Table 1. Reactivities of monoclonal antibodies with 15 SaV VLPs in ELISA

				Reactivity[a]														
				GI[b]			GII									GIV		GV
				1[c]	5	6	2	3	3	3	3	3	3	4	7	1	1	1
Group	MAb	Immunogen	Isotype	Mc114	Yokote1	Nichinan	Mc10	C12	20082029	D1711	Syd53	Kushiro5	Nayoro4	Kumamoto6	20072248	Syd3	Yakumo8	NK24
A	5C9	Nichinan	IgG1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	1A1	Nichinan	IgG1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	5C1	Nichinan	IgG1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	6C4	Nichinan	IgM	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	8127	Yokote1	IgG1	+++	+++	+++	+	+	+	+	+	++	+	+	+	++	++	+++
	6D3	Nichinan	IgG1	++	+++	+++	++	+	+	+	NT	++	++	++	++	++	++	++
	3D2	Nichinan	IgG1	+	++	+++	++	+	+	++	NT	+	++	+	+	+	+	++
	4G7	Nichinan	IgG1	+	NT	+++	+	+	+	+	NT	+	+	+	+	+	+	+
B	616	Yokote1	IgG1	++	+++	++	–	–	–	–	–	–	–	–	–	–	–	–
	1325	Mc114	IgG1	++	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	5001	Mc114	IgG1	++	–	NT	–	–	NT	NT	–	NT	NT	NT	NT	–	NT	–
	4357	Mc114	IgG1	++	–	NT	–	–	NT	NT	–	NT	NT	NT	NT	–	NT	–
	627	Yokote1	IgG1	–	++	NT	–	–	NT	NT	–	NT	NT	NT	NT	–	NT	–
	7F8	Nichinan	IgM	–	–	++	–	–	–	–	NT	–	–	–	–	–	–	–
	1F2	Nichinan	IgM	–	–	++	–	–	–	–	NT	–	–	–	–	–	–	–
C	1803	Syd53	IgG3	–	–	–	++	++	++	++	++	++	++	++	++	–	–	–
	8083	Syd53	IgG3	–	–	NT	++	++	NT	NT	++	NT	NT	NT	NT	–	–	–
	1015	Syd53	IgG2b	–	–	–	–	++	++	++	++	++	+	–	–	–	–	–
D	819	Syd3	IgG1	–	–	NT	–	–	NT	NT	–	NT	NT	NT	NT	++	++	–
	806	Syd3	IgG1	–	–	NT	–	–	NT	NT	–	NT	NT	NT	NT	++	++	–
E	1496	NK24	IgG1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+++
	155	NK24	IgG1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+++
	4971	NK24	IgG1	–	–	NT	–	–	NT	NT	–	NT	NT	NT	NT	–	NT	+++
	1052	NK24	IgG1	–	–	NT	–	–	NT	NT	–	NT	NT	NT	NT	–	NT	+++
	anti-SaV serum			+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	PBS			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a]Reactivities: +++, strong (OD ratio of sample/PBS > 10); ++, moderate (5–9); +, weak (1–4); –, negative (< 1); NT, not tested.

[b]Genogroup

[c]Genotype (22)

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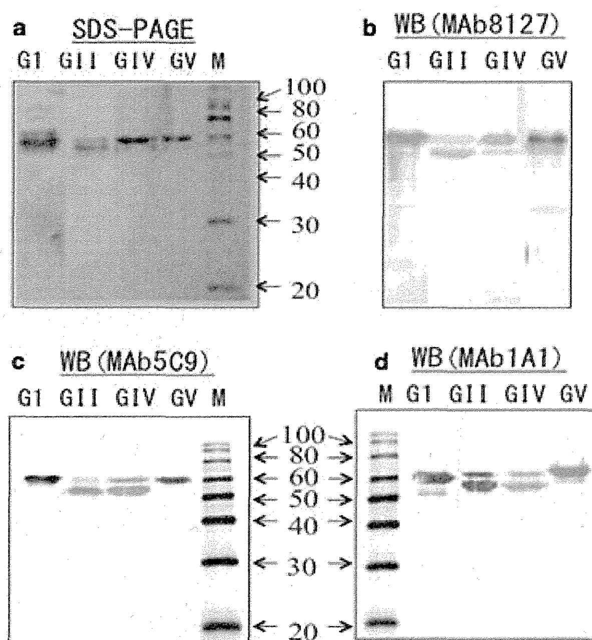

Fig. 2. SDS-PAGE of purified SaV VLPs and western blots. VLPs from (a, b) GI (GI.5 Yokote1) and (c, d) GI.6 Nichinan, GII (20072248), GIV (Yakumo8) or GV (NK24) were separated by SDS-PAGE. The proteins were stained with (a) CBB or transferred to a nitrocellulose sheet which was then incubated with (b) MAbs 8127, (c) 5C9 or (d)1A1. Lane M, molecular mass markers (kDa).

by WB analysis to react with GI, GII, GIV and GV SaV VP1 with an apparent molecular mass of about 60 kDa (Fig. 2). These results indicate that SaV capsid protein carries at least one epitope common to GI, GII, GIV and GV SaV. Protein bands smaller than 60 kDa are likely truncated VP1 as previously described, though direct evidence is lacking (14).

Genogroup- or genotype-specific monoclonal antibodies

Seven group B MAbs reacted exclusively with GI VLPs, but not with any other VLPs from GII, GIV and GV SaVs. MAb 616 bound to three GI SaV VLPs (GI.1 Mc114, GI.5 Yokote1 and GI.6 Nichinan) in a genogroup-specific manner, whereas MAbs 1325, 5001, 4357, 627, 7F8 and 1F2 bound to VLPs in a genotype-specific manner (Table 1). Three group C MAbs (1803, 8083 and 1015) showed reactivity only with GII VLPs. MAb 1803 reacted to all GII VLPs (GII.2, -3, -4, and -7) examined, but not to GI, GIV and GV VLPs. On the other hand, MAb 1015 reacted with all GII.3 VLPs, but not with GII.2, GII.4, GII.7, GI, GIV and GV VLPs, demonstrating that this MAb is likely to be GII.3-specific (Table 1). Two group D MAbs (819 and 806) were specific to GIV (Syd3 and Yakumo8) VLPs and did not bind to GI, GII and GV VLPs. Four group E MAbs (1496, 155, 4971 and 1052) were specific to GV VLPs (Table 1).

Epitopes recognized by cross-reactive monoclonal antibodies

Three MAbs classified as group A (5C9, 1A1 and 8127) had broad reactivity to VLPs derived from all human SaVs genogroups (GI, GII, GIV and GV). The specificity of these MAbs was further examined by competitive ELISA. When GI.6 Nichinan VLP was used as the coating antigen, binding of MAb 5C9 was completely inhibited. In addition, binding of MAb 5C9 was similarly blocked by three heterotypic VLPs (GII.7 20072248, GIV Yakumo8 and GV NK24) (Fig. 3a). Similar results were obtained when GII.7 20072248 VLP was used as the coating antigen. Binding of MAb 5C9 was completely inhibited by homotypic GII.7 VLP and also by three heterotypic VLPs, GI.6 Nichinan, GIV Yakumo8 and GV NK24 (Fig. 3b). The results were similar when two other VLPs, GIV Yakumo8 and GV NK24, were used as the coating antigens (data not shown). Binding of MAb 1A1 to four VLPs (GI.6 Nichinan, GII.7 20072248, GIV Yakumo8 and GV NK24), as well as inhibition by homotypic and heterotypic VLPs was exactly the same as that of MAb 5C9 (Fig. 3c, d), suggesting these two MAbs (5C9 and 1A1) recognize a common epitope. On the other hand, binding of MAb 8127 to GI.5 Yokote1 and GV NK24 VLPs was different. Binding of MAb 8127 to GI VLP was strongly blocked by both homotypic GI VLP and heterotypic GV VLP (Fig. 3e) and binding to GV VLP was similarly inhibited by both homotypic GV VLP and heterotypic GI VLP (Fig. 3f). However, inhibition by two other competitor VLPs, GII.7 20072248 and GIV Yakumo8, was incomplete (Fig. 3e, f), demonstrating that the epitope recognized by MAb 8127 is different from that of MAbs 5C9 and 1A1.

