III

研究成果の刊行に関する一覧表

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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nakagawa-Okamoto R, Arita-Nishida T, Toda S, Kato H, Iwata H, Akiyama M, Nishio O, Kimura H, Noda M, Takeda N, Oka T	Detection of multiple sapovirus genotypes and genogroups in oyster-associated outbreaks.	Jpn J Infect Dis	62(1)	63-66	2009
Sakano C, Morita Y, Shiono M, Yokota Y, Mokudai T, Sato-Motoi Y, Noda A, Nobusawa T, Sakaniwa H, Nagai A, Kabeya H, Maruyama S, Yamamoto S, Sato H, Kimura H	Prevalence of Hepatitis E virus (HEV) infection in wild boar and Pigs in Gunma Prefecture, Japan.	J Vet Med Sci	71(1)	21-25	2009
Iritani N, Seto T, Hattori H, Natori K, Takeda N, Kubo H, Yamano T, Ayata M, Iritani N, Kaida A, Kubo H, Abe N, Murakami T, Venemma H, Koopmans M, Takeda N, Ogura H, Seto Y	Epidemic of genotype GII2 noroviruses during spring 2004 in Osaka City, Japan.	J Clin Microbiol	46	2406-2409	2008
Iritani N, Vennema H, Siebenga JJ, RJ Siezen, B Renckens, Y Seto, A Kaida, and M Koopmans.	Genetic analysis of the capsid gene of genotype GII.2 Noroviruses.	J Virol	82	7336-7345	2008
Nguyen TA, Hoang L, Pham LD, Hoang KT, Okitsu S, Mizuguchi M, Ushijima H	Norovirus and sapovirus infections among children with acute gastroenteritis in Ho Chi Minh City during 2005-2006.	J Trop Pediatr	54(2)	102-113	2008
Malasao R, Maneekarn N, Khamrin P, Pantip C, Tonusin S, Ushijima H, Peerakome S	Genetic diversity of norovirus, sapovirus, and astrovirus isolated form children hospitalized with acute gastroenteritis in Chiang Mai, Thailand.	J Med Virol	80(10)	1749-1755	2008

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
西尾治	ノロウイルスによる食中毒の原因食材	アニムス	14	36-40	2009
西尾治, 秋山美穂	輸入食品のウイルス汚染の実態とその対策	食品衛生研究	58(10)	23-31	2008
西尾治, 中川(岡本)玲子	ノロウイルス感染症と海産物の安全性	臨床とウイルス	36	305-314	2008
片山丘, 宮原香代子, 古屋由美子	神奈川県域で検出されたノロウイルスの解析	神奈川衛研報告	38	8-11	2008
宮原香代子, 片山丘, 原田美樹, 古屋由美子	神奈川県におけるウイルス性胃腸炎の集団発生状況(平成19年度)	神奈川衛研報告	38	69-71	2008
片山丘, 原田美樹, 宮原香代子, 古屋由美子	感染性胃腸炎患者からの原因ウイルスの検出状況(平成19年度)	神奈川衛研報告	38	72-74	2008

IV

研究成果の刊行物・別刷

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Short Communication

Detection of Multiple Sapovirus Genotypes and Genogroups in Oyster-Associated Outbreaks

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SUMMARY: This report describes multiple viruses in stool specimens from oyster-associated gastroenteritis. Eleven outbreaks of oyster-associated gastroenteritis were examined for enteric viruses between January 2002 and March 2006 in Japan. Multiple norovirus genotypes were detected in all outbreaks; moreover, kobuvirus, sapovirus, and astrovirus were also detected in 6, 3, and 1 of the 11 outbreaks, respectively. Notably, multiple sapovirus genogroups were detected in the stool specimens from subjects in two oyster-associated gastroenteritis outbreaks.

Viral agents of gastroenteritis affect millions of people of all ages worldwide. The major viral agents of gastroenteritis include norovirus, sapovirus, rotavirus, astrovirus, and adenovirus (1,2). Kobuvirus, which is now classified into the family *Picornaviridae*, was also recently identified as a possible pathogen for gastroenteritis (3,4). Noroviruses are the dominant cause of gastroenteritis outbreaks worldwide, and are transmitted through the ingestion of contaminated foods, through the air, and by person-to-person contact (5-7). The majority of human noroviruses can be divided into two genogroups (GI and GII) (8). Recent reports revealed sapovirus to be an important cause of gastroenteritis outbreaks (9-13), although foodborne transmission of sapovirus has not been clearly demonstrated. Sapovirus can be divided into five genogroups (GI to GV), among which GI, GII, GIV, and GV are known to be human pathogens (14,15).

