Epidemiological studies on Norovirus in oysters
(カキにおけるノロウイルスの疫学的研究)

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CONTENTS

I. General Introduction

1. Overview of food-borne epidemic gastroenteritis

Epidemic infectious gastroenteritis is caused by mainly bacteria and viruses, which are included in the outbreaks of food-borne poisoning or illness. The outbreaks and sporadic cases of bacterial gastroenteritis of person-to-person and food-borne infection were reported and summarized; the occurrence of these cases intensive in warm season (Honda *et al.*, 2002). Food-borne cases are mainly caused by *Campylobacter jejuni/coli*, *Salmonella* spp., *Vibrio parahaemoliticus*, *Staphylococcus aureus*, *Bacillus cereus*, and pathogenic *Escherichia coli* in Japan (http://idsc.nih.go.jp/ iasr/prompt/s2graph-lj.html). Among these pathogens, *C. jejuni/coli*, *Salmonella* spp., *V. parahaemoliticus*, and *S. aureus* rank prominently of the numbers of outbreaks and patients in recent years (http://www.mhlw.go.jp/topics/syokuchu, Nishio *et al.*, 2005). On the other hand, many kinds of viruses have been found in the stools of persons with viral epidemic gastroenteritis, in which rotaviruses, noroviruses (NVs), sapoviruses, astroviruses and group F adenoviruses are demonstrated to be a casual agent of human gastroenteritis worldwide (Iritani *et al.*, 2003; Fankhauser *et al.*, 2002.; Fretz *et al.*, 2005; Ike *et al.*, 2006; Schnagl *et al.*, 2000).

Among these viruses, NVs are one of the major cause of non-bacterial epidemic gastroenteritis, a disease that usually occurs in family or community-wide outbreaks (Glass *et al.*, 2000; Kaplan *et al.*, 1982). As seen in NVs, Rotaviruses, a member of the family *Reoviridae*, are classified into seven groups of A to G, of which three groups are

known to infect humans (Saif, 1990) and group A rotaviruses are the leading cause of infantile diarrhea worldwide (Kapikian *et al.*, 2001) and more than 6,000,000 deaths annually associated mainly in developing countries (Gentsch *et al.*, 1996). In Japan, rotaviruses were also detected in many cases of gastroenteritis (http://idsc.nih.go.jp/iasr/prompt/graph-kj.html). These viruses are representative pathogens of infantile diarrhea as NVs. They were called traditionally "winter vomiting desease". Pang *et al.* (2000) demonstrated the prevalence of rotaviruses and NVs in winter and their great severity among young children. Other candidates of diarrheal agents have been reported to be coronaviruses (Caul *et al.*, 1975; Doyle *et al.*, 1946), picornaviruses and picotrirnaviruses (Chandra, 1997; Grohman *et al.*, 1993; Ludert *et al.*, 1993), pestiviruses (Baker, 1987; Yolken *et al.*, 1989), and toroviruses (Beards *et al.*, 1984; Beards *et al.*, 1986; Weiss *et al.*, 1983), because they are associated with diarrheal illness in animals and have been found in the stools of humans with gastroenteritis. Non-group F adenoviruses and several enteroviruses (e.g., Coxackie A and B viruses) are also found in the stools of non-ill individuals with frequencies similar to those seen in ill individuals (Kilgore *et al.*, 1997).

2. Family *Caliciviridae*

The family *Caliciviridae* is composed of nonenveloped icosahedral viruses with a single-stranded positive-sense RNA genome. The isometric capsid has a diameter of

35-39 nm. Capsids appear round to hexagonal in outline. The RNA genomes of 7.4-8.3 kiro base of the viruses were organized in two or three major open-reading frames. The 5'-end of the genome has a usually genome-linked protein (VPg), and the 3'-terminus has a poly (A) tract (Green *et al.*, 2001).

The two major groups of human caliciviruses as NV and SV have been classified into distinct genera within the family *Caliciviridae* (Green *et al.*, 2000) and reportedly cause human acute gastroenteriris (Chiba *et al.*, 1979, 2000). The family has two additional genera, *Lagovirus* and *Vesivirus*, each of which includes caliciviruses of veterinary importance such as rabbit hemorrhagic disease virus (RHDV) and feline calicivirus (FCV), respectively (Fastier, 1957; Meyers *et al.*, 1991). It was thought initially that vesiviruses could be the members of the *Picornaviridae* (Melnick *et al.*, 1974). The *Picornaviridae* study group recommended the removal of the CVs from the *Picornaviridae* (Melnick *et al.*, 1978). In the third report of the International Committee Taxonomy of Viruses (ICTV) in 1979, the creation of a new family of positive-strand viruses, named *Caliciviridae*. Although all caliciviruses share a common ancestor in phylogenetic analyses, the NVs and SVs form distinct genetic clusters within the *Caliciviridae* (Berke *et al.*, 1997). In addition, certain features of their RNA genome organization distinguish them from each other and from other genera in the *Caliciviridae*. Generally, it is difficult to distinguish NV from SV by clinical symptoms in patients. After the introduction of direct electron microscopy (EM) for SV, Madeley and Cosgrove (1976) and Flewett and Davies (1976) were the first to find

morphologically typical these viruses of human origin in the stools of children with gastroenteritis as small-round-structured virus (SRSV). They have a typical "Star-of-David" configuration by EM and are antigenically identical to each other by immune EM.

3. Norovirus

1) Overview

NVs have a diameter of 27 to 40 nm by negative-stain EM and RNA genome of approximately 7.6 kiro base (Jiang *et al.*, 1990; 1993). Norwalk virus is a prototype strain of NVs, which was obtained in 1968 from clinical specimens of an outbreak of gastroenteritis among schoolchildren in Norwalk, Ohio (Adler et al., 1969) and identified in 1972 by Kapikian using immune electron microscopy (IEM) (Kapikian *et al.*, 1972). Because of the features of observation with electric microscopy (EM), NVs have been called "small round structured virus (SRSV)". Later studies demonstrated that other small, round-structured viruses (SRSVs) morphologically similar to NV were associated with outbreaks of gastroenteritis (Appleton *et al.*, 1977; Dolin *et al.*, 1982; Thornhill *et al.*, 1977). And other viruses which have features of small round structured virus such as astroviruses and sapoviruses were called "SRSV" in spite of morphologic differences. From the accumulation of investigation, it was found that most of the viruses identified

as "SRSV" in human with gastroenteritis were NV.

The viruses including Norwalk virus were named Norwalk-like viruses in 7th Report of ICTV, and then renamed norovirus in August 2002, ICTV classified in the 8th report SRSV to the genus Norovirus (http://www.ictvdb.rothamsted.ac.uk/Ictv/fr-fst-g.htm).

2) Molecular virology

NVs have three major open reading frames (ORFs) (Clarke *et al.*, 1997). The first ORF encodes a polyprotein precursor to nonstructural proteins. This ORF contains regions analogous to 2C helicase, 3C protease and 3D RNA-dependent RNA polymerase of picornaviruses. The second ORF encodes the capsid protein with an apparent molecular mass of 58kDa. The ORF encodes for a basic protein whose functional properties are still unknown (Venkataram *et al.*, 1996). The RNA-dependent RNA polymerase region has been considered to be functionally the most conserved domain of the NV genome (Green *et al.*, 1993; Ando *et al.*, 1995). Recently, it is demonstrated that capsid region is more suitable for phylogenetic analysis of NVs based on analyzing the sequences of full-length RNA genomes (Kojima *et al.*, 2002). The NVs are divided into genogroup I (GI) and genogroup II (GII), based on genetic diversity of polymerase (Ando *et al.*, 1994; Wang *et al.*, 1994) and capsid region (Ando *et al.*, 1994). The two genogroups can be further divided into 15 genotypes of GI and 18 genotypes of GII (Okada *et al.*, 2005). The frequency distributions of intergenotype distances ranged from 0.122 to 0.356 (mean±3SD, 0.239±0.117) for GI and from 0.118 to 0.464

(0.291±0.173) for GII. Using baculovirus-expressed recombinant NV particles, antigenic epitopes and determinants of immunity to this unique group of viruses were revealed (Prasad *et al.*, 1999).

The NVs have been detected from bovine (Dastjerdi *et al.*, 1999; Liu *et al.*, 1999; Oliver *et al.*, 2003) or swine (Dastjerdi *et al.*, 1999; Sugieda *et al.*, 1998), and most of the strains detected are classified into other genogoups of human. However, several strains are genetically related with humans (Dastjerdi *et al.*, 1999; Sugieda *et al.*, 2002).