DISCUSSION

In this study, we established 65 hybridoma cell lines from six mice immunized with six SaV VLPs, and characterized 24 MAbs in detail. These MAbs were classified into group A (MAbs broadly cross-reactive to all GI, GII, GIV and GV strains), and groups B–E (genogroup-specific or genotype-specific MAbs). We also obtained another 27 MAbs specific to particular strains: five to GI.1 Mc114, three to GI.5 Yokote1, five to GII.3 Syd3, five to GIV Syd3, and nine to GV NK24 (data not shown). In addition, 14 MAbs were positive by ELISA but negative by WB. We did not further characterize these 41 MAbs in this study.

Group A MAbs are broadly reactive to all GI, GII, GIV and GV SaV VLPs by both ELISA and WB (Table 1

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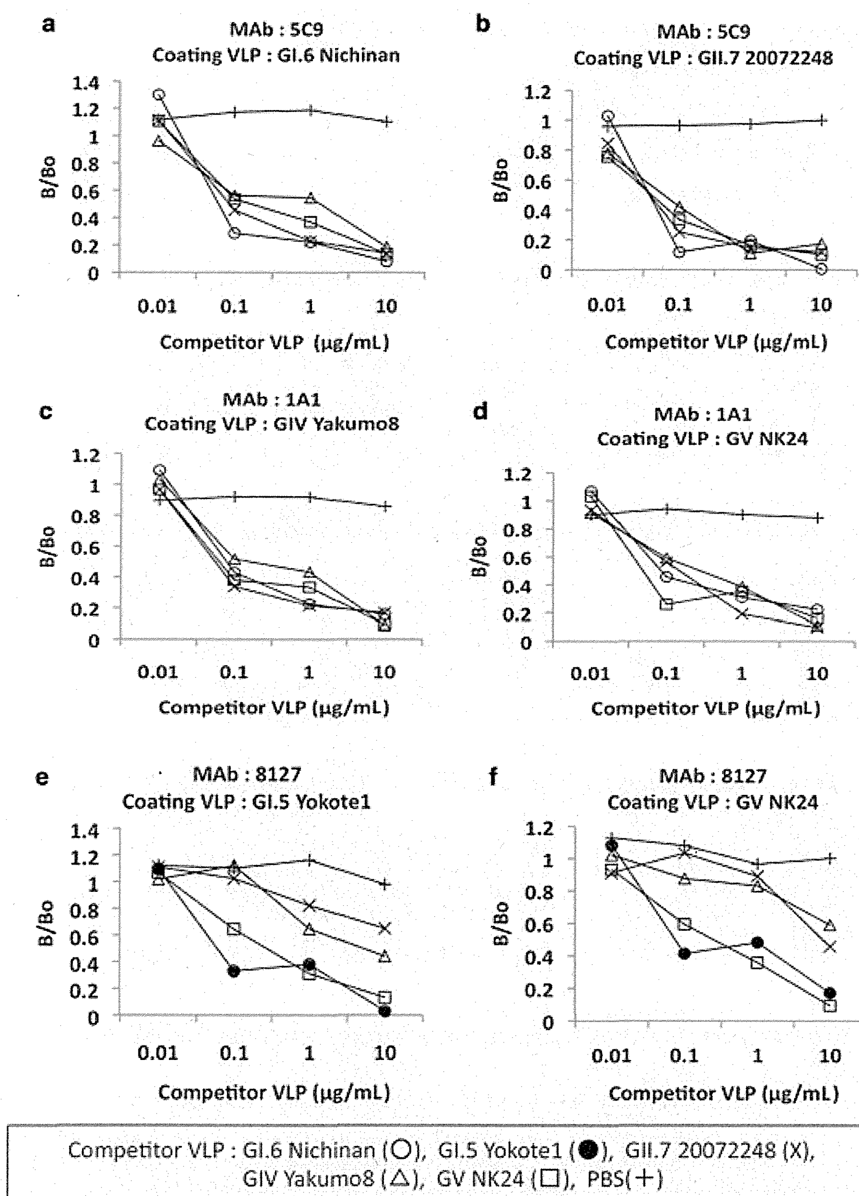

Fig. 3. Competitive ELISA to differentiate epitope recognition. Microplates were coated with VLPs from (a) GI.6 Nichinan, (b) GII.7 20072248, (c) GIV Yakumo8, (e) GI.5 Yokote1, or (d, f) GV NK24. A reaction mixture of the various concentrations of the competitor VLPs and a MAb was added to the plates and binding of the MAb to the coated VLPs was measured as described in Materials and Methods. The competitor VLPs used in this experiments were ○, GI.6 Nichinan; •, GI.5 Yokote1; X, GII.7 20072248; △, GIV Yakumo8; □, GV NK24. PBS(+) was used as a control without competitor. (a, b) MAbs 5C9, (c, d) 1A1 and (e, f) 8127 were incubated with the competitor VLPs.

and Fig. 2), indicating that cross-reactive epitope(s) is/are present on the SaV VP1 in these four genogroups. Based on the competition ELISA, the epitopes recognized by MAbs 5C9 and 1A1 seems to be common to them but different to that of MAb 8127 (Fig. 3). In ELISA, the reactivity of three MAbs (5C9, 1A1 and 5C1) was consistent and strong to GI, GII, GIV and GV VLPs. MAbs 8127 reacted strongly with GI and GV VLPs but weakly with GII and GIV VLPs (Table 1). The different reactivities between different genogroups of SaV VP1 partly supports the possibility of distinct epitopes for MAbs 5C9, 1A1 and MAb 8127.

The X-ray crystallographic structure of human SaV has not been reported, but cryo-electron microscopy has revealed structural similarities between human SaV VLPs and NoV VLPs (8). X-ray crystallographic studies of