The purposes of this study were to detect norovirus, sapovirus, kobuvirus, and astrovirus in stool specimens collected from subjects in oyster-associated outbreaks of gastroenteritis, and then to address the genetic diversity of norovirus and sapovirus.

Stool specimens were collected from 56 patients and 15 food handlers in 11 oyster-associated outbreaks of gastroenteritis (i.e., outbreaks in which oysters were suspected to be the cause, since all affected individuals consumed or handled oysters) between January 2002 and March 2006 in Japan. This included seven restaurants, three private homes, and a monastery (Table 1). Nucleic acids were extracted from 140 μl of a 10% (w/v) stool suspension with a QIAamp Viral RNA kit (QIAGEN K. K., Tokyo, Japan) according to the manufacturer's protocol, and reverse transcription and reverse transcription-polymerase chain reaction (RT-PCR) were performed as previously described (16). Briefly, for norovirus GI PCR, G1SKF and G1SKR primers were used; and for norovirus GII PCR, G2SKF and G2SKR primers were used (16). For sapovirus, F13, F14, R13, and R14 primers were used to amplify the 1st PCR product, whereas for the nested PCR, F22 and R2 primers were used (17). For kobuvirus, C94b and 264K primers were used, and these were designed to amplify the 3C/D junction (3). For astrovirus, PreCAP1 and 12Gr primers were used to amplify the 1st PCR product, and then Mon244 and 82b primers were used for the nested PCR (18,19). Kobuvirus- and astrovirus-positive specimens were directly sequenced, whereas norovirus and sapovirus specimens were cloned into the pCR2.1 vector (Invitrogen Japan K. K., Tokyo, Japan), and at least four clones from each specimen were sequenced. Nucleotide sequences were determined as described earlier (20). The norovirus and sapovirus sequences determined in this study were registered as EF630535-EF630617 in DDBJ.

Forty-nine of 56 (88%) stool specimens from the patients and 6 of 15 (40%) stool specimens from food handlers were positive for at least one type of virus. Interestingly, about one-third of the specimens (21 of 71 [30%]) were positive for two or more types of viruses (Table 1). Noroviruses were detected in all 11 outbreaks, including 52 of 71 (73%) stool specimens. Norovirus GI sequences were detected in 3 of 11 outbreaks, whereas we detected both norovirus GI and GII sequences in the remaining eight outbreaks. The norovirus GI sequences were separated into 10 genotypes (GI/1-5, GI/8, GI/10, and GI/13-15), while the norovirus GII sequences were separated into six genotypes (GII/3-6, GII/8, and GII/12) (Fig. 1A). Two or more genotypes of noroviruses were detected in 20 of 52 (38%) norovirus-positive specimens (Table 1).

Sapoviruses were detected in 3 of 11 outbreaks, including 5 of 71 (7%) specimens. The sapovirus sequences belonged to GI/1, GII/1, GII/2, and GII/3 (Fig. 1B). Interestingly, we detected two sapovirus genogroups in one stool specimen: SAV-H2a (GII/2) and SAV-H2b (GI/1). Kobuviruses were

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Table 1. Details of the outbreaks showing the setting, no. of persons with symptoms and the viruses detected