Recently, Rockx *et al.* (2005) reported that rhesus macaques may yield an animal model to study the immune response and pathogenesis after NV infection. However, these animals are not considered to be conventional animal model of NV infection. Since NVs have not yet been cultivated *in vitro* (Atomar *et al.*, 2001), detection has traditionally relied on direct electron microscopy (EM), which requires virus concentration of at least 10^6 per ml of stool (Danoe, 1994). An enzyme immnoassay (EIA) method for detection of NV antigen was developed using virus like particle by Jiang *et al.* (1995) and several laboratories are improving it to date, but insufficient in sensitivity (requires 10^{4-6} virions). Because of low sensitivity of these methods, they are adopted only in fecal specimens. Nucleic acid detection assays, such as RT-PCR, sequence analysis, and real-time PCR were applied to detect viral RNA in not only fecal specimens but specimens which have a low viral concentration including foods and shellfishes (Nishio *et al.*, 2005).

3) Epidemiology

The NVs are spread by fecal-oral route through contaminated food and water or direct person-to-person contact (CDC, 2001). The vomitus from patient can be also a transmitter of NV infection (Chadwick *et al.*, 1994). Recently air-born suspected cases were reported (Marks *et al.*, 2003; Caul, 1994).

Early volunteer studies examined the ability of different viruses of NVs to induce crossprotection. Based on these studies, Norwalk virus, Hawaii virus, and Snow Mountain virus were defined as separate serotypes (Wyatt *et al.*, 1974; Kapikian *et al.*, 1972). Serologic evidence of past infection with Norwalk virus is present in one-third to three-fourths of adults and varies according to the population studied (Blacklow *et al.*, 1979). Honma *et al.* (1998) and Numata *et al.* (1994) reported that over 70% of people have evidence of past infection with Mexico virus strain in Japan. Nowadays, it is revealed that people can be infected by all types of NVs repeatedly due to existence of multiple antigenic types and lacking of lasting immunity against NVs (CDC, 2001). In biopsy of volunteer study, lesion was found only in superior tunica mucosa intestini tenuis and shrinking and flattening of villus, hypertrophy of crypta and celluler infiltration of uninuclear and polykaryocyte (Agus *et al.*, 1973; Schreiber *et al.*, 1974). The mechanisms of crisis of diarrhea and vomiting are unclear. Recently, it was demonstrated that human histo-blood antigen is the receptor which is combined with NVs (Marionneau *et al.*, 2002).

The clinical features of NV infection including a latent period of 25 to 72 h, are

characterized by acute onset of nausea, vomiting, abdominal cramps and diarrhea that generally lasts for about 48 h (Graham *et al.*, 1994). Symptomatically and asymptomatically infected individuals might still shed virus in stool (Okhuysen *et al.*, 1995). Rockx *et al.* (2002) reported that virus can be excreted for up to 3 weeks. Lavett *et al.* (1996) reported a case of long-term shedding of NVs; approximately 8-month period by immunocompromised patient.

The NV infections begin in infancy; therefore it is very common illness in all ages (Fankhauser *et al.*, 1998), and they are the main cause of outbreaks of gastroenteritis in nursing homes (Calderon-Margalit *et al.*, 2005; Sakon *et al.*, 2005), schools (Buesa *et al.*, 2002), elderly people's home (Schreier *et al.*, 2000), and hospitals (Vinje *et al.*, 1996). NVs are so infectious (CDC, 2001), that they have caused widespread food-borne and waterborne outbreaks (Nishio *et al.*, 2005; Godoy *et al.*, 2005; Nygard *et al.*, 2003; Parashar *et al.*, 1998; Hedberg *et al.*, 1993; Murao, 1991). Because of high resistance against physical and chemical treatments, the ability of the viruses to survive in high levels of chlorine (Keswick *et al.*, 1985) and heating to 60°C (Kapikian *et al.*, 1996) makes the control of NVs difficult.

The number of patients of food-borne cases due to NV occupies first position in Japan in recent years (http://www.mhlw.go.jp/topics/syokuchu). The cases of food-borne outbreaks can be classified by transmitters as food handler-related cases and shellfish-related cases.

4) NV infection associated with shellfishes

There are numerous illnesses transmitted via shellfishes. Bacterial infections such as shigellosis and vibriosis are well known, but in recent years, they are comparatively well controlled in Japan. The majority of these illnesses were attributed to enteric viruses, particularly NVs (Schwab *et al.*, 1998). Because bivalve shellfishes are filter feeding organisms, and it was revealed that sewage can contaminate shellfish-growing waters with various enteric viruses of human origin. Shellfishes accumulate the pathogen to level considerably greater than those in the overlying water, if pathogenic microorganisms are present in the water (Schwab *et al.*, 1998). Very recently, le Guyader (2006) demonstrated that viral particles bind specifically to digestive ducts by carbohydrate structures with a terminal N-acetylgalactosamine residue in an alpha linkage; same binding site used for recognition of human histo-blood group antigens. Oysters tend to be the vehicles of these pathogens because of occasions for raw consumption. Even if the levels of viruses involved in oyster are low, NVs are highly infectious with a minimal infectious dose of 10 virus particles estimated (CDC, 2001).

There are many NV gastroenteritis cases epidemiologically linked to the consumption of raw or mild cooked shellfish (Kohn *et al.*, 1995; Doyle *et al.*, 2004). There are the reports of detection of NVs or other human enteric viruses in river or marine environment (Lodder *et al.*, 1999; 2005), and the route of NV contamination from patients to oysters was demonstrated (Ueki *et al.*, 2005). Therefore NV strains from oyster coincide with prevalent strains from patients living upper stream of oyster farm

whether the strains were able to be detected or not. Although various viruses are accumulated in shellfish body, it is considered that several viruses selected by sensitivity of host (like immune pressure and adaptation to receptor) always increase. Actually, there were patients who were shedding more than one strain (Sugieda *et al.*, 1996). Recent studies revealed that the proportion of larger-sized outbreaks related to school lunches and catered meals increased among food-borne outbreaks in Japan, but more than half of the small-sized outbreaks were related to the consumption of oysters (Inoue *et al.*, 2000). The Japanese government regulates the oyster industry under the Enforcement Regulation of Food Sanitation Law. In Japan, although bacteriological regulation is laid down in oyster for raw consumption and in their growing area, some reports suggest that there is only a weak correlation between bacterial indicators and the presence of enteric viruses in oysters (Leeds, 2000, Richards, 1998, Cook *et al.*, 1986). Especially, low fecal coliform levels in shellfish do not always indicate that shellfish are free of viral contamination, since virus may persist within shellfish for relatively long periods after bacterial levels have been reduced in surrounding waters (Gerba *et al.*, 1978). The batch of oysters must be indicated the growing area and for raw consumption or for cooking, but there is no regulation specified to viruses. However, the batch of the oysters would be recalled by detection of NVs in the oyster or proof of NV infection by the oyster epidemiologically. Without virological regulation, there are nowadays the communities of oyster farm who check NVs in oysters for raw consumption voluntary before shipping.

4. Objectives

Monitoring NVs contamination of oysters continuously would result in improved outbreak prevention and control of NV infection, we attempted to perform phylogenetic analyses of NVs. Quantitating and sequencing NVs in oyster for the purpose of clarifying the epidemiological route of pollution of NV has been one of the major research subjects. It would be useful in understanding oyster-associated illness and enable us to prevent and control shellfish-borne illness. If we can control the outbreaks of shellfish-related gastroenteritis, the outbreaks of food-handler transmission or person-to-person infection by asymptomatic or symptomatic people of shellfish-related NV infection can be controlled. The occurrence of contamination of sewage by their fecal NVs can be reduced, and then the number of population infected by NVs can be decreased. World Health Organization (WHO) and U.S. Food and Drug Administration (FDA) made risk assessment of *Vibrio* spp. and raw consumption of oysters (http://www.who.int/foodsafety/publications/micro/mra8/en/index.html, http://www.cfsan.fda.gov/~dms/vpra-ref.html) and noticed the necessity of assessment for NVs.

We adopted sensitive methods, real-time PCR to quantify copy numbers and semi-nested RT-PCR to determine genome sequences. Viruses are concentrated in

digestive diverticula tissues of shellfishes (Schwab *et al.*, 1998). Then, we removed stomach and digestive diverticula to extract NVs. In these studies, we used capsid region which is considered to be most suitable for phylogenetic analyses, and detection of NV in oysters.