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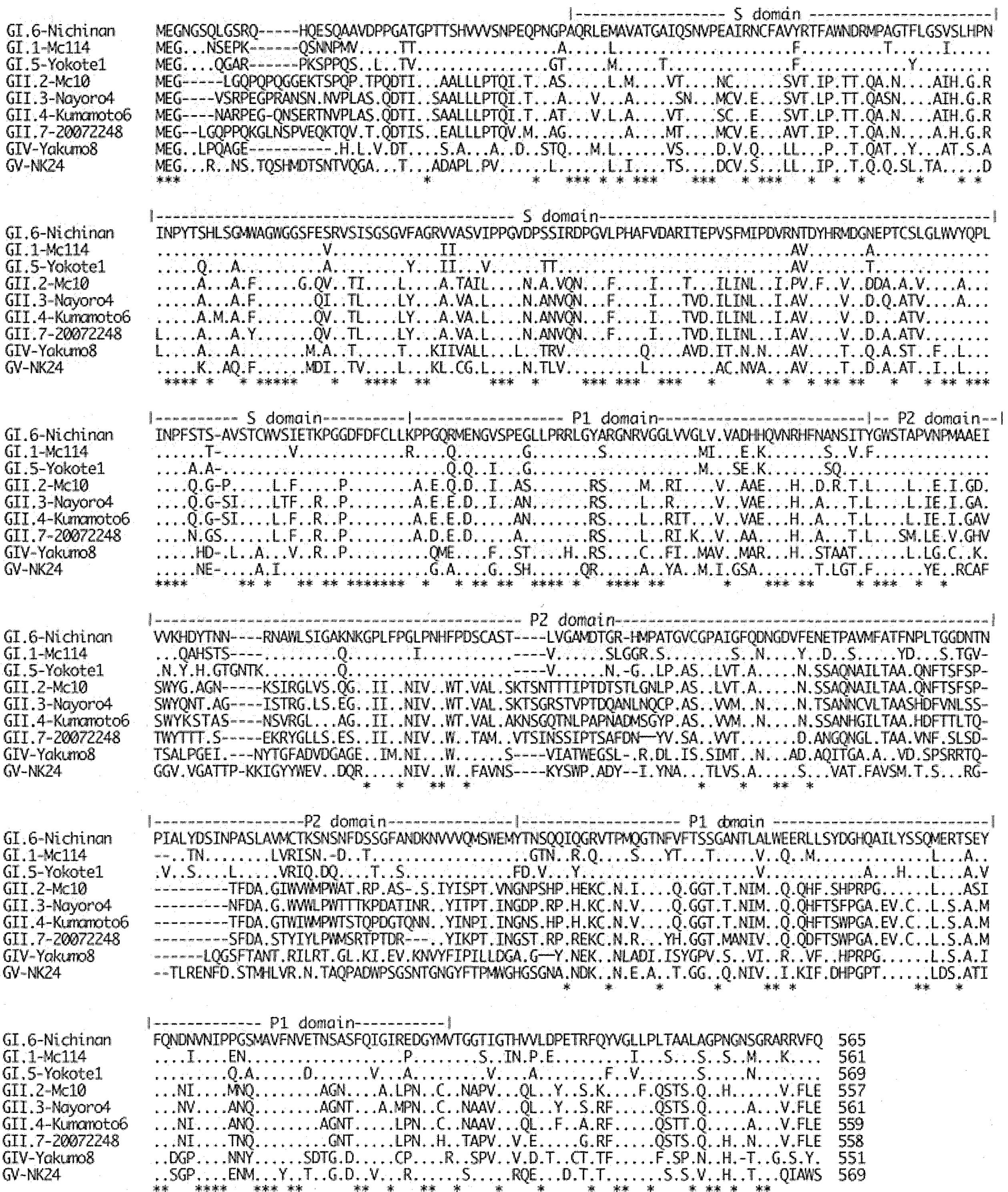

Fig. 4. Amino acid alignment of nine representative SaV VP1 protein sequences. Predicted amino acid sequences of GI.6 Nichinan, GI.1 Mc114, GI.5 Yokote1, GII.2 Mc10, G II.3 Nayoro4, GII.4 Kumamoto6, GII.7 20072248, GIV Yakumo8 and GV NK24 VP1 are shown. Asterisks indicate conserved amino acids among the 15 SaV strains used in this study. Dots indicate identical amino acid residues. The extents of S and P domains indicated in the above sequences were inferred from published information about NoV VP1 (8).

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human NoV VLPs has revealed that NoV VP1 has the following two principal domains: a shell (S) domain, and a protrusion (P) domain that is further divided into three subdomains called N-terminal P1, P2 and C-terminal P1 (36). The NoV S domain and P1 subdomains are highly and moderately conserved, respectively. The P2 domain is highly variable among NoV strains (8, 37), this domain is likely to be the key determinant of strain specificity and antigenicity (37). MAbs broadly cross-reactive with NoV VLPs have been described (31, 34, 38–41), these studies demonstrating that the epitopes are located in the S domain (40, 41) or C-terminal P1 domain (38, 42) in the NoV VP1.

Hyper-immune sera raised against human SaV VLPs has revealed distinct antigenicity among different human SaV genogroups and genotypes (15–17, 20, 21, 23, 43). Genogroup- and genotype-specific MAbs (group B–E MAbs) were also isolated in this study. These MAbs will be useful tools for further study of the antigenic determinant of human SaV. Amino acid sequence homology of VP1 among the 15 SaV strains is 28.1% (data not shown). Despite these significant amino acid sequence variations among different genogroups and genotypes of SaV VP1, the predicted S domain is relatively more conserved than the P2 domains (Fig. 4). From the amino acid sequence alignment, the antigenic determinant is likely to be present in the predicted P2 domain in human SaV, and common epitopes may occur in the predicted S or P1 domains, although further experiments are necessary.

In conclusion, we have established a panel of MAbs that are reactive with human SaV VLPs in a broad, genogroup-specific or genotype-specific manner. The broadly reactive MAbs are of particular interest as possible reagents for the development of human SaV detection or diagnostic assays (i.e., ELISA or immunochromatography) in clinical settings, because ELISA using hyperimmune sera shows narrow reactivity to specific genogroups (15–17, 21, 23, 43). Because human SaVs have also been detected in clams (25,44), oysters (45) and environmental water (46–51), broadly reactive MAbs may also become valuable tools for concentrating or removing human SaVs from food or environmental specimens.

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DISCLOSURE

The authors declare no financial or commercial conflicts of interest.

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NOTE

Detection of Sapovirus in oysters

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ABSTRACT

SaV sequences which are either genetically identical or similar were detected from oysters, feces from gastroenteritis patients, and domestic wastewater samples in geographically close areas. This is the first report of the detection of SaV in oysters which meet the legal requirements for raw consumption in Japan.

Key words commercial oyster, genotyping, human sapovirus, water contamination.

Outbreaks and sporadic infections of acute gastroenteritis caused by SaV have been described all over the world (1–4). *Sapovirus*, a genus of the family *Caliciviridae*, is a non-enveloped, positive-strand RNA virus (5). SaV is genetically classified into at least five genogroups, of which genogroups I, II, IV, and V infect humans (2). SaV has been detected by RT-PCR from a variety of epidemiological sources, including fecal specimens from symptomatic and asymptomatic individuals (6), environmental water (7, 8), and bivalves (9), indicating that SaV can be transmitted via fecal-oral routes through water and contaminated foods, as well as via person-to-person contact. Although several enteric viruses are associated with oyster-related gastroenteritis outbreaks (10), and SaV has been detected in feces from patients in oyster-related gastroenteritis outbreaks (11), the detection of human SaV from oysters has not been reported.

The aim of this study was to determine whether SaV could be detected in oysters for raw consumption, as well as in geographically linked settings, such as primary-treated wastewater, secondary-treated effluent from a wastewater treatment plant, oysters cultivated in an estuary receiving effluent, and fecal specimens from gastroenteritis patients. Phylogenetic analysis was performed to characterize the SaV detected in this study.

Fourteen oysters meant for raw consumption were purchased from local fish markets in Miyagi prefecture, Japan in December 2007. The other 16 oysters, immersed in river water receiving effluent from a wastewater treatment plant, were harvested after being cultivated for 2 months (October–December 2007). The oysters were shucked and digestive diverticula aseptically separated. Viruses in the digestive diverticula were eluted using a method described previously (12). Briefly, a digestive diverticulum in a polyethylene tube was mashed up with two stainless-steel beads and 1 ml of distilled, deionized water using Micro Smash MS-100 (TOMY, Tokyo, Japan) for 1 min at 4500 rpm. The supernatant was recovered by centrifugation at 10 000 rpm for 10 min and stored as a virus concentrate.