Outbreak code	M/D/Y	Setting	No. persons with symptoms	No. specimens collected	Case	Symptoms	Norovirus (genogroup/ genotype)	Sapovirus (genogroup/ genotype)	Kobuvirus	Astrovirus
1	01.23.02	Home	5	3	individual	+	H1 (GI/4)	SAV-H1 (GII/2)	–	–
	01.23.02				individual	+	H2 (GI/4)	–	–	–
	01.23.02				individual	+	H3 (GI/2)	SAV-H2a (GII/2), SAV-H2b (GI/1)	–	–
2	01.23.02	Restaurant	16	14	individual	+	I1 (GII/12)	–	–	–
	01.24.02				individual	+	–	–	+	–
	01.24.02				individual	+	I3a (GI/13), I3b (GI/4)	–	–	–
	01.24.02				individual	+	I4 (GI/13)	–	–	–
	01.24.02				individual	+	–	–	–	–
	01.24.02				individual	+	–	–	–	–
	01.24.02				individual	+	I7 (GII/12)	–	+	–
	01.24.02				individual	+	–	–	–	–
	01.24.02				individual	+	–	–	–	–
	01.24.02				food-handler	–	I10a (GI/4), I10b (GI/13)	–	–	–
	01.24.02				food-handler	–	–	–	–	–
	01.24.02				food-handler	–	–	–	–	–
	01.24.02				individual	+	I13 (GII/12)	–	–	–
	01.24.02				individual	+	I14 (GII/12)	–	–	–
3	01.30.02	Restaurant	39	2	individual	+	J1 (GI/2), J1 (GII/12)	–	–	–
	01.25.02				individual	+	J2 (GII/5)	–	–	–
4	02.26.02	Home	8	4	individual	+	K1 (GII/5)	–	–	–
	02.28.02				individual	+	K2 (GII/5)	–	–	–
	03.01.02				individual	+	K3 (GII/3)	–	–	–
	03.01.02				individual	+	K4 (GI/4)	–	–	–
5	12.25.02	Home	5	4	individual	+	L1a (GI/15), L1b (GI/8), L1a (GII/4), L1b (GII/8)	–	+	–
	12.25.02				individual	+	L2a (GI/10), L2b (GI/13), L2c (GI/4)	–	–	–
	12.25.02				individual	+	L3 (GI/14), L3 (GII/3)	SAV-L3 (GI/1)	–	–
	12.25.02				individual	+	L4 (GI/14), L4 (GII/5)	–	+	–
6	02.07.03	Restaurant	3	4	individual	+	N1 (GI/8)	–	+	–
	02.07.03				individual	+	N2 (GI/4)	SAV-N4 (GII/3)	–	–
	02.07.03				individual	+	N3 (GI/4)	SAV-N5 (GII/1)	–	–
	02.09.03				food-handler	–	–	–	+	–
7	02.16.03	Restaurant	5	3	individual	+	O1 (GI/8), O1 (GII/6)	–	+	–
	02.17.03				food-handler	–	O2a (GI/1), O2b (GI/4)	–	+	–
	02.18.03				individual	+	O3a (GII/8), O3b (GII/6)	–	–	–
8	03.01.03	Restaurant	12	14	individual	+	P1a (GI/4), P1b (GI/8)	–	+	–
	03.01.03				individual	+	P2 (GI/8), P2 (GII/3)	–	+	–
	03.01.03				individual	+	P3 (GII/4)	–	+	–
	03.01.03				individual	+	P4a (GI/2), P4b (GI/8)	–	+	–
	03.01.03				individual	+	P5 (GII/5)	–	+	+
	03.01.03				food-handler	–	–	–	–	–
	03.01.03				food-handler	–	–	–	–	–
	03.01.03				food-handler	–	–	–	+	–
	03.01.03				food-handler	–	–	–	–	–
	03.01.03				food-handler	–	P10 (GI/1)	–	+	–
	03.01.03				food-handler	–	–	–	–	–
	03.01.03				food-handler	–	–	–	–	–
	03.01.03				food-handler	–	–	–	–	–
	03.01.03				individual	+	P14 (GI/2)	–	+	–
9	12.16.04	Monastery	9	4	individual	+	R1 (GI/3)	–	+	–
	12.18.04				individual	+	R2 (GI/3)	–	+	–
	12.17.04				individual	+	R3 (GI/1)	–	+	–
	12.18.04				individual	+	–	–	–	–
10	02.14.06	Restaurant	19	15	food-handler	–	S1 (GI/8)	–	–	–
	02.14.06				individual	+	S2 (GI/8), S2 (GII/4)	–	–	–
	02.14.06				individual	+	S3 (GII/3)	–	–	–
	02.14.06				individual	+	S4 (GI/8), S4 (GII/3)	–	–	–
	02.14.06				individual	+	S5 (GII/3)	–	–	–
	02.14.06				individual	+	S6 (GI/8)	–	–	–
	02.14.06				individual	+	S7 (GI/8)	–	–	–
	02.14.06				individual	+	S8 (GI/8), S8 (GII/6)	–	–	–
	02.14.06				individual	+	S9 (GII/5)	–	–	–
	02.14.06				individual	+	S10 (GI/8), S10 (GII/3)	–	–	–
	02.14.06				individual	+	S11 (GII/4)	–	–	–
	02.14.06				individual	+	S12a (GI/14), S12b (GI/5), S12a (GII/3), S12b (GII/5)	–	–	–
	02.14.06				individual	+	–	–	–	–
	02.14.06				individual	+	S14 (GI/8)	–	–	–
	02.14.06				individual	+	–	–	–	–
11	03.07.06	Restaurant	11	4	food-handler	–	–	–	–	–
	03.07.06				individual	+	T2a (GI/8), T2b (GI/3)	–	–	–
	03.07.06				individual	+	T3 (GI/8)	–	–	–
	03.07.06				individual	+	T4 (GI/8), T4 (GII/3)	–	–	–
Total				71			52	5	19	1