The purpose of these investigations is to elucidate the epidemiological relationships between contamination of oysters for raw consumption and food-borne NV infection, leading to the control and prevention of NV infection. Since there are few reports on quantification and phylogenetic analyses of NV genomes on capsid region, we attempted to detect NVs which contaminate oysters for raw consumption harvested in two areas of Setiouchi sea, from December, 2001 to February, 2002, in chapter II. In chapter III, the change of the rates and degrees of NV contamination in oysters for raw consumption harvested in two distinct sea areas were investigated during three winter seasons from October, 2002 to March, 2005.

II. Detection and quantitation of NVs in oysters

1. Introduction

Noroviruses (NVs), which belong to the family *Calicivilidae*, cause acute gastroenteritis (Wang *et al.*, 1994). According to the Japanese 2001 Food Poisoning Surveillance Report (http://www.mhlw.go.jp/topics/syokuchu), NVs accounted for 28% (7358/25862) of cases of food poisoning overall and 99% (7358/7371) of viral cases. In addition, NVs reportedly cause gastroenteritis in large numbers of patients in many countries, suggesting that NVs are distributed worldwide (Noel *et al.*, 1999).

NVs, enteroviruses, astroviruses, and hepatitis virus type A are likely to be transmitted by shellfish such as oysters and clams (Metcalf *et al.*, 1995). The Japanese, other Asians, and the French eat large amounts of raw fish or shellfish. Raw consumption causes many cases of food poisoning or infectious gastroenteritis, as well as hepatitis (Inoue *et al.*, 2000). Previous epidemiologic studies have linked many cases of NV gastroenteritis to the oyster harvesting season (Mounts *et al.*, 2000). These viruses, known to persist in the environment, can be concentrated by shellfish (le Guyader *et al.*, 2000).

No conventional cell culture method has been developed for propagation of NVs (Atomar *et al.*, 2001). Detection of NVs has relied mainly on reverse transcription polymerase chain reactions (RT-PCR), enzyme-linked immunosorbent assays (ELISA), and electron microscopy (Atomar *et al.*, 2001). NVs can be divided into two distinct genogroups, I (Norwalk virus type) and II (Snow mountain virus type) (Wang *et al.*,1994).

Previous studies using stool specimens from patients with nonbacterial gastroenteritis have demonstrated broad genetic diversity in each genogroup of NVs (Fankhauser *et al.*, 2002; Green *et al.*, 1994; Sugieda *et al.*, 1996). However, epidemiologic and phylogenetic characteristics of NVs in oysters remain obscure. In addition, to our

knowledge, few investigations have quantified NVs in shellfish. This information is important for preventing food poisoning caused by NVs and for determining the infective dose and index of contamination for NVs. We therefore carried out detection, quantitation, and phylogenetic analysis of NV genomes in Japanese oysters.

2. Material and methods

1) Samples and preparation of viral suspension

One hundred ninety-one Japanese oysters (Crassosterea gigas or Crassosterea nippona) were collected from fish distributors handling the harvest from two areas of the Setouchi sea (areas A and B) about 500 km apart. Both areas are located in the part of the west in the Honshu island. Sample numbers and harvesting month in these areas are shown in Table 1. All oysters had been approved for raw consumption according to the Regulation of Food Sanitation Law (< 50 000 standard plate counts of bacteria per gram of oyster with < 230 representing the coliform group). The fresh oysters were shucked, and the stomachs and digestive diverticula of the oysters were removed by dissection on the day of harvest (le Guyader *et al.*, 1996) and then weighed and homogenized in nine times their weight of phosphate-buffered saline (PBS) without magnesium and calcium. In brief, the stomachs and digestive diverticula in PBS with addition of 0.1 ml of antifoam B (Sigma, St. Louis, MO) were homogenized for two 30-s intervals at maximum speed of with an Omni-mixer (OCI Instruments, Waterbury, CT). Six milliliter of choloroform:butanol (1:1, vol/vol) was added to the homogenate. Then the mixture was homogenized for an additional 30 s, and 170 μl of Cat-Floc T (Calgon, Elwood, PA) was added to the homogenate (le Guyader *et al.*, 1996). After homogenate samples were centrifuged at 3000 x g for 30 min at 4°C, all of supernatant was layered onto 3 ml of 30% sucrose solution and ultracentrifuged at 154,000 x g for 3 h at 4°C. Then, the pellet was resuspended in 300 μl of doubly distilled water (DDW) containing 20 units of RNase inhibitor (Promega, Madison, WI) and stored at –80°C until use.

2) RNA extraction, RT-PCR, sequencing, and real-time PCR

Viral RNA was extracted from 140 μl of viral suspension using QIAmp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) and finally suspended in 30 μl of distilled water. Then, the RNA solution was treated with 5 units of DNase I (Takara, Tokyo, Japan). To amplify the partial capsid region of NVs by RT-PCR, we used genogroup-specific primers following reverse transcription as previously described (Kojima *et al.*, 2002). Primers for the first PCR were: G1-SKR and COG1F, G2-SKR and COG2F (Table 2). Primers for the nested PCR were: G1-SKR and G1-SKF, G2-SKF and G2-SKR (Table 2). The PCR protocol included incubation for 3 min at 94°C; then, 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and an additional 15 min for elongation at 72°C after the last cycle. This PCR procedure was repeated using inner primers as a nested PCR (Kojima *et al.*, 2002). We used two types of positive controls and a viral gene-free negative control per five assays for amplification of noroviruses by PCR. Nested PCR is considered to be highly prone to false positive results (Atomar *et al.*, 2001). Therefore, to check for conflicting PCR results, negative controls were included in each PCR round. In addition, first PCR round and nested PCR round were performed independently. The size of the amplified DNA fragment was confirmed by electrophoresis on 1.5% agarose gel. After purification of DNA fragments with a QIAquick PCR Purification Kit (Qiagen), the nucleotide sequence was determined with an automated DNA sequencer (ABI 310 DNA sequencer, Applied Biosystems, Foster City, CA) using a Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) (Kojima *et al.*, 2002). We also quantified NV capsid genes using real-time PCR as previously described (Kageyama *et al.*, 2003). Fifteen microliters of DNase I-treated RNA solution was added to 15 μl of RT solution containing 100 mM Tris-HCl at pH 8.3, 150 mM KCl, 6 mM $MgCl_2$, 1 mM of dNTP mixture, 10 mM dithiothreitol, 75 pmol of random hexamer (Takara), 30 units of RNase inhibitor (Takara),

RNaseH(-); Invitrogen, San Diego, CA). RT mixture was incubated at 42°C for 60 min (RT reaction), and then at 70°C for 15 min (denaturation of reverse transcriptase). The real-time PCR mixture contained 5 μl of cDNA (RT product), 17.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 400 nM of each primer, (primers for genogroup I, COG1R and COG1F; primers for genogroup II, COG2R and COG2F) and fluorogenic probes (probes for genogroup I, 10.5 pmol of RING1(a)-TP and 3.5 pmol of RING1(b)-TP; probe for genogroup II, 3.5 pmol of RING2-TP) (Table 2). The following PCR protocol was used; 2 min at 50°C and 10 min at 95°C, and 50 cycles of 95°C for 15 sec, and 56°C for 60 sec (Kageyama *et al.*, 2003). Data were corrected using an internal standard as previously described (Kageyama *et al.*, 2003). In addition, we tested recovery of the norovirus genome using highly purified norovirus particles obtained by a sucrose gradient method from patients with gastroenteritis.

3) Phylogenetic analysis

Capsid sequences of reference strains of NVs were obtained from GenBank (Katayama *et al.*, 2002). These strains and accession numbers are shown in the legend for Figure 2. Phylogenetic analysis was performed as previously described (Katayama *et al.*, 2002). Briefly, all NV capsid region sequences (238 to 253 nt) were aligned using Genetix-Win version 5.0 software (Software Development, Tokyo, Japan). A phylogenetic tree was constructed by a "neighbor-joining" technique, the Kimura's two-parameter method (N-J method). Reliability of the tree was estimated using 1000 bootstrap replications.

3. Results

1) Detection and phylogenetic analysis of NVs in Japanese oysters

NV capsid gene was amplified and detected in 17 of 191 oysters (9%). Amplicons from oysters were electrophoresed for comparison with corresponding norovirus protostrains (Fig. 1). All amplicons of noroviruses from oysters were sequenced successfully. As shown in Table 4, NV genomes were detected in 13 of 60 oysters in January (9 of 40 from area A, 4 of 20 from area B). NV genomes were detected in 4 of 95 oysters in February (3 of 80 from area A, 1 of 15 from area B). No NV genome was detected in oysters harvested in December. A phylogenetic tree constructed by the N-J method is shown in Figure 2. Of 17 amplicons, 3 were classified into genogroup I (Norwalk virus type), while 14 were classified into genogroup II (Snow mountain virus type). These amplicons could be subdivided into 7 genotypes of genogroup II and 3 genotypes of genogroup I. Both genogroups were detected in area A and B. Several amplicons harvested in area A (A-4, A-5, and A-10; A-1, A-3, and A-11) were closely related genetically (less than 1% genetic diversity). Nucleotide diversity among these amplicons exceeded 10% (see Figure 2), representing wide genetic diversity. Some human NVs in stools from gastroenteritis patients who lived near sampling sites (about 30 km) during the investigation period between December 2001 and February 2002 (cf. AH-1, BH-1, BH-2, in Figure 2) were genetically related to our amplicons from oysters. Approximately 30% to 40% diversity was noted between some animal calicivirus strains and some of our amplicons from oysters.