A total of 13 primary-treated wastewater samples and 13 secondary-treated effluent samples were collected from August 2007 to January 2008 from the wastewater treatment plant located near the river mouth in the oyster-harvesting area. Viruses in primary-treated wastewater and secondary-treated effluents were concentrated by the

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List of Abbreviations: GI, genogroup I; GIV, genogroup IV; SaV, Sapovirus.

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polyethylene glycol precipitation method (12). In addition, SaV-positive fecal specimens from two gastroenteritis outbreaks near the investigation area during the same season (September and October 2007) were included in the analysis. Stool samples were suspended in nine times the weight of distilled, deionized water, and the supernatant collected after being centrifuged at 9200 *g* for 10 min.

Viral RNA was extracted from samples using a QIAamp Viral RNA mini kit (Qiagen, Tokyo, Japan). Complementary DNA was synthesized using a hexa-oligomer random primer with SuperScript III First-Strand Synthesis System (Invitrogen, Tokyo, Japan). The SaV gene was amplified by nested RT-PCR, F13/14 and R13/14 primers being used in the first PCR, and F22 and R2 primers in the nested PCR (13). Nested RT-PCR products were separated with 2% agarose gel electrophoresis, purified with a QIAquick Gel Extraction Kit (Qiagen), and directly sequenced by ABI PRISM 310 (Applied Biosystems, Tokyo, Japan) by using F22 and R2 primers separately. The nucleic acid sequences (approximately 320 nucleotides) were aligned, and a neighbor-joining phylogenetic tree with 1000 bootstrap replicates was generated using ClustalX software (version 2.0.10).

SaV was detected in 1 of the 14 commercial oysters (7.1%), 3 of the 16 cultivated oysters (18.8%), 8 of the 13 primary-treated wastewater samples (61.5%), and 2

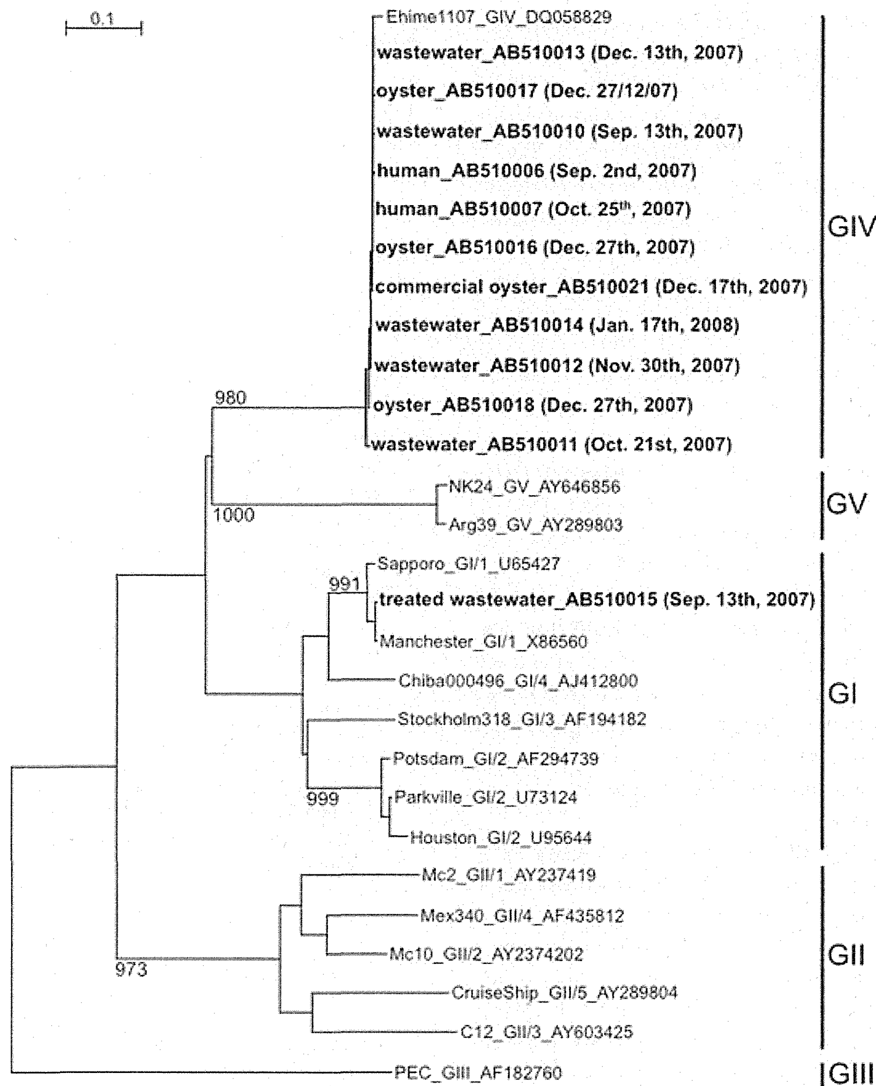

Fig. 1. Neighbor-joining phylogenetic tree of Sapovirus capsid nucleotide sequences acquired in this study. Numbers on each branch indicate bootstrap values for the genotype. Bootstrap values greater than 950 were considered statistically significant for the grouping. The scale bar represents nucleotide substitutions per site. Strains with bold characters were obtained in this study, and the date of sampling is given in the parenthesis of each strain name.

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of the 13 secondary-treated effluent samples (15.4%). The nucleotide sequences of the partial capsid gene were determined from 10 samples in total (all positive oyster samples, five primary-treated wastewater samples and one secondary-treated effluent sample). Sequences in the remaining four positive samples were difficult to determine by the direct sequencing approach, probably because of the presence of mixed sequences. Phylogenetic analysis based on the determined sequences revealed that the SaV detected in the commercial oysters for raw consumption (accession number: AB510021) belonged to GIV (Fig. 1). Genetically similar or identical sequences of SaV GIV (99.4–100% nucleotide identities in 321 nucleotides) were detected from three cultivated oysters (AB510016–AB510018), five primary-treated wastewater samples (AB510010–AB510014), and two fecal specimens (AB510006 and AB510007) collected during the same season (Fig. 1). SaV belonging to GI (AB510015) was also detected in a secondary-treated wastewater sample (Fig. 1). Both GI and GIV genotypes were found in water samples, but only GIV was detected in oysters and human fecal specimens. The prevalence of SaV GIV infections in Japan during the investigation period might explain the frequent detection of this genotype in human feces in this study. The reason for only GIV being detected in oysters is not clear, but differences between SaV genotypes in the efficiency of accumulation in oysters should be investigated in a future study.

To our knowledge, this is the first study to report detection of human SaV in commercial oysters that meet the legal requirements for raw consumption in Japan. Furthermore, our results demonstrate that SaV can accumulate in oysters which are cultivated in an estuary receiving treated wastewater. The presence of very similar or identical nucleotide sequences of SaV GIV in fecal specimens from gastroenteritis patients, wastewater, cultivated oysters, and oysters for raw consumption during the same epidemiological season implies that SaV excreted in feces are released into environmental water, and can contaminate commercial oysters.

The contribution of effluent from wastewater treatment plants and septic tanks to viral contamination of water environments has been reported for several enteric viruses, including norovirus (12, 14, 15). Our investigation shows that wastewater effluent can also lead to SaV contamination. The importance of virus removal during wastewater treatment processes, especially in regions adjacent to oyster cultivation areas, demands investigation in order to prevent contamination of cultivated oysters with enteric viruses. Furthermore, at present enforcement of food sanitation regulations in Japan focus mainly on bacterial contamination. Although further studies are needed in order to determine the health risks associated with eating virus-contaminated oysters, regulations and standards may need to be revised in order to address the problem of viral contamination of oysters. Furthermore, co-contamination with norovirus, a major causative agent of acute gastroenteritis, also should be included in a future study to discuss the regulations regarding oyster cultivation.