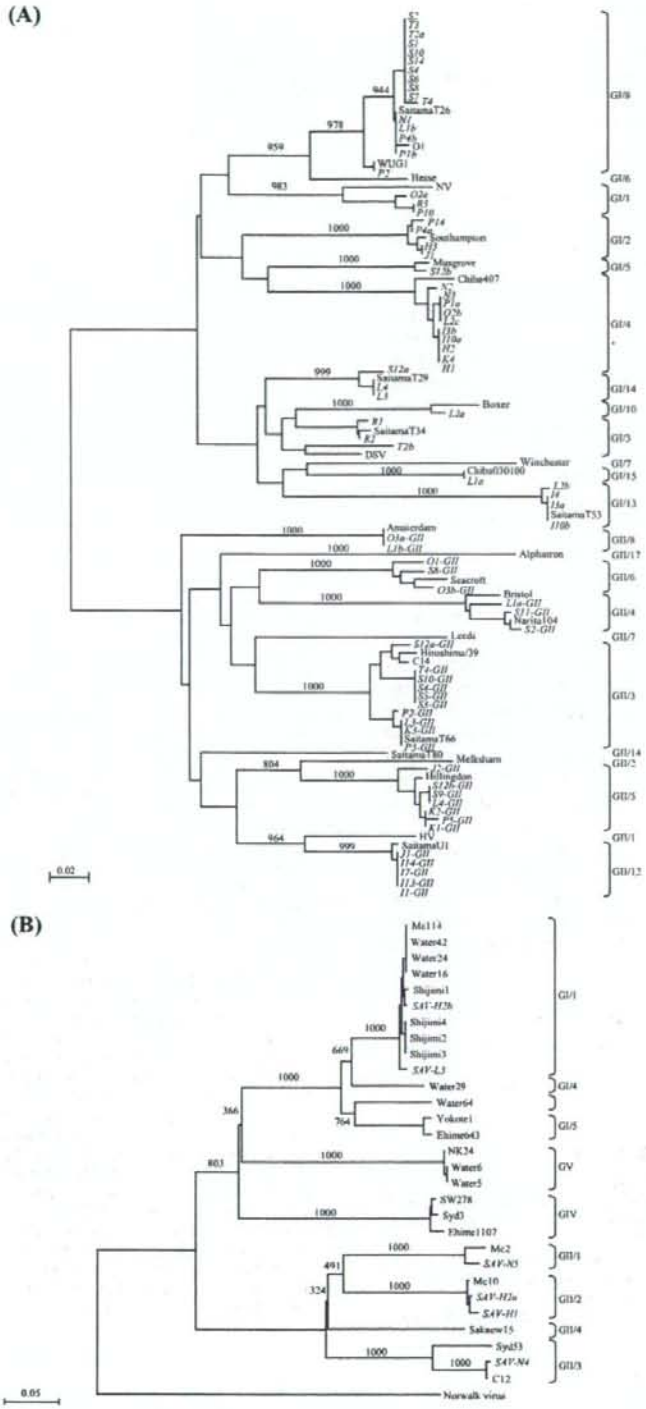

Fig. 1. Phylogenetic tree of the noroviruses (A) and sapoviruses (B) detected in this study. The trees were constructed with the partial N-terminal capsid region. The numbers on the branches indicate the bootstrap values for the clusters. Sequences and accession numbers from references (8) and (26), and Chiba030100 (AJ844469), Hiroshima/39 (AB262170), and C14 (AY845056) were used as the reference sequences.

detected in 6 of 11 outbreaks, including 19 of 71 (27%) specimens (Table 1). The kobuvirus sequences belonged to genotype A and shared greater than 98% nucleotide identity. Interestingly, 16 of 19 kobuvirus-positive specimens were also norovirus-positive, which suggests that co-contamination of these viruses in the natural environment was common. However, astrovirus was detected in only 1 of 11 outbreaks, and its nucleotide sequence was closely related to that of human serotype 4 sequences (data not shown).