2) Quantitation of NV capsid gene by real-time PCR

As shown in Table 4, more than 10^2 copies of NV genome were found in 11 of 191 oysters (6%); 3 from genogroup I and 8 from genogroup II). More than 10^3 copies of NV genome were detected in 4 oysters harvested in area A. In January, about 15% of oysters

harvested in both areas (6 of 40 oysters from area A and 3 of 20 oysters from area B) had large amounts of NV genome. In February, 3 of 80 oysters harvested in area A (4%) and 1 of 15 oysters harvested in area B (7%) had relatively large amounts of NV genome. Thus, over 5% of oysters harvested in the two areas during January and February carried large numbers of NVs. In this study, a greater amount of NV genome was detected in oysters harvested from site A than in oysters from site B. However, no plausible sources of contamination with noroviruses including wastewater treatment plants and combined sewer overflows were present in the vicinity of either site. During the investigation period, no significant difference in seawater quality (< 70 coliform groups/100 ml of seawater) under the Japanese Enforcement Regulation of the Food Sanitation Law for farming of oysters for raw comsamption was observed between sites (data not shown).

4. Discussion

We demonstrated that about 10% of oysters harvested from two areas in January and February for raw consumption had NV genomes, while about 5% had large numbers of NVs (over 10^2 copies). More than 10% nucleotide diversity was observed in our amplicons. No regionally preponderance of any given genogroup was evident in our phylogenetic tree. The results suggested that Japanese oysters harvested in winter in both areas were highly contaminated with a genetically diverse population of NVs.

NV genomes have been detected in shellfish such as oysters and clams in United States (le Guyader *et al.*, 2000; Kingsley *et al.*, 2002; Shieh *et al.*, 2000), United Kingdom (Lees *et al.*, 1995), Canada (Leers *et al.*, 1987), and Japan (Sugieda *et al.*, 1996), and these viruses are associated with gastroenteritis in humans. NVs can be divided into two genogroups, each of which can be further divided into four to six clusters (Fankhauser *et al.*, 1998). Fankhauser *et al.* demonstrated that the polymerase gene of NVs detected during outbreaks of gastroenteritis in the United States can be divided into two clusters within genogroup I and four clusters within genogroup II (Fankhauser *et al.*, 1998). Capsid genes of NVs from stool specimens from patients during outbreaks of gastroenteritis in Japan can be divided into two clusters in genogroup I and four clusters in genogroup II (Katayama *et al.*, 2002). Our phylogenetic analysis revealed that NVs in Japanese oysters can be divided into two clusters in genogroup I and five clusters of genogroup II. This suggests wide genetic diversity, as has been noted in NVs from stool specimens (Fankhauser *et al.*, 1998; Fankhauser *et al.*, 2002; Katayama *et al.*, 2002). Transmission of NVs can occur via contaminated shellfish, especially oysters, and by person-to-person contact (Inoue *et al.*, 2000). Many reports have implicated NVs and enteroviruses in shellfish-associated outbreaks and demonstrated NV gene sequences in shellfish and in patient specimens. Various types of NVs can cause oyster-associated gastroenteritis in Japan (Sugieda *et al.*, 1996).

Although no conventional culture method for NVs is available at present (Atomar *et al.*,. 2001), other NV detection methods such as electron microscopy (EM), ELISA, and RT-PCR method can be used (Atomar *et al.*,. 2001). EM and ELISA are practical for detection of NVs in diarrheal stool specimens containing a large amount of virus (10^5 to 10^6 particles/ml) (Danoe *et al.*, 1994). Our results and previous data suggested that various foods including shellfish contain much smaller numbers of NVs than stool specimens (Jaykus *et al.*, 1996). This hinders detection of NVs in foods by EM and ELISA, since their sensitivity is limited (Glass *et al.*, 2000). In contrast, RT-PCR is not quantitative but is highly sensitive, being able to detect a few copies of NV genome in a sample (Ando *et al.*, 1995). Recently, a quantitative real-time PCR method has been developed (Kageyama *et al.*, 2003). We applied this method to determine copy numbers of NV genomes in Japanese oysters. Some of our oyster-derived amplicons were found to be genetically related to strains infecting patients with gastroenteritis during the period of our investigation (Fig. 2). The results suggested that genetically similar types of NVs from oysters might be associated with NV-associated outbreaks during our investigation period. Some genetic similarities exist between animal calicivirus and human calicivirus (Liu *et al.*, 1999; Sugieda *et al.*, 2002), and animal calicivirues consist of many types (Atomar *et al.*, 2001). Interestingly, some of our amplicons had unique sequences that were genetically related to some animal calicivirus strains (30% to 40% diversity; Fig. 2). Thus, we could not rule out inclusion of animal calicivirus strains in our study. In addition, norovirus genome was detected in abundance in oysters harvested in January and February, but not in December. Japanese epidemiologic data for the interval between October and December (http://www.mhlw.go.jp/topics/syokuchu) suggest that incidence of oyster- or clam-associated gastroenteritis outbreaks was low at that time, although these data are not directly linked to our own. The population of norovirus-contaminated Japanese

oysters from both areas in December indeed may have been low. Yearlong month- to -month studies regarding of noroviruses in oysters may be needed.

III. Investigation of contamination of NVs in oysters in distinct areas in three seasons.

1. Introduction

Norovirus (NV) is a member of the *Caliciviridae* family (Atomar *et al.*, 2001; Kapikian *et al.*, 1996) and a major causative pathogen of acute nonbacterial gastroenteritis worldwide (CDC, 2001; Fankhauser *et al.*, 2002; Kapikian *et al.*, 1996; Sakon *et al.*, 2005). The NV causes fecal-oral infection and is highly infectious (CDC, 2001). According to the data from 2002 to 2004 in Japan, NV is the most common viral agent of food poisoning, this virus accounted for approximately 30% to 45% of all the food poisoning cases (http://www.mhlw.go.jp/topics/syokuchu). The prevalence of NV in other developing countries as well as U.S. and France is higher (Fankhauser *et al.*, 1998; Bon *et al.*, 2005). In 1994, Wang *et al.* (1994) showed that NVs isolated from humans were classified into two major genogroups: genogroup I (GI, Norwalk-type viruses) and genogroup II (GII, Snow mountain-type viruses). Each genogroup is further subdivided into many genotypes; thus NVs exhibit a wide genetic diversity (Katayama *et al.*, 2002; Kageyama *et al.*, 2004). It is suggested that NVs expelled from the patients with gastroenteritis are condensed in shellfishes such as oysters and clams (Schwab *et al.*, 1998; Burkhardt III *et al.*, 2000; Ueki *et al.*, 2005). 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 (Le Guyader *et al.*, 1996, 2003; Shieh *et al.*, 2000). Previous epidemiological studies showed linkage between gastroenteritis due to NV and the oyster-harvesting season (http://idsc.nih.go.jp/iasr). In several studies, NVs were detected in approximately 5% to 20% of oysters and clams (Formiga-Cruz *et al.*, 2002; le Guyader *et al.*, 2000; Myrmel *et al.*, 2004). Thus, these shellfishes are one of the infectious sources of NV infections (Metcalf *et al.*, 1995). Person-to-person infection is another common mode of NV

infection (CDC, 2001). 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 (Chapter II). Furthermore, most of the oysters had relatively large amounts of NV viral genomes with more than 100 copies of the capsid gene (Chapter II). However, the molecular study on NVs in oysters is not well known in Japan. To investigate the geographically and seasonally difference of NV detection, 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) in three seasons from October 2002 to March 2005.