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BRIEF REPORT

A foodborne outbreak of sapovirus linked to catered box lunches in Japan

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Abstract Sapovirus (SaV) is a common cause of acute viral gastroenteritis worldwide, and SaV outbreaks have become more frequent in recent years. In January 2010, an outbreak of acute gastroenteritis due to SaV occurred in Aichi, Gifu and Mie Prefectures, Japan. The illness was strongly associated with eating a delivered box lunch prepared by one catering company. In total, 655 (17.1 %) of 3827 individuals developed gastroenteritic symptoms. SaV was detected in seven of the nine people who became ill and in seven of the 52 food handlers at the catering company, but all the tested samples were negative for norovirus and enteropathogenic bacteria. Sequence analysis of RT-PCR products indicated that the nucleotide sequences of SaV strains from the people who became ill and the food handlers were identical. The detected SaV strains were genogrouped as SaV genotype I.2. This was the largest foodborne outbreak of sapovirus in Japan.

Sapovirus (SaV), a member of family *Caliciviridae*, is known as one of the major causative agents of viral gastroenteritis affecting both children and adults [1, 2]. SaV has a single-stranded positive-sense RNA genome of approximately 7.3 to 7.5 kb in length, containing two or three open reading frames (ORF). ORF1 encodes the nonstructural proteins and a capsid protein. ORF2 and ORF3 encode putative proteins with unknown functions [1, 3]. On the basis of capsid gene sequences, SaV strains can be divided into at least five genogroups, GI–GV, of which the GI, GII, GIV, and GV strains infect humans, while the GIII strains infect porcine species [3, 4].

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The SaV prototype strain, Sapporo virus (Hu/ Sapovirus/ Sapporo virus/1977/JP), was first identified in an outbreak of gastroenteritis in an infant home in Sapporo, Japan, in 1977. Sapovirus was primarily known as a common cause of viral gastroenteritis in infants and young children [5]. Outbreaks of SaV are less frequent than those of NoV. However, in recent years, SaV has increasingly been identified as a cause of outbreaks of nonbacterial gastroenteritis in various epidemiological settings, including a kindergarten [6], an elementary school [7], a junior high school [8], a college [9], a hospital [10], a senior residence [2], a hotel [11] and a wedding hall [12]. The age groups affected in these settings have ranged from children to the elderly.

This report describes a large-scale foodborne outbreak of SaV linked to catered box lunches in Japan. In January 2010, an outbreak of acute gastroenteritis occurred simultaneously in Aichi, Gifu and Mie Prefectures in central Japan. Of the 3827 individuals who had eaten a delivered box lunch prepared by a catering company in Aichi Prefecture, 655 (17.1 %) developed gastrointestinal illness. Epidemiologic studies revealed that illness was significantly associated with eating a box lunch on January 21. The main symptoms of the 655 people who became ill were diarrhea (92.8 %), abdominal pain (62.7 %), nausea (59.8 %), chills (39.2 %), vomiting (37.1 %), malaise (35.4 %), fever (32.5 %), and headache (20.2 %). Of the 655 who became ill, 350 (53.4 %) developed gastroenteritic symptoms within 24 to 48 h after the suspicious lunch on January 21. The epidemic curve showed characteristics of a single exposure route. The mean incubation time was estimated to be 43 h (Fig. 1).

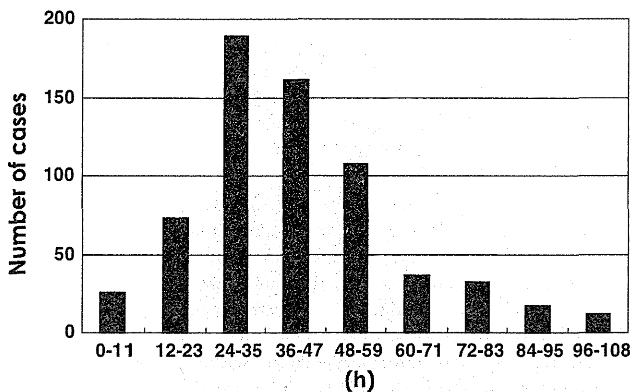

Fig. 1 Epidemic curve of gastroenteritic patients after eating suspicious lunch (n = 655)

To investigate the etiologic agent, stool samples were collected from nine of the individuals who became ill and 52 food handlers from the catering company. Routine microbiological cultures for enteric bacterial pathogens were performed, and all of the samples gave negative results. The stool samples were tested for norovirus (NoV) and SaV by real-time reverse-transcription polymerase chain reaction (RT-PCR). Viral RNA was extracted from 200 μl of 10 % stool sample suspensions in distilled water using a High Pure Viral RNA Kit (Roche Applied Science, Tokyo Japan) according to the manufacturer's instructions. RNA was then recovered in 50 μl of nuclease-free water. A real-time RT-PCR assay was performed separately using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Tokyo, Japan) with NoV-specific primers/probes [13] and SaV-specific primers/probes [14]. SaV was detected in seven of the nine people who became ill and in seven of the 52 food handlers, but all samples were negative for NoV. The viral loads in stool samples were calculated based on a standard curve with serial 10-fold dilutions (10^7 to 10^1 copies) of cDNA plasmid that was supplied from National Institute of Infectious Diseases in Tokyo, Japan [14]. The viral loads in seven samples from the patients ranged from 2.0×10^8 to 2.9×10^{10} copies/g stool, and those from the seven food handlers ranged from 1.5×10^7 to 7.5×10^9 copies/g stool. Of the seven SaV-positive food handlers, two showed symptoms of gastroenteritis, and the other five were asymptomatic during the outbreak. Similar viral loads were observed for symptomatic and asymptomatic food handlers.

Real-time RT-PCR-positive samples were genotyped by direct sequencing after amplification of the partial capsid gene by universal nested RT-PCR with primers SV-F13, SV-F14, SV-R13, and SV-R14 for the first PCR, and SV-F22 and SV-R2 for the second PCR [15]. PCR products were purified using the Wizard SV Gel and PCR Clean up system (Promega, Tokyo, Japan), and the purified DNAs were sequenced directly using a BigDye Terminator v3.1Cycle Sequencing Kit (Applied Biosystems) and an automated sequencer (3130 Genetic Analyzer, Applied Biosystems). Nucleotide sequences were aligned with Clustal W, and then a phylogenetic tree was constructed by the neighbor-joining method.

