters accumulated a relatively large number of NVs.**

Key words: **Oyster, Norovirus, Phylogenetic analysis, Real-time PCR**

Introduction

Norovirus (NV) is a member of the *Caliciviridae* family (1, 11) and a major causative pathogen of acute nonbacterial gastroenteritis worldwide (4, 6, 8, 11). NV causes fecal-oral infection and is highly infectious (4). According to the data from 2002 to 2004 in Japan, NV is the most common viral agent of food poisoning, and this virus accounted for approximately 30% to 45% of all food poisoning cases (http://www.mhlw.go.jp/topics/syokuchu). The prevalence of NV in other developed countries including the U.S.A. and France is even higher (2, 5). In 1994, Wang et al. showed that NVs isolated from humans are classified into two major genogroups—genogroup I (GI; Norwalk-type viruses) and genogroup II (GII; Snow mountain-type viruses) (26). Each genogroup is further subdivided into many genotypes; thus NVs exhibit wide genetic diversity (10, 12). It is suggested that NVs expelled from patients with gastroenteritis are condensed in shellfishes such as oysters and clams (3, 22, 25). The Japanese and other Asians consume large amounts of raw shellfish, and Europeans and North Americans are also eating increasing amounts of raw shellfish. Raw consumption of shellfishes causes large outbreaks of food poisoning and infectious gastroenteritis (14, 16, 23). Previous epidemiological studies showed a linkage between gastroenteritis due to NV and the oyster-harvesting season (http://idsc.nih.go.jp/iasr) (8). In several studies, NVs were detected in approximately 5% to 20% of oysters and clams (7, 15, 19). Thus, these shellfishes are one of the sources of NV infections (4, 18). Person-to-person infection is another common mode of NV infection (4, 18). In our previous study, we used reverse transcription polymerase chain reaction (RT-PCR) and showed that the NV capsid gene was detected in approximately 10% of the oysters for raw consumption (20). Furthermore, most of the oysters had relatively large amounts

*Address correspondence to Dr. Tomoko Nishida, Yamaguchi Prefectural Research Institute of Public Health, 2–5–67 Aoi, Yamaguchi, Yamaguchi 753–0821, Japan. Fax: +81–83–922–7632. E-mail: nishida.tomoko@pref.yamaguchi.lg.jp

Abbreviations: GI, genogroup I; GII, genogroup II; N-J, neighbor-joining; NV, norovirus; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR.

of NV viral genomes with more than 100 copies of the capsid gene (20). However, the molecular study on NVs in oysters is not well known in Japan. In this study, we performed genetic analysis of NVs obtained from 1,512 Japanese oysters for raw consumption which were harvested from two distinct sea areas (Sanriku Sea and Setouchi Sea areas) from October 2002 to March 2005.

Materials and Methods

Samples and preparation of viral suspension. A total of 1,512 Japanese oysters (*Crassostrea gigas*) were collected from the fish distributors that manage harvesting in the Sanriku Sea (483 oysters; Area A) and Setouchi Sea (1,029 oysters; Area B). The distance between these areas is approximately 1,000 km. The Sanriku and Setouchi Seas are located in the northeastern and western parts of the Honshu Island, respectively. The number of samples and the months during which the oysters were harvested in these areas are listed in Table 1. All of the oysters harvested had been approved for raw consumption according to the Food Sanitation Law Enforcement Regulations (in which the standard plate count of bacteria in 1 g of an oyster should be <50,000 with <230 coliforms). The fresh oysters were shucked, and their stomachs and digestive tracts were removed by dissection and then weighed and homogenized in 10 mM phosphate-buffered saline, pH 7.4 without magnesium or calcium to prepare a 10% suspension. The suspension received 0.1 ml antifoam B (Sigma, St. Louis, Mo., U.S.A.) and then was homogenized twice at a 30 sec interval at the maximum speed by using an Omni-mixer (OCI Instruments, Waterbury, Conn., U.S.A.). Six milliliters of chloroform:butanol (1:1 vol) was added to the homogenate. The mixture was then homogenized for an additional 30 sec and 170 µl Cat-Floc T (Calgon, Elwood, Pa., U.S.A.) was added to the homogenate (14). In addition, to monitor for efficiency of the RNA extraction, we added echovirus type 9 to the homogenate samples. After the homogenate samples were centrifuged at 3,000×*g* for 30 min at 4 C, their supernatants were layered onto 1 ml of 30% sucrose solution and ultracentrifuged at 154,000×*g* for 3 hr at 4 C. Subsequently, the pellet was resuspended in 138 µl of double distilled water (DDW) and stored at −80 C until use.

RNA extraction, RT-PCR, sequencing, and real-time PCR. Viral RNA was extracted with the QIAamp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany) from 138 µl of the viral suspension. Two microliters of echovirus type 9 (Hill strain; corresponding to approxi-

Table 1. Sample numbers and positive rate of capsid gene of noroviruses in oysters in this study

Month/year	Area A		Area B	
	Sample No.	Positive for RT-PCR (%)	Sample no.	Positive for RT-PCR (%)
Oct/2002	12		6	
Nov/2002	27	1 (3.7)	39	
Dec/2002	45	1 (2.2)	138	6 (4.3)
Jan/2003	48	6 (12.5)	93	12 (12.9)
Feb/2003	39		36	2 (5.6)
Mar/2003	18		12	
Subtotal	189	8 (4.2)	324	20 (6.2)
Oct/2003	15		12	
Nov/2003	18		45	
Dec/2003	81	17 (21)	147	7 (4.8)
Jan/2004	60	7 (11.7)	126	1 (0.8)
Feb/2004	45		78	2 (2.6)
Mar/2004	9		21	1 (4.8)
Subtotal	228	24 (10.5)	429	11 (2.6)
Oct/2004	12		12	
Nov/2004	6	1 (16.7)	51	1 (2)
Dec/2004	12		57	
Jan/2005	9		84	3 (3.6)
Feb/2005	15		48	7 (14.6)
Mar/2005	12		24	
Subtotal	66	1 (1.5)	276	11 (4)
Total	483	33 (6.8)	1,029	42 (4.1)
		75/1,512 (5.0)		