2. Materials and Methods

1) Samples and preparation of viral suspension

A total of 1,512 Japanese oysters (*Crassosterea gigas*) were collected from the fish distributors that manage harvesting in the Sanriku Sea (483 oysters; Area C) and Setouchi Sea (1,029 oysters; Area D). 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 5. All of the oysters harvested had been approved for raw consumption according to the Regulation of Food Sanitation Law (in which the standard plate count of bacteria in one gram 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 10mM phosphate-buffered saline, pH 7.4 without magnesium and calcium to prepare a 10% suspension. The suspension received 0.1 ml antifoam B (Sigma, St. Louis, MO) and then homogenized twice at a 30 s interval at the maximum speed by using an Omni-mixer (OCI Instruments, Waterbury, CT). Six ml of choloroform:butanol (1:1 vol/vol) was added to the homogenate. The mixture was then homogenized for an additional 30 s and 170 μl Cat-Floc T (Calgon, Elwood, PA) was added to the homogenate (Le Guyader, 1996). In addition, to monitor for efficiency the RNA extraction, we added echovirus type 9 to the homogenate samples. After the homogenate samples were centrifuged at 3000 × *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 h at 4°C. Subsequently, the pellet was resuspended in 138 μl of double distilled water (DDW) and stored at –80°C until use.

2) 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. Samples were finally suspended in 30 μl of DNase- and RNase-free water. In order to prevent carryover contamination by NV cDNA and to reduce nonspecific amplicons, the RNA solution was treated with 2 U of RNase-free DNase I (Takara, Tokyo, Japan) 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, CA) 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, Tokyo, Japan) with GI, GI-SKR and COG1F, and GII, G2-SKR and COG2F (Table 2).

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 primers: G1-SKR and G1-SKF; GII primers: G2-SKR and G2-SKF (Table 2). 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, CA) 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 (Kageyama *et al.*, 2003). The real-time PCR mixture contained 5 μl of cDNA, 2 μl of the LightCycler master mix (Roche, Penzberg, Germany), 0.7 μM of primers for GI, COG1R and COG1F; primers for GII, COG2R and COG2F, and fluorogenic probes for GI, 2 pmol of RING1(a)-TP and 2 pmol of RING1(b)-TP; probes for GII, 4 pmol of RING2-TP (Table 2). The amplification was performed by using LightCycler (Roche). The following PCR protocol was employed: 10 min at 95°C followed by 50 cycles at 95°C for 10 s, 60°C for 25 s and 40°C for 30 s. The data were corrected using internal standards as described previously (Kageyama *et al.*, 2003).

3) 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 database. The strains and accession numbers of these sequences have been provided in the legend for Figure 1. Phylogenetic analysis was performed as described previously (Katayama *et al.*, 2002). In brief, all of the NV capsid region sequences (nt 244 to 313) were aligned using Clustal W program (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 1000 bootstrap replications.

4) Statistical analysis

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

3. Results

1) 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 area of Sanriku (483 oysters) and Setouchi Seas (1,029 oysters). Detailed data regarding these oysters have been listed in Table 5. In total, 33 of the 483 oysters from area C (6.8%) and 42 of the 1,029 oysters from area D (4.1%), NV capsid genome were detected from oysters in the two distinct areas during all the investigation periods. During the 2003-2004 harvesting season, a higher number of NVs was detected in the oysters from area C than that from area D ($p<0.05$). However, during 3 harvesting seasons (2002-2003 and 2004-2005 harvesting seasons), the detection of NVs in oysters from area D was higher than that from area C ($p<0.05$). In both areas, most of the NVs were detected during December to next January at the investigation periods. We also quantified the copy number of the NV capsid gene in all oysters by real-time PCR (Table 6,7,8). The sensitivity (limit of detection) for this method is more than 100 copies of the NV genomes/oyster (Nishida *et al.*, 2003). Interestingly, the number of oysters from area C, which harbored the NVs genome, was significantly greater than that from area D. Oysters harvested from area D had a relatively large number of capsid genomes with more than 100 copies/oyster, while those harvested from area C had less than 100 copies of the NV genome (Table 6,7,8).

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, there are cases where the peaks of the nucleotide sequences in the chromatogram overlap and determination of the sequence is impossible, when the shellfishes have accumulated several kinds of viruses.

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. 3). 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. 3). 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 C were classified into GII/2 (Melksham-type). In area D, 6 amplicons were classified into GI/4 (Chiba407-type). In this study, many NV genotypes were detected in oysters, but 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 (Okada *et al.*, 2005). This classification method was based on the range of genetic distance (Kageyama *et al.*, 2004). However, our 2 amplicons, i.e., D-2 and D-3, were not included in any genotype because of the genetic distances exceeded the range for a same cluster, suggesting that these strains may be new NV genotypes (Fig. 3). In addition, the sequences of some amplicons matched completely each other, 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 oyster (cf. D-6 in Fig. 3) and animals (cf. Sw/NLV/Sw43/

1997/JP and Sw/NLV/Sw918/1997/JP in Fig. 3) were closely related, indicating that the NV capsid genes in oysters exhibit a wide genetic diversity, but some of them were genetically related to swine noroviruses (Sugieda *et al.*, 1998).

4. 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 D, 50% carried relatively large amounts of the NV genomes which were not observed in oysters from area C. In addition, the capsid gene exhibited a 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 (Le Guyader *et al.*, 2003; Myrmel *et al.*, 2004, Lodder-Verschoor *et al.*, 2005). For example, Le Guyader *et al.* (2003) showed that the polymerase gene of NVs was detected in approximately 20% of French oysters and showed wide genetic diversity. Myrmel *et al.* (2004) demonstrated approximately 7% of the shellfishes from the Norwegian coast contained the NV gene. 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 (Nishida *et al.*, 2003). On the other hand, Lodder-Verschoor *et al.* (2005) 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. 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 (le Guyader *et al.*, 2003; Myrmel *et al.*, 2004; Lodder-Verschoor *et al.*, 2005). Recent data suggested that the infectivity of NVs is relatively strong and less than 100 particles of NVs can easily cause gastroenteritis in humans (CDC, 2001). 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 (Nishida *et al.*, 2003). 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 C 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 both 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 and 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 NV 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 (Ueki *et al.*, 2005). Therefore, it is important to clarify the genetic characteristics of NVs detected from both shellfishes and humans, to get a better understanding of the epidemiology of NV infection (Kageyama *et al.*, 2004).

Our data indicated that most GI and GII strains detected in oysters and humans were genetically related to a considerable extent (Fig. 3).

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.

IV. Conclusion

To prevent oyster-associated gastroenteritis, the necessity of monitoring risk of raw oyster consumption has been increased. In this study, we attempted to quantify and analyze phylogenetically NVs in oysters for the prevention. In the chapter II, the detection and quantification of NVs in oysters with the method developed for stool samples was carried out. The method was revealed to be practicable to detect and quantify NVs in oysters sufficiently, and the degree of contamination of NVs in oysters was recognized. In the Chapter III, we attempted to investigate the differences of degree of contamination and phylogenetic analyses of NVs in oysters from two areas in three seasons in order to elucidate of epidemiology of NV contamination in oysters for raw consumption. Consequently, we demonstrated that the degree of contamination and phylogenetic features of amplicons detected in oysters differ by seasons and areas.

In four winter seasons, we detected approximately 5% to 10% of oysters involving NVs. Further, we demonstrated that the amplicons detected from oysters in both GI and GII showed a wide genetic diversity. In addition, we detected NV genomes in oysters from January to February 2001, from November 2002 to February 2003, from December 2003 to March 2004, and from November 2004 to February 2005. High levels of NV contamination as more than 10^2 copies of the viruses occurred in January and February 2003, from December 2002 to February 2003, in December 2003, and February 2005. These evidences should agree with the reports that viral contamination in oysters was found mainly during cold seasons (Metcalf *et al.*, 1995). The month in winter when NVs were detected differed in earch year but a high level contamination of NVs was

occurred from December to next February. In these months, many outbreaks of oyster-related gastroenteritis occurred during our investigation periods (http://www.mhlw.go.jp/topics/ syokuchu/index.html). The oyster-associated outbreaks of viral gastroenteritis occur mainly in December and next January also in Japan, while oyster-unassociated outbreaks take place a broader period as almost the same period as pediatric cases with a peak in January (Inoue *et al.*, 2000). The seasonal occurrence of shellfish-related illnesses by enteric viruses was considered to be the result of seasonal prevalence (Burkhardt *et al.*, 2000), seasonal physiological changes of ability to accumulate viral particles (Burkhardt *et al.*, 2000), and viral survival in estuarine water which has been modulated by temperature and sunlight exposure (Chung *et al.*, 1993, Lo *et al.*, 1976). This bioaccumulation of viruses during feeding is probably assisted by the ionic bonding of viral particles to the mucopolysaccharide moiety of shellfish mucus (di Girolamo *et al.*, 1977). Since the level of mucus production generally corresponds to the glycogen content of the connective tissue (Galtstoff, 1964), which is the highest in oysters from November through March (Burkhardt III *et al.*, 2000), there could be a connection to the fact that viral contamination is mainly observed during the winter months (le Guyader *et al.*, 2000). These findings have shown that winter gastroenteritis outbreaks have an impact on the environment and can be responsible for the increased viral contamination observed in those seasons (Miossec *et al.*, 1998). Since the months when a high level contamination of NVs occurred were revealed in this study, the attention for raw consumption of oysters is needed during these months.