In 7 of the 11 outbreaks (Outbreaks 1, 2, 5, 6, 7, 8, and 9), two or more types of viruses were detected, whereas only noroviruses were detected in the remaining four outbreaks (Outbreaks 3, 4, 10, and 11). Moreover, multiple norovirus genogroups and/or genotypes were detected in all outbreaks. It is noteworthy that we detected two sapovirus genogroups (GI/1 and GII/2) and two norovirus genotypes (GI/2 and GI/4) in one outbreak (Table 1, Outbreak 1). Although multiple norovirus genotypes were previously found, as were kobuviruses in oyster-associated outbreaks (3,4,8,21,22), this is the first report to detect multiple genotypes and genogroups of human sapoviruses in stool specimens from subjects with oyster-associated gastroenteritis. In addition, we detected two sapovirus genogroups in the same outbreak for the first time. Recently, we detected sapoviruses in the clam *Corbicula japonica* (bivalve mollusk), which is used for human consumption, and the sequences were closely related to those from patients with gastroenteritis (20). The results described in this study suggest that multiple sapovirus genotypes were concentrated in oysters, as were norovirus genotypes (23-25), which may be transmitted to humans, causing gastroenteritis. Unfortunately, no oyster samples were available for screening. The detection of sapovirus in oysters is an issue to be addressed in the future. It would also be interesting to determine whether or not the clinical symptoms of patients infected with multiple species of viruses were different from those infected with a single species of a virus.

In conclusion, sapovirus and kobuvirus were frequently detected with multiple genotypes of norovirus in stool specimens from subjects in oyster-associated outbreaks, suggesting that examination of not only norovirus but also these enteric viruses is needed in order to confirm the causative agents.