mately 10^4 viral genes) was used to test the adequacy of RNA extraction using the QIAamp Viral RNA Mini kit, and samples was finally suspended in 30 μl of DNase- or RNase-free water. In order to prevent carryover contamination by NV cDNA and to reduce the nonspecific amplicon, the RNA solution was treated with 2 U of RNase-free DNase I (TaKaRa, Tokyo) for 30 min at 37 C followed by inactivation of the enzyme at 75 C for 5 min. RT-PCR was performed in 15 μl of DNase I-treated RNA solution and 15 μl of the RT solution that contained 1 mM dNTP mixture, 10 mM dithiothreitol, 0.75 μg random hexamers (TaKaRa), 33 U RNase inhibitor (TaKaRa), 300 U reverse transcriptase (Superscript II, RNaseH(−); Invitrogen, San Diego, Calif., U.S.A.) and 4.5 μl Superscript II buffer. The RT mixture was incubated at 42 C for 75 min and subsequently at 99 C for 5 min. Five microliters of cDNA was added to 45 μl of the PCR mixture containing 5 μl of 10× Ex *Taq* buffer, 2.5 mM $MgCl_2$ and 20 μM of dATP, dGTP, dTTP and dCTP. The PCR was carried out using 2.5 U of TaKaRa Ex *Taq* (TaKaRa) with GI, antisense primer (G1-SKR): 5'-CCA ACC CAR CCA TTR TAC A-3' and sense primer (COG1F): 5'-CGY TGG ATG CGN TTY CAT GA-3'; GII, antisense primer (G2-SKR): 5'-CCR CCN GCA TRH CCR TTR TAC AT-3' and sense primer (COG2F): 5'-CAR GAR BCN ATG TTY AGR TGG ATG AG-3' (13). For the Alphatron-type strains, the antisense primer (G2AL-SKR) 5'-CCA CCA GCA TAT GAA TTG TAC AT-3' and the sense primer (ALPF) 5'-TTT GAG TCC ATG TAC AAG TGG ATG CG-3' were used.

The amplification was performed under the following conditions: initial denaturation at 94 C for 3 min, 40 cycles of denaturation at 94 C for 1 min, annealing at 50 C for 1 min, extension at 72 C for 2 min and final extension at 72 C for 15 min. Seminested PCR was performed under identical conditions by using the following primers: GI antisense primer: G1-SKR and sense primer (G1-SKF): 5'-CTG CCC GAA TTY GTA AAT GA-3'; GII antisense primer: G2-SKR and sense primer (G2-SKF): 5'-CNT GGG AGG GCG ATC GCA A-3', and Alphatron-type antisense primer: G2AL-SKR and sense primer: ALPF. PCR was performed for 35 cycles at 94 C for 3 min followed by a 15-min incubation at 72 C. Each amplicon was analyzed on 1.5% agarose gels. The products were visualized by 0.5 μg per ml of ethidium bromide staining. The amplicons were purified using the QIAquick PCR purification kit (Qiagen) and the nucleotide sequence was determined by an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, Calif., U.S.A.) by using the Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

We also quantified the NV capsid genes by real-time PCR as described previously (9). The real-time PCR mixture contained 5 μl of cDNA, 2 μl of the LightCycler master mix (Roche, Penzberg, Germany), 0.7 mM of each primer and fluorogenic probes (probes for GI, 2 pmol of RING1(a)-TP and 2 pmol of RING1(b)-TP; probe for GII, 4 pmol of RING2-TP). The fluorogenic probes used for real-time PCR were as follows: 5'-AGA TYG CGA TCY CCT GTC CA-3' (RING1(a)-TP) and 5'-AGA TCG CGG TCT CCT GTC CA-3' (RING1(b)-TP) and 5'-TGG GAG GGC GAT CGC AAT CT-3' (RING2-TP) (9). The amplification was performed by using a LightCycler (Roche). The following PCR protocol was employed: 10 min at 95 C followed by 50 cycles at 95 C for 10 sec, 60 C for 25 sec and 40 C for 30 sec. The data were corrected using internal standards as described previously (9).

Phylogenetic analysis. The capsid sequences were compared with those of the strains detected in gastroenteritis patients living near oyster farms and the reference strains from GenBank. The strains and accession numbers of these sequences have been provided in the legend for Fig. 1. Phylogenetic analysis was performed as described previously (12). In brief, all of the NV capsid region sequences (nt 244 to 313) were aligned using Clustal W (http://www.ddbj.nig.ac.jp/search/clustalw-j.html). A phylogenetic tree was constructed by the neighbor-joining (N-J) technique, Kimura's two-parameter method, by using the Tree Explorer software (ver. 2.12). The reliability of the tree was estimated using 1,000 bootstrap replications.

Statistical analysis. Statistical analysis was performed using Fisher's exact test. A P value of <0.05 was considered significant.

Results

Detection of NV Capsid Gene and Determination of Copy Number in Oysters

We detected the NV capsid gene from a total of 1,512 oysters that were for raw consumption and harvested from the Sanriku (483 oysters) and Setouchi Seas (1,029 oysters). Detailed data regarding these oysters have been listed in Table 1. In total, for 33 of the 483 oysters from area A (6.8%) and 42 of the 1,029 oysters from area B (4.1%), the NV capsid genome was detected during the entire investigation periods. During the 2003–2004 harvesting season, a higher number of NVs was detected in the oysters from area A than in those from area B ($P<0.05$). However, during the 2002–2003 and the 2004–2005 harvesting seasons, the detection of NVs in oysters from area B was higher than that from area A ($P<0.05$). In both areas, most of