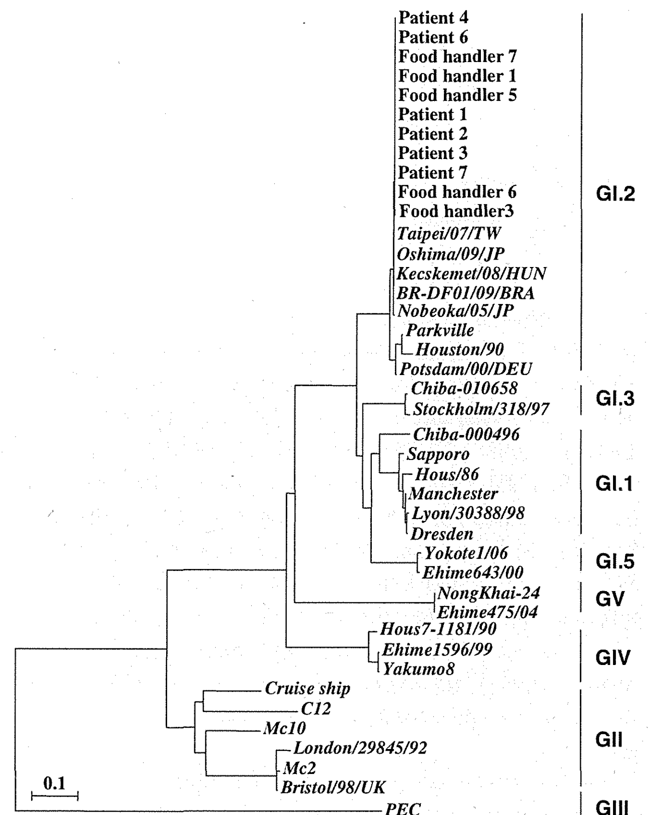

Fig. 2 Phylogenetic analysis of partial capsid gene sequences of SaV strains detected from ill patients and food handlers using the neighbor-joining method. Reference strains of SaV used in this analysis are shown in italics. The GenBank accession numbers for the reference strains of SaV are as follows: Taipei/07/TW, EU124657; Oshima/09/JP, AB518056; Kecskemet/08/HUN, FJ844411; BR-DF01/09/BRA, AB614356; Nobeoka/05/JP, AB455801; Parkville, U73124; Houston/90, U95644; Potsdam:/00/DEU, AF294739; Chiba-010658, AJ606696; Stockholm/318/97, AF194182, Chiba-000496, AJ606693; Sapporo, U65427; Hous/86, U65643; Manchester, X86560; Lyon/30388/98, AJ251991; Dresden, AY694184; Yokote1/06, AB253740; Ehime643/00, DQ366345; NongKhai-24, AY646856; Ehime475/04, DQ366344; Hou7-1181/90, AF435814; Ehime1107, DQ058829; Yakumo8, AB455795; CruiseShip, AY289804; C12, AY603425; Mc10, AY237420; London/29845/92, U95645; Mc2, AY237419; Bristol/98/UK, AJ249939; PEC, AF182760

Three of 14 real-time RT-PCR-positive samples were found to be negative by conventional RT-PCR, probably because real-time RT-PCR may be more sensitive than the RT-PCR used in this study. Sequence analysis of capsid SaV partial nucleotide sequences of the nested RT-PCR product (approximately 420 nt) showed that 11 sequences from this outbreak had 100 % nucleotide identity,

indicating that a single SaV strain caused this outbreak. Based on the phylogenetic analysis with the partial capsid nucleotide sequences, strains from this outbreak were clustered into genogroup I/genotype 2 (Fig. 2).

BLAST sequence analysis revealed that the SaV strains from our outbreak showed 99.5 % nucleotide sequence identity to the strain Hu/Oshima1/2009/JP (accession no. AB518056), which was detected in a gastroenteritis outbreak in school children in Hokkaido, Northern Japan. In this outbreak, a causative food was not identified, and SaV infection via human-to-human transmission may be a possible source [7]. Closely matching sequences (99.5 %) were also observed in Hu/BR-DF01 /2009/BRA (AB6 14356) and Hu/MN/ME08-4902/2008/US (HM800 917). SaV GI.2 has been quite frequently detected from outbreak cases in Europe, including the Netherlands, Sweden, Slovenia, and Hungary during 2007–2009 [16]. SaV GI.2 was also detected in an outbreak of gastroenteritis among college students in Taiwan in 2007, but the source of the outbreak was not determined [9]. These results suggest the worldwide circulation of SaV GI.2, though the main source of SaV infection still remains unclear.

In conclusion, we indicate that our outbreak was probably caused by contaminated food from food handlers who were shedding the virus, though the specific food item was not identified. This was the largest foodborne SaV outbreak in Japan. This study shows the importance of SaV as one of the etiological agents for large-scale foodborne outbreaks of nonbacterial gastroenteritis.

Nucleotide sequence accession number: The nucleotide sequence of SaV determined in this study was deposited in the DNA Data Bank of Japan under accession number AB607855.

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Key words
ノロウイルス
サポウイルス
掃除機
ダスト

経験

ノロウイルスおよびサポウイルスの掃除機内ダスト中の汚染実態調査

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要旨　ノロウイルス感染症の感染様式の一つとして塵埃感染が知られている．筆者らは，結婚式披露宴会場において，この塵埃感染が疑われた事例に遭遇した．その際，掃除機内のダストから患者便由来のノロウイルス株と同一株が検出されたことから，掃除機内のダストの検査が感染経路の推定に有用であった．この事例をきっかけとして，一般家庭の掃除機内ダストについて，ノロウイルスおよびサポウイルスの汚染実態調査を実施した．59検体について検査した結果，ノロウイルスは2検体（3.4%），サポウイルスは1検体（1.7%）から検出され，その汚染ウイルス量はダスト1gあたり10^6個を超えるものも存在したことから，汚染ダストは重要な感染源の一つになると考えられた．

はじめに

ノロウイルスおよびサポウイルスは，ともにカリシウイルス科に属し，乳幼児から高齢者まで幅広い年齢層に感染する．これらのウイルスに感染した患者は，嘔吐や下痢などの胃腸炎症状を呈し，患者の嘔吐物や糞便中には大量のウイルス粒子が存在することが知られている[1)2)]．また，これらウイルスの感染力は強く，ノロウイルスについてはわずか10～100個のウイルス粒子で感染が成立すると考えられている[3)]．

ノロウイルスおよびサポウイルス感染症は，本ウイルスに感染したヒトからヒトへ，直接あるいは間接的に接触することによって拡散するほか，ノロウイルスに汚染された塵埃を嚥下することにより感染する塵埃感染（dust transmission）により広がることが知られている[4)]．筆者らは結婚式披露宴会場において，この塵埃感染の疑われたノロウイルスによる集団感染性胃腸炎事例に遭遇した[5)]．その際，感染経路を推定する決め手となったのは，当該会場専用の掃除機内のダストからノロウイルスが検出されたことであった．

本稿では，ノロウイルスおよびサポウイルスの性状やこれら感染症の臨床症状ならびに感染経路について概説するとともに，われわれが厚生労働科学研究費補助金食品の安心・安全確保推進事業「食品中のウイルスの制御に関する研究」の助成を一部受けながら調査を行った，掃除機内ダストにおけるウイルスの汚染実態について述べることにする．

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I　ノロウイルスおよびサポウイルス

1．分類

ノロウイルスおよびサポウイルスは，ともにカリシウイルス科に属する直径 30〜40 nm の球形ウイルスで，以前は小型球形ウイルス（small round structured virus：SRSV）とよばれていた．これらのウイルスは 7.5〜7.7 kb のプラス 1 本鎖 RNA のゲノムを保有する．ノロウイルスは 5 つの遺伝子群（GⅠ〜GⅤ）に分類され，ヒトに病原性を示すのは GⅠ，GⅡ および GⅣ である．さらに，GⅠ は 14 の遺伝子型に，GⅡ は 21 の遺伝子型にそれぞれ少なくとも分類されている[6]．一方，サポウイルスは 5 つの遺伝子群（GⅠ〜GⅤ）に分類され，ヒトに病原性を示すのは GⅠ，GⅡ，GⅣ および GⅤ で，GⅠ と GⅡ はそれぞれ複数の遺伝子型に分類されている[7]．