Four winter seasons in our investigation periods, we detected amplicons which had wide genetic diversity and were divided into 8 genotypes of GI and 9 genotypes of GII including unidentified ones. The numbers of genotypes detected is greater than the results of Cheng *et al.* (2005), Formiga-Cruz *et al.* (2002), and le Guyader *et al.* (2000). The same strains of NVs were detected in geographically the same and distant point in the same time (Noel *et al.*, 1999), some amplicons were matched completely each other in spite of the differences of oceans and seasons in our present study. This evidence would indicate that extensive and long-term prevalence of identical strains of NVs were occurred.

Although the sensitive limit of detection of viral genomes in the present studies was 10^2 copies, not all of the viral genomes we detected were infectious (de Medici *et al.*, 2001). The minimum dose of NVs was estimated at less than 10^2 virions (CDC, 2001), and then the results of no detection usually protect persons from NV infection. Evaluating safety of oyster is limited because of difficulty of NV detection, alternative indicators that closely correlate with level of NV contamination in shellfish and environments or additional regulation based on viral sanitation may be needed. Up to this point, there are reports for candidates of indicator predicting the existence of the enteric viruses, such as fecal specific coliphage (Burkhardt III *et al.*, 2000), but it was demonstrated that these indicators do not predict in the marine environment and shellfish (Hernroth *et al.*, 2002). Since many postharvest controls are not effective in completely eliminating viruses from contaminated shellfish (Shieh *et al.*, 2000), preharvest controls

should be very important. In view of the route of infection, improvement of sewage treatment and surveillance of the prevalence on NV infection in people living near oyster farms would be one of the most important subjects.

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VII. Appendix

Table 1. Oyster samples collected for this study

Month/Year	No. of samples from:	
	Area A	Area B
Dec/2001	20	16
Jan/2002	40	20
Feb/2002	80	15
Total	140	51

Table 2. Primer and Probe oligonucleotides used for NV RT-PCR and Real-time PCR

	Primer or Probe	Sequence (5'→3')[c]	Polarity[d]	Location
Primer	COG1F[a]	CGY TGG ATG CGN TTY CAT GA	+	5291[f]
	COG1R[a]	CTT AGA CGC CAT CAT CAT TYA C	−	5375[f]
	COG2F[a]	CAR GAR BCN ATG TTY AGR TGG ATG AG	+	5003[h]
	COG2R[a]	TCG ACG CCA TCT TCA TTC ACA	−	5100[h]
	G1-SKF[b]	CTG CCC GAA TTY GTA AAT GA	+	5342[f]
	G1-SKR[b]	CCA ACC CAR CCA TTR TAC A	−	5671[f]
	G2-SKF[b]	CNT GGG AGG GCG ATC GCA A	+	5058[g]
	G2-SKR[b]	CCR CCN GCA TRH CCR TTR TAC AT	−	5401[g]
Probe	RING1(a)-TP[a]	FAM-AGA TYG CGA TCY CCT GTC CA-TAMUF	+	5340[f]
	RING1(b)-TP[a]	FAM-AGA TCG CGG TCT CCT GTC CA-TAMUF	+	5340[f]
	RING2-TP[a]	TGG GAG GGC GAT CGC AAT CT	+	5048[h]

[a]Kageyama et al.(2003)

[b]Kojima et al.(2002)

[c]Mix bases in degenerated primers and probes are as follows: Y=C/T, S=G/C, R=A/G, B=not A, N=any.

[d]+, sense; −, antisense

[e]6-Carboxyfluorescein (FAM) as the reporter dye is coupled in the 5' end of the oligonucleotide, and 6-carboxy-tetramathylrhodamine (TAMURA) as the quencher dye in the 3' end of the oligonucleotide

[f]Corresponding nucleotide position of Norwalk/68 virus (M87661) of the 5' end.

[g]Corresponding nucleotide position of Camberwell virus (AF145896) of the 5' end.

[h]Corresponding nucleotide position of Lordsdale virus (X86557) of the 5' end.

Table 3. Reference strains of NVs for phylogenetic analysis

strains	Gene Bank accession NO.
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
Bovine enteric calici-like virus Newbury agent-2	AF097917
Bo/Thisk10/00/UK	AY126468
OUTGROUP Manchester Sapovirus	X86560
AH-1	AB111894
AH-2	AB111895
BH-1	AB111896
CH-1	AB262166
CH-2	AB262167
CH-3	AB262168
DH-1	AB262169
DH-2	AB262170
DH-3	AB262171
DH-4	AB262172
DH-5	AB262173
DH-6	AB262174

Table 4. Genogroups and NV genome copy numbers in Japanese oysters

Month/Year	Area	amplicons [a]	Genogroup/genotype	NV genome copy number	Gene Bank accession NO.
Jan/2002	A	A-1	GII/8	2.7×10^3	AB097905
	A	A-2	GII/1	2.9×10^3	AB097906
	A	A-3	GII/8	ND[b]	AB097907
	A	A-4	GII/6	ND	AB097908
	A	A-5	GII/6	7.5×10^3	AB097909
	A	A-6	GII/14	1.6×10^2	AB097910
	A	A-7	GI/14	2.9×10^4	AB097911
	A	A-8	GII/3	ND	AB097912
	A	A-9	GI/4	2.2×10^2	AB097913
	B	B-1	GII/7	ND	AB097917
	B	B-2	GII/3	6.2×10^2	AB097918
	B	B-3	GII/6	1.5×10^2	AB097919
	B	B-4	GI/7	2.4×10^2	AB097920
Feb/2002	A	A-10	GII/6	ND	AB097914
	A	A-11	GII/8	1.4×10^2	AB097915
	A	A-12	GII/7	ND	AB097916
	B	B-5	GII/5	4.8×10^2	AB097921

[a] amplicon; A and B refer to the areas of harvest.

[b] ND; not detected (<100copies/oyster)

Table 5. Sample numbers and positive rate of capsid gene of noroviruses in Japane

Month/Year	Area C		Area D	
	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)	1029	42 (4.1)
	75/1512 (5.0)			

Table 6. Genotypes and copy numbers of NVs in Japanese oysters

Month/Year	Area	amplicons[a]	Genogroup/genotype	NV genome copy number	Gene Bank accession NO.
Nov/2002	C	C-1	II/5	ND[b]	AB262091
	C	C-2	II/4	ND	AB262092
	D	D-1	I/12	5.0×10^2	AB262093
	D	D-2	II/untypable	9.1×10^2	AB262094
Dec/2002	D	D-3	II/untypable	8.3×10^2	AB262095
	D	D-4	II/6	3.8×10^2	AB262096
	D	D-5	II/4	2.1×10^2	AB262097
	D	D-6	II/14	5.8×10^2	AB262098
	C	C-3	II/3	ND	AB262099
	C	C-4	II/3	ND	AB262100
	C	C-5	II/3	ND	AB262101
	C	C-6	II/3	ND	AB262102
	C	C-7	II/3	ND	AB262103
	C	C-8	II/3	ND	AB262104
	D	D-7	II/6	1.7×10^3	AB262105
	D	D-8	I/2	1.4×10^2	AB262106
	D	D-9	I/2	2.0×10^2	AB262107
Jan/2003	D	D-10	I/12	1.8×10^3	AB262108
	D	D-11	II/4	1.8×10^3	AB262109
	D	D-12	I/4	4.1×10^2	AB262110
	D	D-13	I/4	ND	AB262111
	D	D-14	I/4	ND	AB262112
	D	D-15	I/4	ND	AB262113
	D	D-16	II/8	ND	AB262114
	D	D-17	II/5	ND	AB262115
	D	D-18	II/8	ND	AB262116
Feb/2003	D	D-19	I/3	4.8×10^2	AB262117
	D	D-20	I/3	ND	AB262118

[a] amplicon; A and B refer to the areas of harvest.