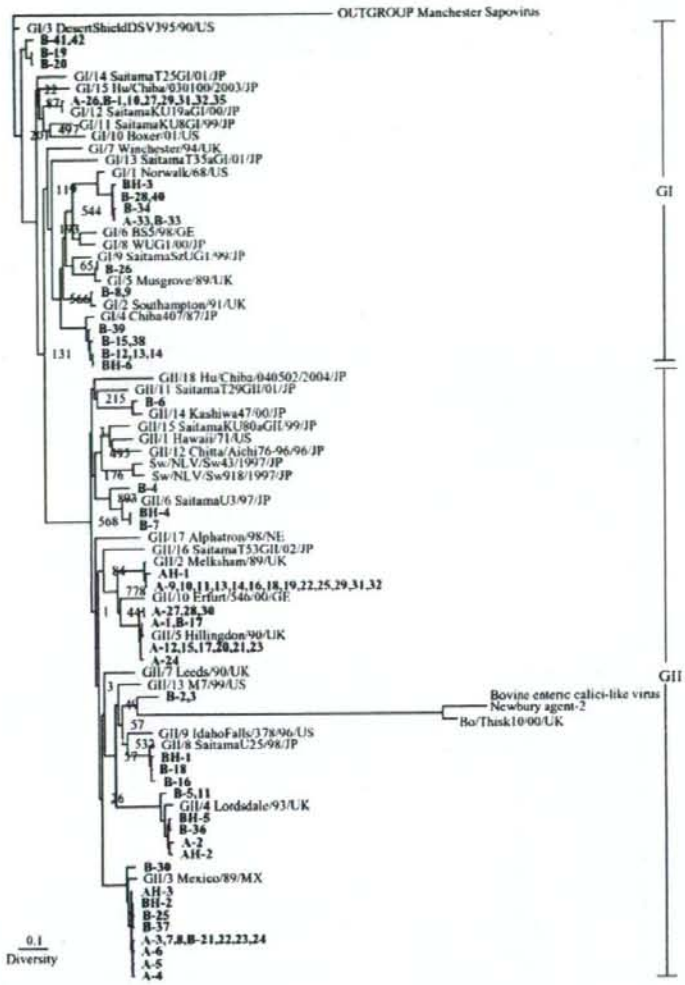

Fig. 1. Phylogenetic tree constructed on the basis of the partial sequences of the Norovirus (NV) capsid gene. The distance was calculated using Kimura's two-parameter method, and the tree was plotted using the neighbor-joining (N-J) method. The numbers at each branch indicate the bootstrap values for the clusters supported by that branch. As an outgroup, the sapovirus strain Manchester: X86560 was used. A and B indicate the harvest areas, and positive samples in this study are designated in bold face. AH and BH refer to human NV strains detected in gastroenteritis patients living near areas A and B. GI: genogroup I; GII: genogroup II. The GenBank accession numbers of the reference strains are as follows: GI/1 Norwalk/68/US (M87661), GI/2 Southampton/91/UK (L07418), GI/3 DesertShieldDSV395/90/US (U04469), GI/4 Chiba407/87/JP (AB042808), GI/5 Musgrove/89/UK (AJ277614), GI/6 BS5/98/GE (AF093797), GI/7 Winchester/94/UK (AJ277609), GI/8 WUG1/00/JP (AB081723), GI/9 SaitamaSzUG1/99/JP (AB039774), GI/10 Boxer/01/US (AF538679), GI/11 SaitamaKU8GI/99/JP (AB058547), GI/12 SaitamaKU19aGI/00/JP (AB058525), GI/13 SaitamaT35aGI/01/JP (AB112132), GI/14 SaitamaT25GI/01/JP (AB112100), GI/15 Hu/Chiba/030100/2003/JP (AJ865494), GII/1 Hawaii/71/US (U07611), GII/2 Melksham/89/UK (X81879), GII/3 Mexico/89/MX (U22498), GII/4 Lordsdale/93/UK (X86557), GII/5 Hillingdon/90/UK (AJ277607), GII/6 SaitamaU3/97/JP (AB039776), GII/7 Leeds/90/UK (AJ277608), GII/8 SaitamaU25/98/JP (AB067543), GII/9 Idaho Falls/378/96/US (AY054299), GII/10 Erfurt/546/00/GE (AF427118), GII/11 SaitamaT29GII/01/JP (AB112221), GII/12 Chitta/Aichi76-96/96/JP (AB032758), GII/13 M7/99/US (AY130761), GII/14 Kashiwa47/00/JP (AB078334), GII/15 SaitamaKU80aGII/99/JP (AB058582), GII/16 SaitamaT53GII/02/JP (AB112260), GII/17 Alphatron/98/NE (AF195847), GII/18 Hu/Chiba/040502/2004/JP (AJ865586), AH-1 (AB262166), AH-2 (AB262167), AH-3 (AB262168), BH-1 (AB262169), BH-2 (AB262170), BH-3 (AB262171), BH-4 (AB262172), BH-5 (AB262173), BH-6 (AB262174), Bovine enteric calici-like virus Newbury agent-2 (AF097917), Bo/Thisk10/00/UK (AY126468), and OUTGROUP Manchester Sapovirus (X86560).

Table 2. Genotypes and copy numbers of NVs in Japanese oysters

Month/year	Area	Amplicons[a]	Genogroup/genotype	NV genome copy number	GenBank accession No.
Nov/2002	A	A-1	II/5	ND[b]	AB262091
Dec/2002	A	A-2	II/4	ND	AB262092
	B	B-1	I/12	5.0×10^{2}	AB262093
	B	B-2	II/untypable	9.1×10^{2}	AB262094
	B	B-3	II/untypable	8.3×10^{3}	AB262095
	B	B-4	II/6	3.8×10^{2}	AB262096
	B	B-5	II/4	2.1×10^{2}	AB262097
	B	B-6	II/14	5.8×10^{2}	AB262098
Jan/2003	A	A-3	II/3	ND	AB262099
	A	A-4	II/3	ND	AB262100
	A	A-5	II/3	ND	AB262101
	A	A-6	II/3	ND	AB262102
	A	A-7	II/3	ND	AB262103
	A	A-8	II/3	ND	AB262104
	B	B-7	II/6	1.7×10^{3}	AB262105
	B	B-8	I/2	1.4×10^{2}	AB262106
	B	B-9	I/2	2.0×10^{2}	AB262107
	B	B-10	I/12	1.8×10^{3}	AB262108
	B	B-11	II/4	1.8×10^{3}	AB262109
	B	B-12	I/4	4.1×10^{2}	AB262110
	B	B-13	I/4	ND	AB262111
	B	B-14	I/4	ND	AB262112
	B	B-15	I/4	ND	AB262113
	B	B-16	II/8	ND	AB262114
	B	B-17	II/5	ND	AB262115
	B	B-18	II/8	ND	AB262116
Feb/2003	B	B-19	I/3	4.8×10^{2}	AB262117
	B	B-20	I/3	ND	AB262118
Dec/2003	A	A-9	II/2	ND	AB262119
	A	A-10	II/2	ND	AB262120
	A	A-11	II/2	ND	AB262121
	A	A-12	II/5	ND	AB262122
	A	A-13	II/2	ND	AB262123
	A	A-14	II/2	ND	AB262124
	A	A-15	II/5	ND	AB262125
	A	A-16	II/2	ND	AB262126
	A	A-17	II/5	ND	AB262127
	A	A-18	II/2	ND	AB262128
	A	A-19	II/2	ND	AB262129
	A	A-20	II/5	ND	AB262130
	A	A-21	II/5	ND	AB262131
	A	A-22	II/2	ND	AB262132
	A	A-23	II/5	ND	AB262133
	A	A-24	II/5	ND	AB262134
	A	A-25	II/2	ND	AB262135
	B	B-21	II/3	1.4×10^{3}	AB262136
	B	B-22	II/3	4.0×10^{2}	AB262137
	B	B-23	II/3	9.6×10^{2}	AB262138
	B	B-24	II/3	1.0×10^{3}	AB262139
	B	B-25	II/3	9.9×10^{2}	AB262140
	B	B-26	I/5	ND	AB262141
	B	B-27	I/12	ND	AB262142
Jan/2004	A	A-26	I/12	ND	AB262143
	A	A-27	II/5	ND	AB262144
	A	A-28	II/5	ND	AB262145
	A	A-29	II/2	ND	AB262146
	A	A-30	II/5	ND	AB262147
	A	A-31	II/2	ND	AB262148
	A	A-32	II/2	ND	AB262149
	B	B-28	I/1	ND	AB262150
Feb/2004	B	B-29	I/12	ND	AB262151
	B	B-30	II/3	ND	AB262152
Mar/2004	B	B-31	I/12	ND	AB262153
Nov/2004	A	A-33	I/1	ND	AB262154
	B	B-32	I/12	ND	AB262155
Jan/2005	B	B-33	I/1	ND	AB262156
	B	B-34	I/1	ND	AB262157
	B	B-35	I/12	ND	AB262158
Feb/2005	B	B-36	II/4	9.9×10^{2}	AB262159
	B	B-37	II/3	4.1×10^{2}	AB262160
	B	B-38	I/4	3.1×10^{2}	AB262161
	B	B-39	I/4	ND	AB262162
	B	B-40	I/1	ND	AB262163
	B	B-41	I/3	ND	AB262164
	B	B-42	I/3	ND	AB262165