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REFERENCES

1. Bon, F., Fascia, P., Dauvergne, M., et al. (1999): Prevalence of group A rotavirus, human calicivirus, astrovirus, and adenovirus type 40 and 41 infections among children with acute gastroenteritis in Dijon, France. J. Clin. Microbiol., 37, 3055-3058.
2. Sdiri-Loulizi, K., Gharbi-Khelifi, H., de Rougemont, A., et al. (2008): Acute infantile gastroenteritis associated with human enteric viruses in Tunisia. J. Clin. Microbiol., 46, 1349-1355.
3. Yamashita, T., Sugiyama, M., Tsuzuki, H., et al. (2000): Application of a reverse transcription-PCR for identification and differentiation of Aichi virus, a new member of the Picornavirus family associated with gastroenteritis in humans. J. Clin. Microbiol., 38, 2955-2961.
4. Ambert-Balay, K., Lorrot, M., Bon, F., et al. (2008): Prevalence and genetic diversity of Aichi virus strains in stool samples from community and hospitalized patients. J. Clin. Microbiol., 46, 1252-1258.
5. Gotz, H., de, J.B., Lindback, J., et al. (2002): Epidemiological investigation of a food-borne gastroenteritis outbreak caused by Norwalk-like virus in 30 day-care centres. Scand. J. Infect. Dis., 34, 115-121.
6. Marks, P.J., Vipond, I.B., Regan, F.M., et al. (2003): A school outbreak of Norwalk-like virus: evidence for airborne transmission. Epidemiol. Infect., 131, 727-736.
7. Centers for Disease, Control and Prevention (2008): Norovirus outbreak in an elementary school—District of Columbia, February 2007. Morb. Mortal. Wkly. Rep., 56, 1340-1343.
8. Kageyama, T., Shinohara, M., Uchida, K., et al. (2004): Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to *Norovirus* in Japan. J. Clin. Microbiol., 42, 2988-2995.
9. Noel, J.S., Liu, B.L., Humphrey, C.D., et al. (1997): Parkville virus: a novel genetic variant of human calicivirus in the Sapporo virus clade, associated with an outbreak of gastroenteritis in adults. J. Med. Virol., 52, 173-178.
10. Johansson, P.J., Bergentoft, K., Larsson, P.A., et al. (2005): A nosocomial sapovirus-associated outbreak of gastroenteritis in adults. Scand. J. Infect. Dis., 37, 200-204.
11. Hansman, G.S., Saito, H., Shibata, C., et al. (2007): Outbreak of gastroenteritis due to sapovirus. J. Clin. Microbiol., 45, 1347-1349.
12. Hansman, G.S., Ishida, S., Yoshizumi, S., et al. (2007): Recombinant sapovirus gastroenteritis, Japan. Emerg. Infect. Dis., 13, 786-788.
13. Wu, F.T., Oka, T., Takeda, N., et al. (2008): Acute gastroenteritis caused by GI/2 sapovirus, Taiwan, 2007. Emerg. Infect. Dis., 14, 1169-1171.
14. Farkas, T., Zhong, W.M., Jing, Y., et al. (2004): Genetic diversity among sapoviruses. Arch. Virol., 149, 1309-1323.
15. Hansman, G.S., Oka, T., Katayama, K., et al. (2007): Human sapoviruses: genetic diversity, recombination, and classification. Rev. Med. Virol., 17, 133-141.
16. Kojima, S., Kageyama, T., Fukushi, S., et al. (2002): Genogroup-specific PCR primers for detection of Norwalk-like viruses. J. Virol. Methods, 100, 107-114.
17. Okada, M., Yamashita, Y., Oseto, M., et al. (2006): The detection of human sapoviruses with universal and genogroup-specific primers. Arch. Virol., 151, 2503-2509.
18. Matsui, M., Ushijima, H., Hachiya, M., et al. (1998): Determination of serotypes of astroviruses by reverse transcription-polymerase chain reaction and homologies of the types by the sequencing of Japanese isolates. Microbiol. Immunol., 42, 539-547.
19. Yan, H., Yagyu, F., Okitsu, S., et al. (2003): Detection of norovirus (GI, GII), Sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR. J. Virol. Methods, 114, 37-44.
20. Hansman, G.S., Oka, T., Okamoto, R., et al. (2007): Human sapovirus in clams, Japan. Emerg. Infect. Dis., 13, 620-622.
21. Gallimore, C.I., Cheesbrough, J.S., Lamden, K., et al. (2005): Multiple norovirus genotypes characterised from an oyster-associated outbreak of gastroenteritis. Int. J. Food Microbiol., 103, 323-330.
22. Le Guyader, F.S., Bon, F., DeMedici, D., et al. (2006): Detection of multiple noroviruses associated with an international gastroenteritis outbreak linked to oyster consumption. J. Clin. Microbiol., 44, 3878-3882.
23. Costantini, V., Loisy, F., Joens, L., et al. (2006): Human and animal enteric caliciviruses in oysters from different coastal regions of the United States. Appl. Environ. Microbiol., 72, 1800-1809.
24. Nishida, T., Kimura, H., Saitoh, M., et al. (2003): Detection, quantitation, and phylogenetic analysis of noroviruses in Japanese oysters. Appl. Environ. Microbiol., 69, 5782-5786.
25. Nishida, T., Nishio, O., Kato, M., et al. (2007): Genotyping and quantitation of noroviruses in oysters from two distinct sea areas in Japan. Microbiol. Immunol., 51, 177-184.
26. Hansman, G.S., Sano, D., Ueki, Y., et al. (2007): Sapovirus in water, Japan. Emerg. Infect. Dis., 13, 133-135.

FULL PAPER *Public Health*

Prevalence of Hepatitis E Virus (HEV) Infection in Wild Boars (*Sus scrofa leucomystax*) and Pigs in Gunma Prefecture, Japan

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ABSTRACT. The prevalence of hepatitis E virus (HEV) infection in wild boars and pigs in Gunma Prefecture, Japan, was serologically and genetically examined. The positive detection rates of anti-HEV IgG and HEV RNA in the wild boars were 4.5% (4/89) and 1.1% (1/89), whereas those in the pigs were 74.6% (126/169) and 1.8% (3/169), respectively. The positive rates of anti-HEV IgG and HEV RNA on the 17 pig farms in the present study ranged from 20% to 100%, respectively. One male wild boar approximately 5 years of age was positive for HEV RNA but was negative for anti-HEV IgG. Three pigs from 2 farms were positive for HEV RNA; 2 of these pigs were negative for HEV IgG, and the other was positive. A phylogenetic analysis revealed that all of the HEV ORF1 genes detected in the present study belonged to genotype III. In Gunma Prefecture, HEV is highly prevalent and widespread, and uncooked wild boar and pig meat may have the potential to transmit HEV to humans.