2．臨床症状

ノロウイルスは，乳幼児から高齢者まで幅広い年齢層に急性の胃腸炎を引き起こす主要なウイルスであり，主な臨床症状は，吐き気，嘔吐，下痢，腹痛，発熱などである．潜伏期間は通常 1〜2 日で，発病後 2〜3 日で軽快し一般的に予後は良好である．患者は糞便中に多量のウイルス粒子を排泄し，症状が治まっても 10 日間程度はウイルスの排泄が続くことが知られている[1]．

サポウイルスの場合，症状はノロウイルスと同じかやや軽い傾向があるものの，それ以外はノロウイルスの場合と同様である．

なお，両ウイルスとも不顕性感染が存在する．

3．感染経路

ノロウイルスおよびサポウイルス感染症は，これらウイルスに感染したヒトから接触感染や飛沫感染によって感染する（person-to-person）ほか，感染者によって汚染された食品あるいはウイルスを中腸腺に蓄積した二枚貝を生もしくは加熱不足で経口摂取することで感染する食品媒介感染（いわゆる食中毒：foodborne）が知られている．

ノロウイルスは乾燥に強く，4℃では 60 日間，室温（20℃）では 20 日間以上生残可能と考えられている[8]．このため，ノロウイルス感染者の嘔吐物などによって環境が汚染された後乾燥すると，塵埃とともにウイルス粒子が空中に舞い上がり，それを経食道的に嚥下することで感染を起こす塵埃感染に関する報告もされている[1]．

4．ウイルス検出報告数

2008 年第 36 週〜2011 年第 22 週に全国の地方衛生研究所において感染性胃腸炎患者便等から検出された，ノロウイルスおよびサポウイルスの週別検出報告数を図 1 に示した．ノロウイルスの検出報告数は 1 シーズンあたり 3,000 件前後で，そのほとんどが遺伝子群 GⅡ である．一方，サポウイルスの検出報告数は 1 シーズンあたり 200 件前後とノロウイルス報告数の 1/10 未満である．

一般的にウイルス性の感染性胃腸炎は，冬期に多いとされており，2008/2009〜2010/2011 シーズンの検出報告数も 11 月から 2 月にかけて，とくにノロウイルスにおいてその傾向が顕著である．一方，サポウイルスはノロウイルス検出報告数が減少しかける 3 月頃から増加する傾向が認められる．

II　掃除機内ダスト中のノロウイルスおよびサポウイルス汚染状況

1．ダストからのウイルス RNA の抽出方法とウイルスの定量方法

ダストからのウイルス粒子の回収は図 2 に示すとおり行い[10]，RNA 抽出用試料とした．ウイルス RNA は抽出用キットを用いて抽出し，その後 DNase 処理および逆転写反応により cDNA を合成した．ノロウイルスおよびサポ

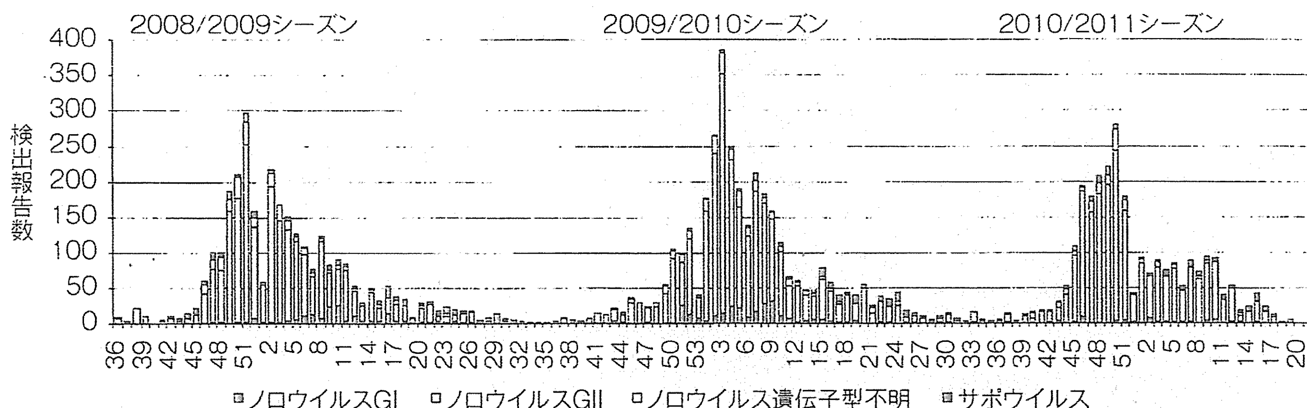

図1 ノロウイルスおよびサポウイルスの週別報告数（2008年36週～2011年22週）（検体採取日に基づく集計）（文献9）より）

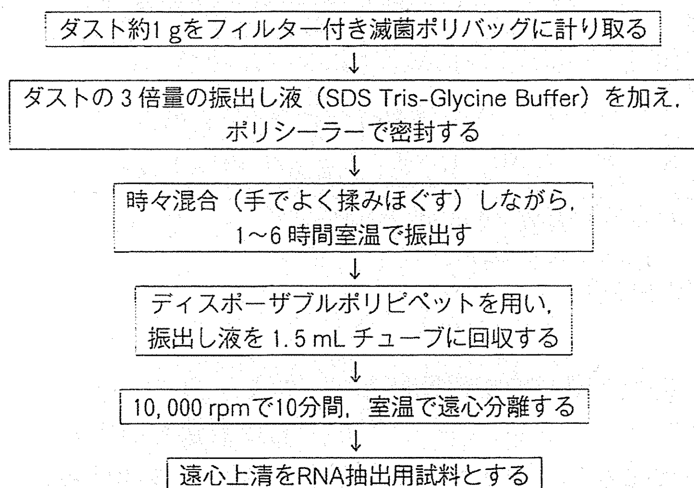

図2 ダストからのノロウイルスおよびサポウイルス回収方法

ウイルスの検出および定量は，リアルタイムRT-PCR（reverse transcriptase-polymerase chain reaction）法で行った[11)12)].

2．一般家庭のダストにおける汚染率

ウイルス性胃腸炎の流行時期である2008年12月～2009年3月（2008/2009シーズン）および2009年11月～2010年3月（2009/2010シーズン）に，一般家庭で使用されている掃除機内から採取されたダスト59検体（47家庭）を試料とした．その結果，ダスト59検体中2検体（3.4%）がノロウイルス陽性，1検体（1.7%）がサポウイルス陽性であった（表1）．ノロウイルスおよびサポウイルスがともに陽性となったダストは認められなかった．シーズン別にみると2008/2009シーズンは35検体中ノロウイルスあるいはサポウイルス陽性がそれぞれ1検体（2.9%），2009/2010シーズンは24検体中1検体（4.2%）がノロウイルス陽性であった（表1）．

表1 一般家庭ダストのノロウイルスおよびサポウイルス汚染実態調査結果

試料採取シーズン	供試検体数	陽性数（%）	
		ノロウイルス	サポウイルス
2008/2009	35	1 (2.9)	1 (2.9)
2009/2010	24	1 (4.2)	0 (0.0)
計	59	2 (3.4)	1 (1.7)

表2 ノロウイルス陽性家庭のダストにおけるウイルス量の推移

家庭（シーズン）	経過日数[a]					家庭内における胃腸炎患者発生の有無
	0	18	30	50	60	
A (2008/2009)	＋ (5.7)[b]	NT[c]	＋ (4.3)	NT	−	有（14日前）
B (2009/2010)	＋ (3.5)	＋ (2.5)	NT	−	NT	有（27日前）

[a]：最初に試料を採取した日を0とした.
[b]：$\log_{10}$ (copies/g of dust)
[c]：試験実施せず.