[a] Amplicon; A and B refer to the areas of harvest.
[b] ND; not detected (<100 copies/oyster).

the NVs were detected during the December to January investigation periods. We also quantitated the copy number of the NV capsid gene in all oysters by real-time PCR (Table 2). The sensitivity (limit of detection) for this method is more than 100 copies of the NV genomes/oyster (20). Interestingly, the number of oysters from area A, which harbored the NVs genome, was significantly greater than that from area B. Oysters harvested from area B had a relatively large number of capsid genomes with more than 100 copies/oyster, while those harvested from area A had less than 100 copies of the NV genome (Table 2).

Phylogenetic Analysis of NVs from Oysters

We sequenced all the amplicons of the NV capsid genes in oysters by using the direct sequencing method. In the direct sequencing method, if the shellfishes have accumulated several kinds of viruses, there are cases where the peaks of the nucleotide sequences in the chromatogram overlap and determination of the sequence is impossible. In this study, the main fluorescence peak of each nucleotide sequence was observed without overlapping in all the sequence data (data not shown). The capsid gene amplicon was considered to have been amplified from homogeneous NVs in the samples. Based on these sequences, we performed a phylogenetic analysis of the NV capsid gene by using the N-J method (Fig. 1). The genetic distance between the clusters of GI and GII in the phylogenetic tree was observed to be 0.35 and 0.27, respectively. Of 75 amplicons, 26 were classified as GI and the remaining 49 as GII. The GI amplicons were further classified into 6 genotypes and those of GII into 8 genotypes (Fig. 1). In GI, there were 8, 6, and 5 amplicons in the GI/12 (SaitamaKU19a-type), GI/4 (Chiba407-type) and GI/1 (Norwalk-type), respectively. In GII, there were 13 amplicons each in the GII/3 (Mexico-type) and GII/2 (Melksham-type), and 12 amplicons in the GII/5 (Hillingdon-type). We also characterized the geographical and seasonal features of the genotypes. Throughout the investigation period, 12 amplicons in the oysters from area A were classified under GII/2 (Melksham-type). In area B, 6 amplicons were classified under GI/4 (Chiba407-type). In this study, many NV genotypes were detected in oysters, but only 5 genotypes, GI/1, GI/12, GII/3, GII/4 (Lordsdale-type) and GII/5 were common to both areas.

Very recently, all the GI and GII genogroups were classified into 15 and 18 genotypes, respectively (21). This classification method was based on the range of genetic distance (10). However, our 2 amplicons, i.e., B-2 and B-3, were not included in any genotype because the genetic distances exceeded the range for a single cluster, suggesting that these strains may be new NV genotypes (Fig. 1). In addition, the sequences of some amplicons matched each other completely, although most of these were detected in different seasons and different areas. The sequences of some amplicons detected in oysters and humans showed high similarity. Moreover, the sequences from oysters (cf. B-6 in Fig. 1) and animals (cf. Sw/NLV/Sw43/1997/JP and Sw/NLV/Sw918/1997/JP in Fig. 1) were closely related, indicating that the NV capsid genes in oysters exhibit wide genetic diversity but some of them were genetically related to swine NVs (24).

Discussion

In this study, we demonstrated that the NV capsid gene was detected in approximately 5% of the Japanese oysters for raw consumption. Of the oysters from area B, 50% carried relatively large amounts of the NV genomes which were not observed in oysters from area A. In addition, the capsid gene exhibited wide genetic diversity in both genogroups, GI and GII. The nucleotide sequences of the capsid gene of some strains from both areas matched completely, suggesting that some strains of oysters were genetically related to a considerable extent although the oysters were harvested far apart.

Recent studies in some countries showed that approximately 0% to 20% of oysters contained NVs and these viruses are associated with gastroenteritis in humans (16, 17, 19). For example, Le Guyader et al. showed that the polymerase gene of NVs was detected in approximately 20% of French oysters and showed wide genetic diversity (16). Myrmel et al. demonstrated approximately 7% of the shellfishes from the Norwegian coast contained the NV gene (19). In addition, we demonstrated that 10% of Japanese oysters contain the NV capsid genomes and show wide genetic diversity, although these oysters were harvested in areas different from those used in this study (20). On the other hand, Lodder-Verschoor et al. demonstrated that NV genes were not detected in Dutch oysters although the enterovirus genes were detected in these oysters, suggesting that NV does not always contaminate oysters (17). The genetic characteristics of NVs contaminating shellfishes are poorly understood. Therefore, we conducted a molecular typing study on NVs in oysters obtained from two distinct sea areas in Japan, in order to make an accurate comparison of the prevalence patterns of NVs in Japanese oysters. The present data indicates that the NVs detected in Japanese oysters from different sea areas showed wide genetic diversity. This is consistent with the results of earlier reports (16, 17, 19).

Recent data suggested that the infectivity of NVs is relatively strong and less than 100 particles of NVs can easily cause gastroenteritis in humans (4). Our previous data showed that approximately 5% of the oysters for raw consumption from two distinct areas in the Setouchi Sea contained more than 100 copies of the NV genomes in each oyster (20). In contrast, we found that only 1.4% of the oysters had more than 100 copies of NVs in this study. In addition, the oysters from area A had less than 100 copies of NVs. During our investigation periods, 34,000–37,000 tons of oysters were harvested per year (http://www.maff.go.jp/www/info/bun08.html). More than 70% of the oysters were harvested from these two areas. We detected NVs in approximately 5% of the oysters for raw consumption; however, this rate did not directly reflect the possibilities of NV infection. In order to prevent food poisoning caused by microorganisms present in raw oysters, the Japanese government regulates the oyster industry via the Enforcement Regulation of the Food Sanitation Law. However, this regulation mainly focuses on bacteriology. To our knowledge, there is no universal risk assessment of viruses, such as NVs or the hepatitis A virus, which are contained in shellfishes. Ensuring the virological safety of oysters would have an enormous effect. If we can control outbreaks of oyster-related gastroenteritis, the number of patients with gastroenteritis may be reduced. Accurate risk assessment of raw oysters with regard to NVs infections and regulation based on viral sanitation is needed.