[b] ND; not detected (<100copies/oyster)

Table 7. Genotypes and copy numbers of NVs in Japanese oysters

Month/Year	Area	amplicons[a]	Genogroup/genotype	NV genome copy number	Gene Bank accession NO.
Dec/2003	C	C-9	II/2	ND	AB262119
	C	C-10	II/2	ND	AB262120
	C	C-11	II/2	ND	AB262121
	C	C-12	II/5	ND	AB262122
	C	C-13	II/2	ND	AB262123
	C	C-14	II/2	ND	AB262124
	C	C-15	II/5	ND	AB262125
	C	C-16	II/2	ND	AB262126
	C	C-17	II/5	ND	AB262127
	C	C-18	II/2	ND	AB262128
	C	C-19	II/2	ND	AB262129
	C	C-20	II/5	ND	AB262130
	C	C-21	II/5	ND	AB262131
	C	C-22	II/2	ND	AB262132
	C	C-23	II/5	ND	AB262133
	C	C-24	II/5	ND	AB262134
	C	C-25	II/2	ND	AB262135
	D	D-21	II/3	1.4×10^2	AB262136
	D	D-22	II/3	4.0×10^2	AB262137
	D	D-23	II/3	9.6×10^2	AB262138
	D	D-24	II/3	1.0×10^3	AB262139
	D	D-25	II/3	9.9×10^2	AB262140
	D	D-26	I/5	ND	AB262141
	D	D-27	I/12	ND	AB262142
Jan/2004	C	C-26	I/12	ND	AB262143
	C	C-27	II/5	ND	AB262144
	C	C-28	II/5	ND	AB262145
	C	C-29	II/2	ND	AB262146
	C	C-30	II/5	ND	AB262147
	C	C-31	II/2	ND	AB262148
	C	C-32	II/2	ND	AB262149
	D	D-28	I/1	ND	AB262150
Feb/2004	D	D-29	I/12	ND	AB262151
	D	D-30	II/3	ND	AB262152
Mar/2004	D	D-31	I/12	ND	AB262153

[a] amplicon; A and B refer to the areas of harvest.

[b] ND; not detected (<100copies/oyster)

Table 8. Genotypes and copy numbers of NVs in Japanese oysters

Month/Year	Area	amplicons[a]	Genogroup/genotype	NV genome copy number	Gene Bank accession NO.
Nov/2004	C	D-33	I/1	ND	AB262154
	D	D-32	I/12	ND	AB262155
Jan/2005	D	D-33	I/1	ND	AB262156
	D	D-34	I/1	ND	AB262157
	D	D-35	I/12	ND	AB262158
Feb/2005	D	D-36	II/4	9.9×10^2	AB262159
	D	D-37	II/3	4.1×10^2	AB262160
	D	D-38	I/4	3.1×10^2	AB262161
	D	D-39	I/4	ND	AB262162
	D	D-40	I/1	ND	AB262163
	D	D-41	I/3	ND	AB262164
	D	D-42	I/3	ND	AB262165

[a] amplicon; A and B refer to the areas of harvest.

[b] ND; not detected (<100copies/oyster)

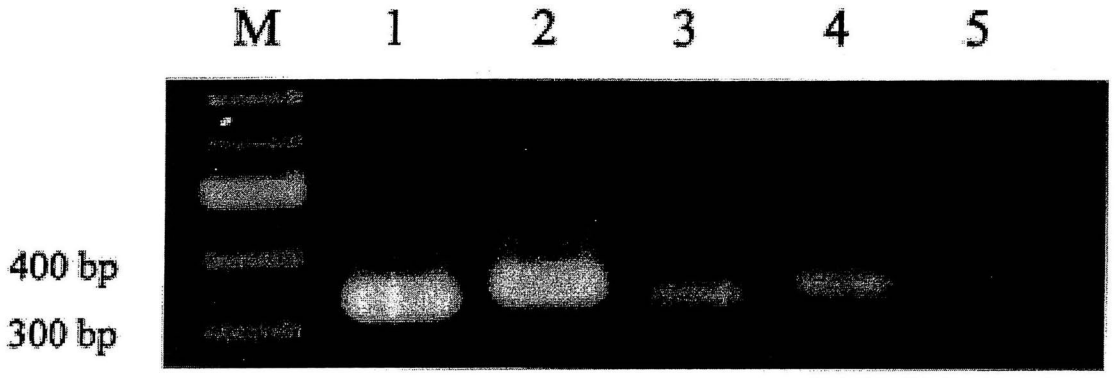


FIG. 1. Detection of NV capsid gene from oysters. Nested PCR products were electrophoresed on a 1.5% agarose gel. M, marker; lane 1, positive control for genogroup I (DSV395/90/Saudi Arabia, accession no. U04469); lane 2, positive control for genogroup II (Arg320, accession no. AF190817); lane 3, genogroup I amplicon from an oyster (A-9 strain in Fig. 2); lane 4, genogroup II amplicon from an oyster (B-3 strain in Fig. 2); lane 5, negative control (free of viral RNA). RT-PCR, nested PCR, and electrophoresis procedures are described in detail in Materials and Methods.

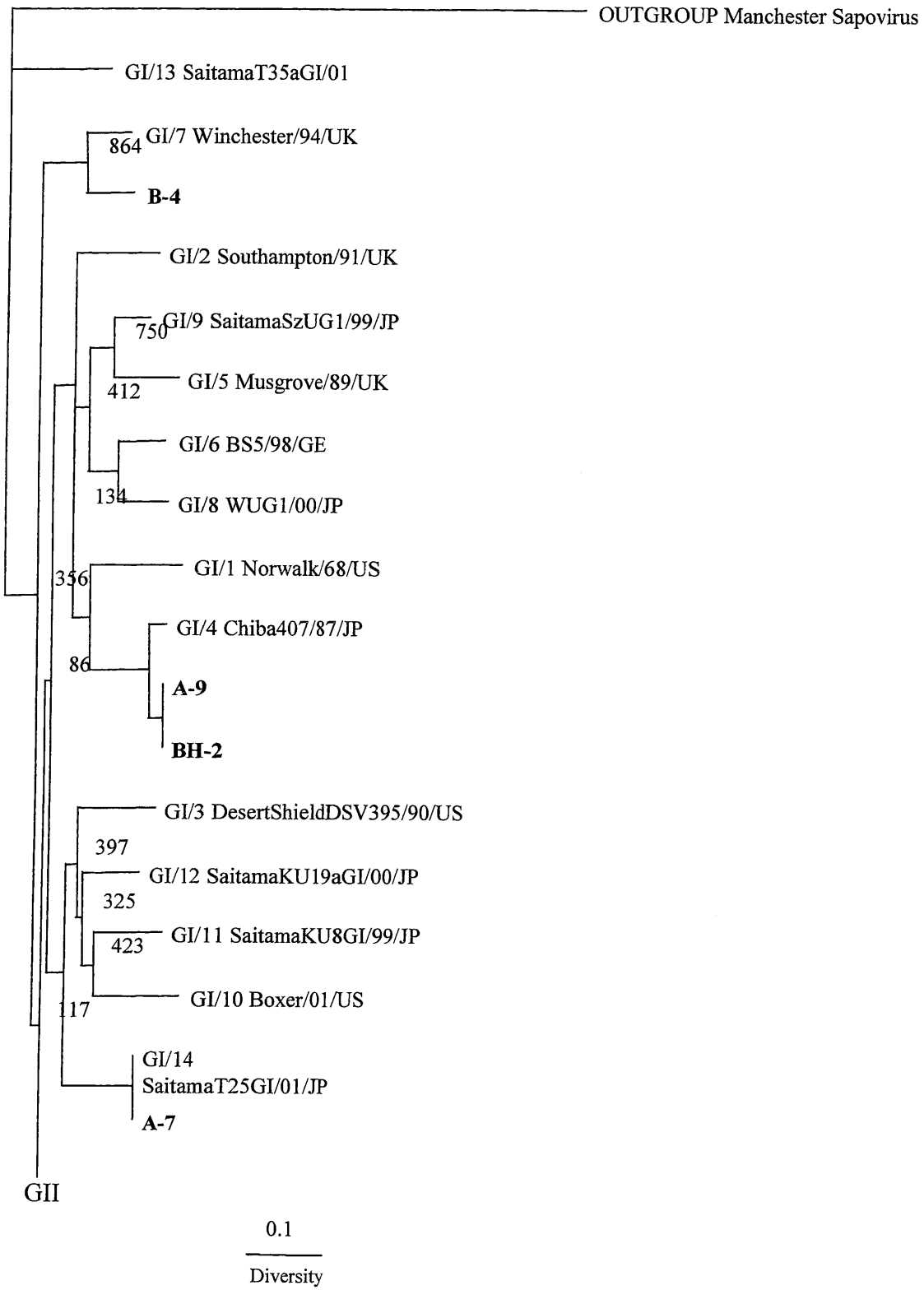
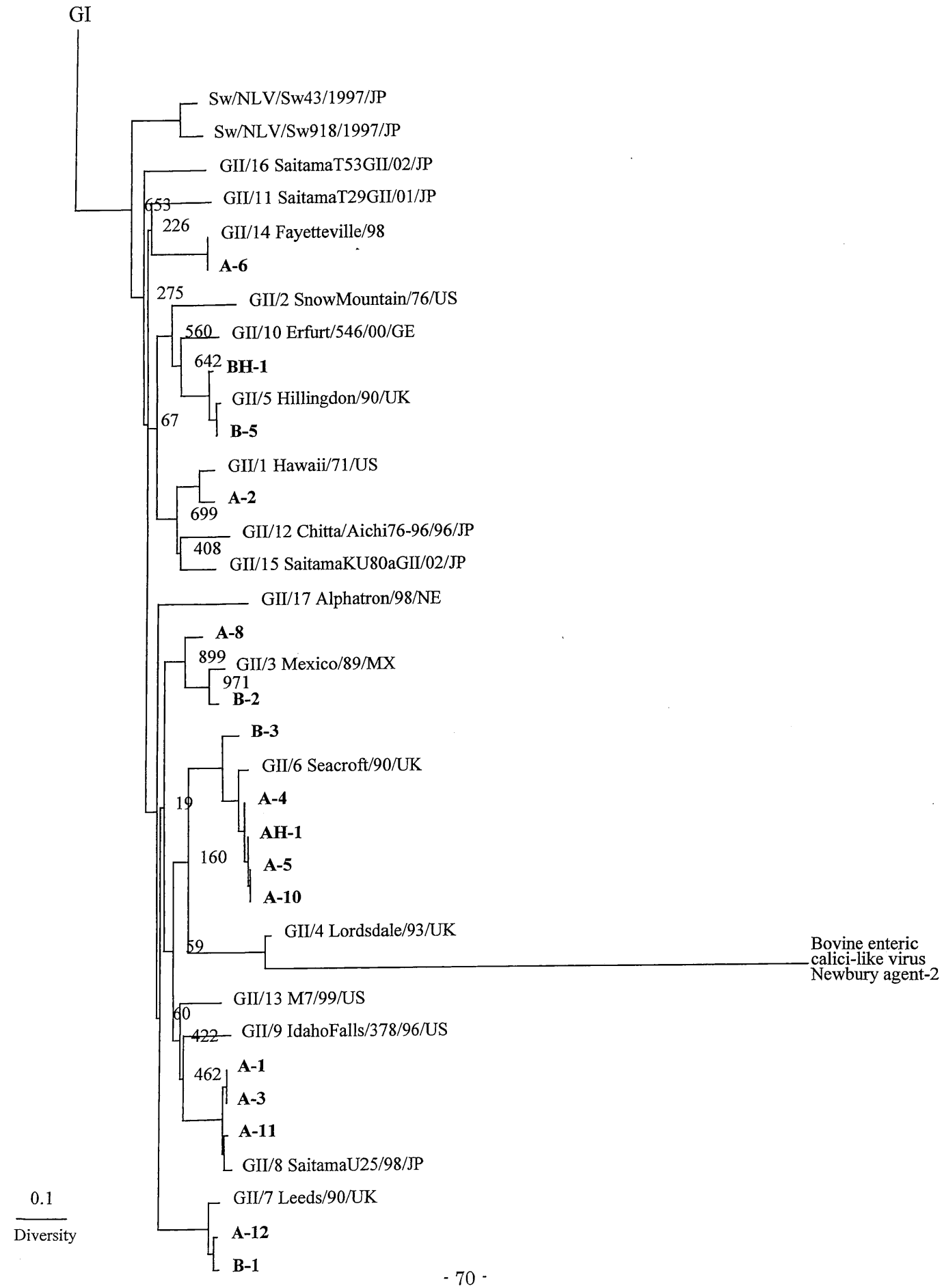