表3 サポウイルス陽性家庭のダストにおけるウイルス量の推移

ダスト採取箇所	経過日数[a]							
	0	34	45	68	77	92	105	107
集塵器内	＋ (6.6)[b]	＋ (5.2)	＋ (5.2)	NT[c]	−	−	NT	NT
集塵器奥	NT	NT	NT	＋ (6.2)	NT	＋ (4.4)	＋ (5.4)	−

[a]：最初に試料を採取した日を0とした
[b]：$\log_{10}$ (copies/g of dust)
[c]：試験実施せず

3．ダストからウイルスが検出された家庭における汚染状況の推移

ダストがノロウイルスあるいはサポウイルス陽性となった場合は，当該家庭のダスト試料を継続して採取し，ウイルス量の推移を調査するとともに，家庭内における胃腸炎患者の発生などについての聴き取り調査を実施した．

ノロウイルスが検出された2家庭（AおよびB）におけるダスト中のウイルス量の推移をみると（表2），A家庭の初回採取時（0日）のノロウイルス量は$10^{5.7}$コピー/gで，30日に$10^{4.3}$コピー/gに減少し，60日で定量下限未満となった．また，0および30日に採取された試料から検出した株は，いずれも遺伝子型GII.6に属し，さらにカプシド領域の一部塩基配列(287nt)は100％相同であった．このことから，同一株によってA家庭内の環境が少なくとも30日間汚染されていたことが示唆された．B家庭については，0日のノロウイルス量が$10^{3.5}$コピー/gで，18日に$10^{2.5}$コピー/gとなり，50日で定量下限未満となった．

聴き取り調査の結果，A家庭においては，初回採取時の14日前に家族4名中2名が嘔吐・下痢症状を示していたことが判明した．一方，B家庭においては，初回採取時の27日前に5名中1名が嘔吐症状を呈し，その嘔吐物によって寝具を汚染していたことがわかった．以上のことから，胃腸炎症状を示した家族がノロウイルスに感染し，これらの感染者によってそれぞれの家庭内の環境がノロウイルスに汚染されたものと推察された．

一方，サポウイルス陽性家庭で使用されていた掃除機からは，集塵器内ダスト（集塵器内）および集塵器奥のフィルター付着ダスト（集塵器奥）の2種類を試料として採取した．0日の集塵器内サポウイルス量は$10^{6.6}$コピー/gで，34および45日に$10^{5.2}$コピー/gに減少し，77日に定量下限未満となった（表3）．集塵器奥か

ら採取した試料では，68日でも$10^{6.2}$コピー/gで，定量下限未満になったのは107日であった．0，45，68および105日の試料から検出された株について遺伝子解析を行ったところ，いずれも遺伝子群GⅠに属し，カプシド領域の一部の塩基配列（399nt）は100％相同であった．このことから，約3カ月にわたり家庭内環境あるいは掃除機のフィルター面が，同一のウイルス株によって継続して汚染されていたことが明らかとなった．なお，当該家庭においても胃腸炎症状を呈する家族の存在について聴き取り調査を実施したが，明確な回答は得られなかった．

以上のように，家庭内のダストからノロウイルスおよびサポウイルスが検出され，その汚染ダストの中にはウイルス量が10^{6}コピー/gを超えるものも存在していたことが明らかになった．さらに，長期間にわたりノロウイルスなどによる汚染が継続したことから，ダストが感染源となる可能性も示唆され，その取扱いに対する注意喚起が必要と考えられた．

おわりに

ノロウイルスおよびサポウイルスの性状，ならびに両ウイルス感染症の感染経路や症状などについて概要を述べた．さらに，これらのウイルスによる一般家庭の掃除機内ダスト中における汚染実態について，われわれの行った調査結果に基づき示した．その結果，ノロウイルスは3.4％，サポウイルスは1.7％のダストから検出された．さらに，ウイルスが検出された家庭のダストは，長期間継続して同一ウイルス株によって汚染されることが明らかになった．これはダストが長期間感染源になりうることを示唆するものであり，ダストを取り扱う際は屋外の風通しのよい場所で，使い捨ての手袋・マスクをして，ポリ袋へ廃棄したらすぐに密封する，などの注意を払う必要があることを推奨するものである．

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Overestimation of nonsynonymous/synonymous rate ratio by reverse-translation of aligned amino acid sequences

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In the analysis of protein-coding nucleotide sequences, the ratio of the number of nonsynonymous substitutions to that of synonymous substitutions (d_N/d_S) is used as an indicator for the direction and magnitude of natural selection operating at the amino acid sequence level. The d_S and d_N values are estimated based on the comparison of homologous codons, which are often identified by converting (reverse-translating) aligned amino acid sequences into codon sequences. In this method, however, homologous codons may be mis-identified when frame-shifts occurred or amino acid sequences were mis-aligned, which may lead to overestimation of the d_N/d_S ratio. Here the effect of reverse-translating aligned amino acid sequences on the estimation of d_N/d_S ratio was examined through a large-scale analysis of protein-coding nucleotide sequences from vertebrate species. Apparently, 1–9% of codon sites that were identified as homologous with reverse-translation contained non-homologous codons, where the d_N/d_S ratio was unduly high. By correcting the d_N/d_S ratio for these codon sites, it was inferred that the ratio was 5–43% overestimated with reverse-translation. These results suggest that caution should be exerted in the study of natural selection using the d_N/d_S ratio by reverse-translating aligned amino acid sequences.

Key words: nonsynonymous/synonymous rate ratio, reverse-translation, alignment, negative selection, positive selection

INTRODUCTION

Point mutations occurring in protein-coding nucleotide sequences are synonymous or nonsynonymous according to whether they retain or alter coding amino acids, respectively (Miyata and Yasunaga, 1980; Perler et al., 1980). Synonymous mutations are considered to be selectively neutral or nearly neutral, where the rate of synonymous substitution (r_S) may reflect the mutation rate. In contrast, nonsynonymous mutations are considered to be subject to natural selection operating at the amino acid sequence level. Since fixation probabilities of advantageous and deleterious mutations are higher and lower than that of neutral mutations, respectively, the rate of nonsynonymous substitution (r_N) may be greater and smaller than the mutation rate when positive and negative selection operates, respectively. Therefore, natural selection can be detected by comparing r_S and r_N, where $r_S < r_N$, $r_S > r_N$, and $r_S = r_N$ indicate positive, negative, and no selection, respectively (Kimura, 1977).

The comparison of r_S and r_N can be performed by comparing the numbers of synonymous (d_S) and nonsynonymous (d_N) substitutions that have accumulated during the same evolutionary time period (t), because it is expected that $d_S = r_S t$ and $d_N = r_N t$ (Hughes and Nei, 1988). The nonsynonymous/synonymous rate ratio (r_N/r_S) is estimated by d_N/d_S, which reflects the direction and magnitude of natural selection. In haploid organisms, $d_N/d_S = 2N_e s/(1 - e^{-2N_e s})$, where N_e and s denote the effective population size and the selection coefficient, respectively (Nielsen and Yang, 2003). The estimation of d_S and d_N is based on the comparison of homologous codons (Nei and Kumar, 2000; Suzuki and Gojobori, 2003). In the real data analysis, homologous codons are often identified by making a multiple alignment of amino acid sequences and converting it into codon sequences (Suyama et al., 2006; Wong et al., 2008; Schneider et al., 2009; Fletcher and Yang, 2010). This conversion process is called reverse-translation in this paper. The alignment of codon sequences obtained is usually treated as an observation without errors (Wong et al., 2008).

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