It is suggested that various genotypes of NVs in oysters are associated with the outbreaks of gastroenteritis in humans (25). Therefore, it is important to clarify the genetic characteristics of NVs detected from both shellfishes and humans, to gain a better understanding of the epidemiology of NV infection (10). Our data indicated that most GI and GII strains detected in oysters and humans were genetically related to a considerable extent (Fig. 1).

Interestingly, some NVs that are usually detected in swine are also genetically related to the NVs in oysters. Therefore, we could not exclude the possibility that some NV strains detected in oysters were derived from other animals such as swine. In conclusion, further molecular studies of NVs and quantitative real-time PCR methods are needed for a better understanding of the NV infection, which may provide more accurate assessment of the risk factors for shellfish-associated diseases.

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Detection and Phylogenetic Analysis of Norovirus in *Corbicula fluminea* in a Freshwater River in Japan

Mika Saitoh[1,2], Hirokazu Kimura[1], Kunihisa Kozawa[1], Osamu Nishio[3], and Akira Shoji[*,2]

[1]Gunma Prefectural Institute of Public Health and Environmental Sciences, Maebashi, Gunma 371–0052, Japan, [2]Department of Biological Sciences, Faculty of Engineering, Gunma University, Kiryu, Gunma 376–8515, Japan, and [3]National Institute of Infectious Diseases, Musashimurayama, Tokyo 208–0011, Japan

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***Abstract*: To study the molecular epidemiology of noroviruses (NoVs) in bivalves residing in freshwater rivers, we detected, quantified and phylogenetically analyzed the NoV genome in purified concentrates obtained from the gills and digestive diverticula of *Corbicula fluminea* in a freshwater river in Gunma Prefecture, Japan. We detected the NoV genome in 35 of the 58 *C. fluminea* samples. Based on our phylogenetic analysis, the NoV genome detected in the samples was classified into 4 genotypes (GI/1, GI/2, GI/3 and GI/4) in genogroup I and 5 genotypes (GII/3, GII/4, GII/5, GII/8 and GII/12) in genogroup II. The phylogenetic tree showed wide genetic diversity among the genogroups. In addition, more than 10^4 copies of the NoV genome were detected in 2 of 35 samples. These results suggest that the freshwater bivalve *C. fluminea* is a reservoir for NoVs, similar to seawater bivalves such as oysters.**

***Key words*: Phylogenetic analysis, Norovirus, *Corbicula fluminea*, Freshwater**

Norovirus (NoV) belongs to the family *Caliciviridae* and is a causative agent of acute gastroenteritis in humans. NoV has been the main cause of human food-borne and water-borne non-bacterial acute gastroenteritis (3, 13, 20). In Japan, approximately 45% (12,537 of 28,175 patients) of the total gastroenteritis and food-borne cases in 2004 were attributed to NoV. River water, sewage and seawater have been polluted with the excreta of infected patients, and NoV gastroenteritis outbreaks have been associated with the consumption of bivalves, including oysters (22). Furthermore, viral particles can accumulate in various types of freshwater or seawater bivalves (21, 28). Cooking with inadequate heating or the consumption of raw bivalves has resulted in several instances of NoV food-borne and infectious gastroenteritis in Japan, other Asian countries and France (7, 9, 24). Additionally, person-to-person transmission of NoV occurs more easily than any other mode of transmission. This is because infected patients shed a large amount of NoVs in their feces and/or vomit, while a small NoV load can cause gastroenteritis in humans (as few as 10–100 viral particles can cause infection) (3, 19).

The NoV is classified into two genogroups: genogroup I (GI) and genogroup II (GII). Each group is further subdivided into 14 (GI/1–14) and 17 (GII/1–17) genotypes, respectively (12, 14). Recently, numerous outbreaks due to NoV GII/4 were reported worldwide including in European countries, Japan and the U.S. Data from these outbreaks suggest that the NoV GII/4 detected in patients is related to the NoV GII/4 found in seawater bivalves (7, 25, 27).

In Japan, there are three primary domestic *Corbicula* species: *Corbicula japonica* (*C. japonica*), which is widely distributed in brackish waters, *Corbicula sandai*, which is found in Lake Biwa, and *Corbicula leana*, which lives in freshwater. *C. japonica* has been primarily caught for food. In recent years, however, the fishing of *C. japonica* has been decreasing in Japan, and therefore various corbicula clams are now being imported from foreign countries (10). *Corbicula fluminea* (*C. fluminea*: taiwan shijimi) is variable in its shape, and

*Address correspondence to Dr. Akira Shoji, Department of Biological Sciences, Faculty of Engineering, Gunma University, 1–5–1 Tenjincho, Kiryu, Gunma 376–8515, Japan. Fax: +81–277–30–1443. E-mail: shoji@bce.gunma-u.ac.jp

Abbreviations: GI, genogroup I; GII, genogroup II; N-J, neighbor-joining; NoV, norovirus; OTU, operational taxonomic unit; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR.

naturally inhabits mostly freshwater in southeastern Asia, China, Korea and southeastern Russia. *C. fluminea* has been imported into Japan as food, and is now widely distributed in Japanese rivers (Ministry of the Environment, Invasive Alien Species Act (http://www.env.go.jp/index.html); Gulf States Marine Fisheries Commission, *Corbiculla fluminea* (Muller 1977) (http://www.gsmfc.org/)). These corbicula clams might become a source of NoV infection, as seawater bivalves are. In addition, phylogenetic analysis data of the NoV isolated from corbicula clams might be useful to investigate the prevalence of NoV. To examine these issues from a molecular epidemiological approach, we collected *C. fluminea* from river water in Gunma Prefecture in Japan every month in 2004, detected the concentrated NoV, and then analyzed the NoV genomes phylogenetically.

Materials and Methods

Sampling site. The sampling site was a tributary of the Kanna River flowing through Fujioka City in Gunma Prefecture in Japan (Fig. 1). The river stream, including our sampling site, flows along the residential areas of Fujioka City, whose population is approximately 71,000. The river is approximately 2 m wide, and sewage water that is conventionally treated in septic tanks in the houses is discharged into the river.