FIG. 2. Phylogenetic tree based on partial sequences of the capsid gene of NVs. The distance was calculated by Kimura's two-parameter method, and the tree was plotted by using the neighbor-joining method. Numbers at each branch indicate bootstrap values for the clusters supported by that branch. Accession numbers of our strains are indicated. A and B refer to the areas of harvest, and positive samples from this study are designated in boldface type. AH and BH refer to human NV strains detected in patients with gastroenteritis living near sites A and B. GI, genogroup I; GII, genogroup II.

(Continued, Fig. 2)



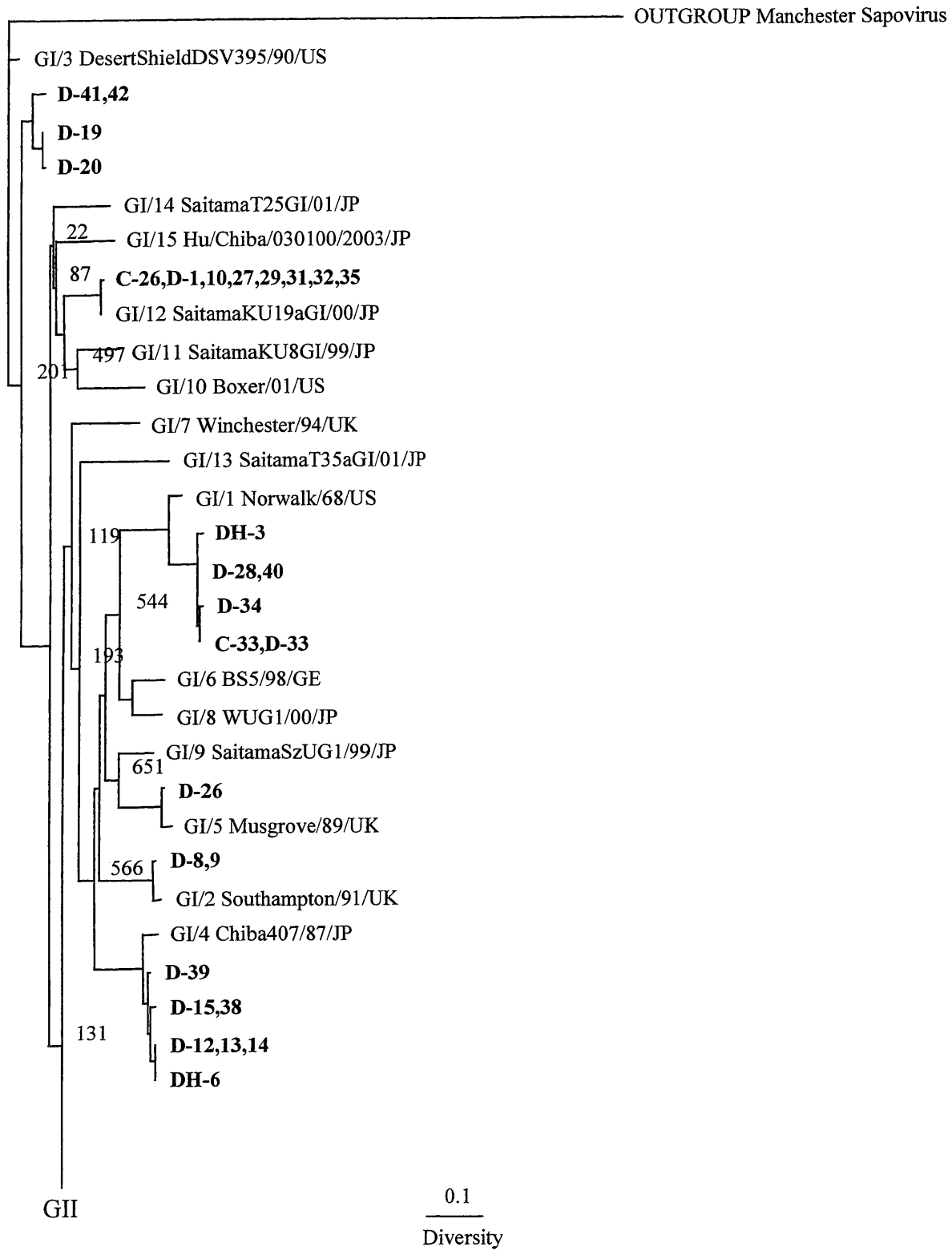


FIG. 3. Phylogenetic tree based on partial sequences of the capsid gene of NVs. The distance was calculated by Kimura's two-parameter method, and the tree was plotted by using the neighbor-joining method. Numbers at each branch indicate bootstrap values for the clusters supported by that branch. Accession numbers of our strains are indicated. C and D refer to the areas of harvest, and positive samples from this study are designated in boldface type. CH and DH refer to human NV strains detected in patients with gastroenteritis living near sites C and D. GI, genogroup I; GII, genogroup II.

(Continued, Fig. 3)