Samples and preparation of viral suspension. A total of 406 corbicula clams (*C. fluminea*) were collected monthly from January to December 2004, and included 35 clams every month except January, when 21 clams were collected. Since each sample consists of 7 clams, the number of samples was 5 every month except 3 in January. On the day of collection, the clams were shucked and their gills and digestive diverticula were dissected. Subsequently, 1–1.3 g of gills and digestive diverticula (giving a total weight corresponding to that of 7 clams) were homogenized and 10% suspension was prepared with phosphate-buffered saline (PBS) solution without magnesium or calcium. The homogenate was mixed with 0.1 ml of antifoam B (Sigma, St. Louis, Mo., U.S.A.) and homogenized for two 30-sec intervals, then centrifuged with a maximum speed of 18,000 rpm using an Omni-mixer (OCI Instruments, Waterbury, Conn., U.S.A.). Six milliliters of a chloroform:butanol (1:1, vol vol^{-1}) mixture were added to the supernatant of the homogenate (8, 24). The mixture was then homogenized for an additional 30 sec, and 170 ml of Cat-Floc T (Calgon, Elwood, Pa., U.S.A.) was added to the homogenate. The homogenate samples were centrifuged at 3,000 $\times g$ for 30 min at 4 C, and 7 ml of the supernatant was layered on 2 ml of 30% sucrose solution, and ultracentrifuged at 201,000 $\times g$ for 2 hr at 4 C. The pellet was resuspended in 200 µl of DNase/RNase-free water and then stored at −80 C until use.

RNA extraction, RT-PCR and sequencing. Viral RNA was extracted from 138 µl of viral suspension using a QIAamp Viral RNA Mini Kit (Qiagen, Germantown, Md., U.S.A.), and 2 µl of solution containing poliovirus type II (Sabin strain, corresponding to approximately

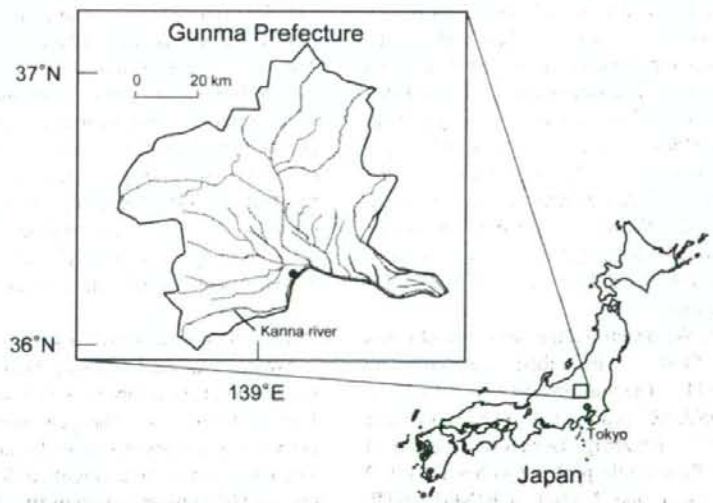

Fig. 1. Location of the sampling site in Gunma Prefecture, Japan. The dotted lines indicate the main river streams. The closed circle indicates the sampling site.

10^4 viral genes) was used as a control RNA extraction. The RNA was suspended in 60 μl of DNase/RNase-free water and then treated with 2 units of DNase I (TaKaRa, Tokyo). After reverse transcription, we used genogroup-specific primers (24) to amplify the partial capsid region of NoVs by reverse transcription-polymerase chain reaction (RT-PCR). The RT reaction was performed in 15 μl of DNase I-treated RNA solution and 15 μl of RT solution containing 1 mM of dNTP mixture, 10 mM of dithiothreitol, 0.05 g/liter of random hexamer (TaKaRa), 33 units of RNase inhibitor (TaKaRa), 300 units of reverse transcriptase (Superscript II, RNaseH (−); Invitrogen, San Diego, Calif., U.S.A.), and 4.5 μl of Superscript II buffer. The RT mixture was incubated at 42 C for 75 min and then at 99 C for 5 min. The following primers were used for the first PCR: 5′-CGY TGG ATG CGN TTY CAT GA-3′ (COG1F, sense), 5′-CCA ACC CAR CCA TTR TAC A-3′ (G1-SKR, antisense), 5′-CAR GAR BCN ATG TTY AGR TGG ATG AG-3′ (COG2F, sense), and 5′-CCR CCN GCA TRH CCR TTR TAC AT-3′ (G2-SKR, antisense). The following primers were used for the nested PCR: 5′-CTG CCC GAA TTY GTA AAT GA-3′ (G1-SKF, sense), 5′-CCA ACC CAR CCA TTR TAC A-3′ (G1-SKR, antisense), 5′-CNT GGG AGG GCG ATC GCA A-3′ (G2-SKF, sense), and 5′-CCR CCN GCA TRH CCR TTR TAC AT-3′ (G2-SKR, antisense) (16). We amplified the poliovirus type II VP1 gene using the following specific primers: 5′-AGC AAG CAC CGT ATT GAG CC-3′ (sense) and 5′-GTT TCA TGT CTG CTC CGT CTG-3′ (antisense) (24). The PCR protocol included incubation for 3 min at 94 C. This was followed by 40 cycles under each of the following sets of conditions: 94 C for 60 sec, at 50 C for 60 sec, and at 72 C for 2 min. Elongation was performed for 15 min at 72 C after the last cycle. This PCR procedure was repeated for the nested PCR using the inner primers (16). The size of the amplified DNA fragment was confirmed by 1.5% agarose gel electrophoresis. The DNA fragments was purified with a QIAquick PCR Purification Kit (Qiagen), and the nucleotide sequence was determined with an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, Calif., U.S.A.) using a Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Real-time PCR. We quantified the NoV capsid genes using real-time PCR, as described previously by Kageyama et al. (11). The real-time PCR mixture contained 5 μl of cDNA (RT product), 2 μl of LightCycler Master Mix (Roche, Penzberg, Germany), 0.7 μM of each primer, and fluorogenic probes (probes for GI, 2 pmol of RING1(a)-TP and 2 pmol of RING1(b)-TP; probe for GII, 4 pmol of RING2-TP). The fluorogenic probes for real-time PCR were as follows: 5′-AGA TYG CGA TCY CCT GTC CA-3′ (RING1-TP(a)), 5′-AGA TCG CGG TCT CCT GTC CA-3′ (RING1-TP(b)), and 5′-TGG GAG GGC GAT CGC AAT CT-3′ (RING2-TP) (11). PCR amplification was performed with a LightCycler (Roche). The following PCR protocol was employed: 10 min at 95 C followed by 50 cycles under each of the following sets of conditions: 95 C for 10 sec, at 60 C for 25 sec, and at 40 C for 30 sec. Data were corrected using the NoV GI or GII cDNA plasmid standards described by Kageyama et al. (11).

Phylogenetic analysis. The capsid sequences of the reference strains of the NoVs were obtained from GenBank. The strains and accession numbers of these sequences are provided in the legend of Fig. 2. Phylogenetic analysis was performed as previously described by Katayama et al. (14). In brief, all of the NoV capsid region sequences (291 to 302 nt) were aligned using Clustal W (http://www.ddbj.nig.ac.jp/search/clustalw-j.html). A phylogenetic tree was constructed using the neighbor-joining (N-J) technique (26), and Kimura's two-parameter method (15) using the TreeExplorer (ver. 2.12) software (http://evolgen.biol.metro-u.ac.jp/). The reliability of the tree was estimated using 1,000 bootstrap replications.

Results

Detection and Quantification of NoV in C. fluminea

The NoV capsid genes detected by RT-PCR are shown in Table 1. NoV genomes were detected in 35 of the 58 samples (60%). Of these 35 samples, 6 (17%) samples contained GI amplicons alone and 15 (43%) samples contained GII amplicons alone. The other 14 (40%) samples contained both GI and GII amplicons. The detection rate was 40% to 100% every month except September, when all of the collected samples were negative. Predominance of any specific NoV genogroups detected in each tested month was not observed. We also quantified the number of copies of the NoV capsid gene using real-time PCR. In the 2 samples collected in July and December, 2.9×10^4 and 3.0×10^4 copies, respectively, of the GII genome were detected, while no GI genome was detected.

Phylogenetic Analysis of NoVs Detected in C. fluminea

We constructed the phylogenetic tree on the basis of the nucleotide sequence of the capsid genes using the N-J method (Fig. 2). The genogroup and genotype of norovirus genomes in corbiculas are shown in Table 1. The phylogenetic tree classified 20 and 29 strains into GI and GII clusters, respectively. In the present study,

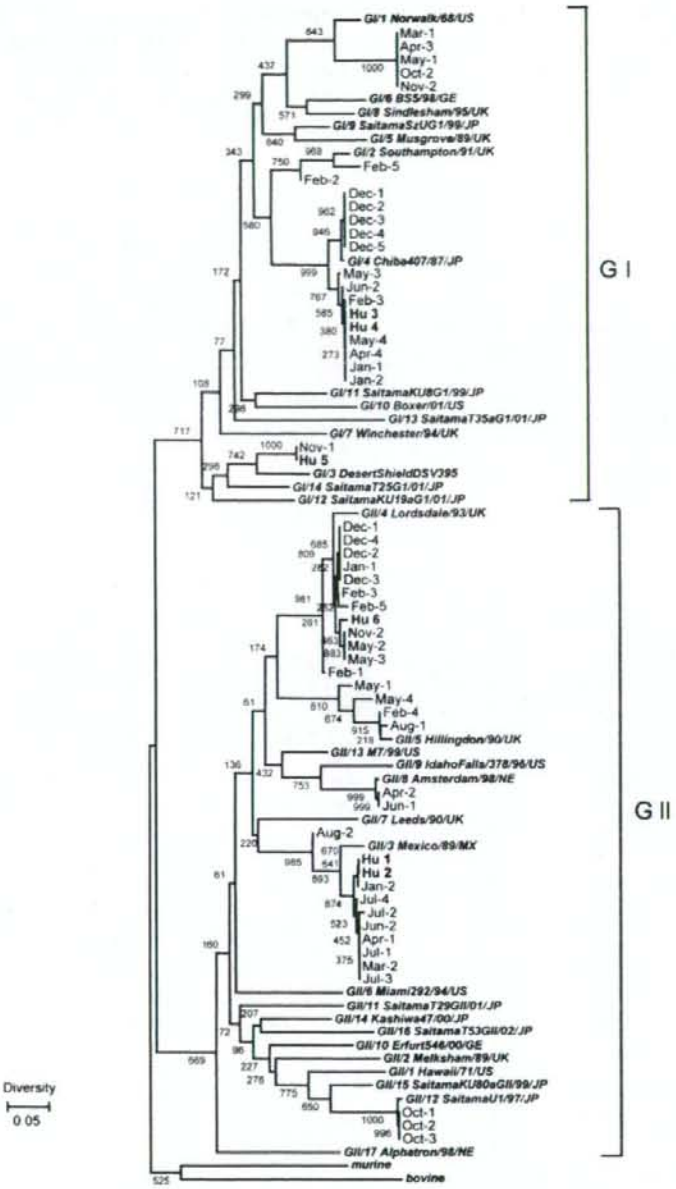

Fig. 2. Phylogenetic tree constructed based on the partial sequences of the norovirus (NoV) capsid gene. The distance was calculated using Kimura's two-parameter method, and the tree was plotted using the neighbor-joining (N-J) method. The numbers at each branch indicate the bootstrap value for the clusters supported by that branch. The accession numbers of our strains are also indicated in Table 1. "Hu1-6" refers to NoVs isolated from children with gastroenteritis who lived near the sampling site and are designated in boldface type. GI: genogroup I; GII: genogroup II. The following are the strain and GenBank accession numbers: Norwalk/68/US, M87661; BS5/98/GE, AF093797; Sindlesham/95/UK, AJ277615; SaitamaSzUG1/99/JP, AB039774; Musgrove/89/UK, AJ277614; Chiba407/87/JP, AB042808; Southampton/91/UK, L07418; Winchester/94/UK, AJ277609; SaitamaT35aGI/01/JP, AB112132; Boxer/01/U, AF538679; SaitamaKU8GI/99/J, AB058547; SaitamaK19aGI/01/J, AB058525; DesertShieldDSV395, U04469; SaitamaT25GI/01/JP, AB112100; Lordsdale/93/UK, X86557; Miami/292/94/US, AF414410; Alpha-

the GI strains were subdivided into 4 clusters, and the GII strains were subdivided into 5 clusters. The maximum genetic diversity (evolutionary distances calculated on the basis of substitutions per site of pairwise strains) within the GI and GII clusters was 0.36 and 0.38, respectively, while that between the GI and GII strains represented as an operational taxonomic unit (OTU) (26) was 0.47; the intercluster OTUs including the GI and GII strains from *C. fluminea* ranged from 0.005 to 0.112. Among the GI strains, 5 and 12 strains shared a

Table 1. Genogroup and genotype of norovirus genomes in corbiculas

Month	Samples	Genogroup(s)	Genotype(s)	GenBank accession nos.	
				GI	GII
January	Jan-1	GI + GII	GI/4, GII/4	AB246115	AB246135
	Jan-2	GI + GII	GI/4, GII/3	AB246116	AB246136
February	Feb-1	GII	GII/4	—	AB246137
	Feb-2	GI	GI/2	AB246117	—
	Feb-3	GI + GII	GI/4, GII/4	AB246118	AB246138
	Feb-4	GII	GII/5	—	AB246139
	Feb-5	GI + GII	GI/2, GII/4	AB246119	AB246140
March	Mar-1	GI	GI/1	AB246120	—
	Mar-2	GII	GII/3	—	AB246141
April	Apr-1	GII	GII/3	—	AB246142
	Apr-2	GII	GII/8	—	AB246143
	Apr-3	GI	GI/1	AB246121	—
	Apr-4	GI	GI/4	AB246122	—
May	May-1	GI + GII	GI/1, GII/5	AB246123	AB246144
	May-2	GII	GII/4	—	AB246145
	May-3	GI + GII	GI/4, GII/4	AB246124	AB246146
	May-4	GI + GII	GI/4, GII/5	AB246125	AB246147
June	Jun-1	GII	GII/8	—	AB246148
	Jun-2	GI + GII	GI/4, GII/3	AB246126	AB246149
July	Jul-1	GII	GII/3	—	AB246150
	Jul-2	GII	GII/3	—	AB246151
	Jul-3	GII	GII/3	—	AB246152
	Jul-4 [a]	GII	GII/3	—	AB246153
August	Aug-1	GII	GII/5	—	AB246154
	Aug-2	GII	GII/3	—	AB246155
October	Oct-1	GII	GII/12	—	AB246156
	Oct-2	GI + GII	GI/1, GII/12	AB246127	AB246157
	Oct-3	GII	GII/12	—	AB246158
November	Nov-1	GI	GI/3	AB246128	—
	Nov-2	GI + GII	GI/1, GII/4	AB246129	AB246159
December	Dec-1	GI + GII	GI/4, GII/4	AB246130	AB246160
	Dec-2 [a]	GI + GII	GI/4, GII/4	AB246131	AB246161
	Dec-3	GI + GII	GI/4, GII/4	AB246132	AB246162
	Dec-4	GI + GII	GI/4, GII/4	AB246133	AB246163
	Dec-5	GI	GI/4	AB246134	—
Total	35	49			

Samples collected in September were negative.

[a] The genome in the 2 samples were quantified: Jul-4, 2.9×10^4 copies (GII) and Dec-2, 3.0×10^4 copies (GII). Units are copy numbers of NoV genome /g of the gills and digestive diverticula.

tron/98/NE, AF195847; Mexico/89/MX, U22498; Leeds/90/UK, AJ277608; Amsterdam/98/NE, AF195848; Idaho-Falls/378/96/US, AY054299; M7/99/US, AY130761; Kashiwa47/00/JP, AB078334; SaitamaT53GII/02/JP, AB112260; SaitamaT29GII/01/JP, AB112221; Hawaii/71/US, U07611; SaitamazU1/97/JP, AB039775; SaitamaKU80aGII/99/JP, AB058582; Melksham/89/UK, X81879; Erfurt/546/00/GE, AF427118; Hillingdon/90/UK, AJ277607; HU1, AB246164; HU2, AB246165; HU3, AB246166; HU4, AB246167; HU5, AB246168; HU6, AB246169; Murine norovirus 1, AY228235; Bovine enteric calicivirus, AJ011099.

close genetic relationship with GI/1 and GI/4, respectively. In addition, two GI/2 and one GI/3 strains were found. The GII strains from *C. fluminea* were classified into the following 5 clusters: GII/3–5, GII/8 and GII/12. Most (20 strains) of the GII strains belonged to either GII/3 or GII/4.

During the investigation period, we observed that NoVs from children with gastroenteritis who lived near the sampling site (within approximately 5 km) were genetically related to the strains isolated from *C. fluminea* (Fig. 2). Thus, these results suggest that the NoVs isolated from *C. fluminea* showed a wide diversity in their capsid genes, and that human strains were genetically related to the strains detected in *C. fluminea*.

Discussion

NoVs have been detected in seawater bivalves such as oysters and clams in France (7), the United States (2, 8), the United Kingdom (4, 17), Spain, Sweden (5), and Japan (24). These viruses have been associated with human gastroenteritis. NoV was detected in approximately 5%–20% bivalves, including oysters and clams. We found that approximately 60% of the corbicula clams collected from the river in Gunma Prefecture in 2004 had the NoV genome, and more than 10^4 copies of the NoV genome were detected in approximately 5% of the clams. We previously reported that NoVs were detected in approximately 10% of Japanese oysters, and that 5% of the oysters showed relatively high concentrations of the NoV genome (24). Thus, it is suggested that the prevalence of NoV in the corbicula clams in rivers in Japan is higher than that in seawater bivalves.

It is well known that NoV detected in infected patients and bivalves can be classified into 2 major genogroups (GI and GII), and that each genogroup can be further subdivided into many clusters. The genetic diversity ranges from 0.12 to 0.36 among GI strains, from 0.12 to 0.46 among GII strains and from 0.46 to 0.49 between GI-GII strains (12, 14). The phylogenetic tree showed that the NoV in clams can be classified into 4 GI clusters and 5 GII clusters. Genetic diversity among the GI strains, among the GII strains, and between the GI-GII strains was 0.36, 0.38 and 0.47, respectively (Fig. 2). Furthermore, the rate of nucleotide substitutions in each intercluster of GI and GII was in the range of 0.50 and 11.2%. This suggests a wide genetic diversity of NoV in the corbicula clams in rivers, similar to that in oysters and clams (1, 16, 30).

The NoV capsid genes from 6 stools of gastroenteritis patients who lived near the sampling site were genetically related to the strains detected in corbicula clams, even though the NoV capsid genes were only partially sequenced. Previous studies have shown that the NoV genogroups detected in oysters and humans are slightly different. For example, although both GI and GII have been detected in oysters, only GII has been detected in humans (6, 24). A recent study demonstrated the existence of GII/4 in oysters during several outbreaks of NoV infection in various countries, including the Netherlands, the United States and Japan (7, 25, 27). In this study, we detected many NoV genotypes from the corbicula clams in the river. They were similar to those detected in other seawater bivalves. Especially, it is remarkable that 4 genotypes including GII/4 were also detected in patients who lived near the sampling site. Thus, it is suggested that the corbicula clams collected from the river in Gunma Prefecture were highly contaminated with genetically diverse NoV strains. The possibility of the existence of 2 or more NoVs of the same genogroup in a single specimen cannot be completely excluded. A cloning might be required for a detailed analysis of the NoV genotype in bivalves.

Several viruses such as NoVs or hepatitis virus type A might be present in the stools of gastroenteritis or hepatitis patients. Seawater, sewage and river water may have been polluted with these viruses (18, 21, 29). Because various types of bivalves are capable of indiscriminately accumulating viruses and may concentrate them (2, 5, 7), it is possible that these bivalves cause the main infection. Asian clams including *C. fluminea* are marketed as fresh food imports into Japan for human consumption. In Japan, various corbicula clams are almost always consumed after heating and boiling. However, the boiling required to open the bivalves is not necessarily sufficient to inactivate viruses (23).

The present study suggests that *C. fluminea* in the river might be a source of NoV infection, as are other seawater bivalves such as oysters and clams, and that this finding identifies an important etiological factor of gastroenteritis.

The prevalence of NoV in various bivalves has not yet been elucidated. To the best of our knowledge, there are no reports regarding the detection, quantification or phylogenetic analysis of NoVs in clams. Thus, molecular epidemiologic studies on NoVs in bivalves, including corbicula clams in rivers, and in humans are necessary to provide important data for a more detailed assessment of NoV infections and their regional prevalence, and the risk factors of NoV, as well as to provide an estimate of NoV pollution in rivers.

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