KEY WORDS: hepatitis E virus, Japan, swine, wild boar.

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Hepatitis E virus (HEV), which belongs to the genus *Hepevirus*, is the causative agent of hepatitis E. Hepatitis E infection has been found in many developing countries in Asia, Africa and Latin America, where the disease is an important public health concern [15]. HEV is primarily transmitted by the fecal-oral route such as in waterborne epidemics.

Recent studies have suggested that HEV is divided into 4 genotypes designated as G I, G II, G III, and G IV [17]. The HEV infections in Asia and Africa are mainly caused by G I, and the majority of the GII infection have been reported in Mexico and Nigeria. On the other hand, only a single case of infection with GIII or GIV has been described in the United States, European counties, Argentina, Taiwan and China [17, 21, 22]. In Japan, most imported cases with G I have derived from epidemic areas such as Asia and Africa [2]; however, G III or G IV has also been detected in acute hepatitis patients who have never traveled to HEV epidemic areas [6, 8, 13, 14, 20, 21, 24, 29]. These patients often have a history of consuming uncooked wild boar (*Sus scrofa leucomystax*) and sika deer (*Cervus nippon*) meat and liver [5, 27, 28]. Also, HEV strains belonging to G I, G III or G IV have been detected in Japanese patients with sporadic acute or fulminate hepatitis E [8, 9, 19–22, 24, 31]. In addition, Yazaki *et al.* [31] reported that HEV RNA has been detected in 2% (7/363 packages) of sold pig liver on the market by reverse transcription-polymerase chain reaction (RT-PCR).

In Japan, it has been suggested that the transmission route of HEV remains unclear in approximately 60% of infected patients [1]; zoonotic food-borne transmissions account for 30%, imported infection accounts for 8% and blood transfusion is responsible for 2%. In Gunma Prefecture, Japan, approximately 3,000 wild boars are annually slaughtered for meat [unpublished data], and the number of breeding pigs in the prefecture was approximately 6 million in 2005. According to the Gunma Prefectural Statistics Report (http://www.pref.gunma.jp/cts/PortalServlet?DISPLAY_ID=DIRECT&NEXT_DISPLAY_ID=U000004&CONTENTS_ID=43375), Gunma Prefecture is one of the major pork-producing areas in Japan. However, to the best of our knowledge, there have been no reports on the prevalence of HEV infection in wild boars and pigs in the prefecture to date. Here in, we report the seroprevalence of anti-HEV IgG detected by enzyme-linked immunosorbent assay (ELISA) and HEV RNA by RT-PCR among wild boars and pigs in Gunma Prefecture, Japan.

MATERIALS AND METHODS

Samples: From September 2004 to March 2006, blood samples from 89 wild boars were kindly provided by hunters, and these samples were placed in sterile tubes, stored at approximately 4°C and sent to the laboratory within 12 hr. The ages of the wild boars were estimated by the hunters. From September to December 2004, we collected 169 pig blood samples from 17 pig farms during viscera inspections

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at G slaughterhouse in Gunma Prefecture, with 9 to 10 samples obtained from each farm. All pigs were approximately 6 months old. The blood samples were placed in sterile tubes, stored at approximately 4°C and sent to the laboratory within 3 hr. All blood samples were centrifuged at 1,900 × *g* for 20 min, and the serum was stored at –20°C until analyses.

Serologic analysis: Anti-HEV IgG was measured by ELISA as previously described with some modifications [4]. The antigen used in the ELISA was HEV-like particles composed of a truncated open reading frame 2 (ORF2) protein of genotype I HEV expressed by a recombinant baculovirus in insect cells and was suspended with 0.5 M carbonate buffer (pH 12.5) at a concentration of 1 μg/ml [3]. The antigen solution (100 μl) was added to duplicate wells of 96-well microplates (Sumiron ELISA plate type H, Sumitomo Bakelite, Tokyo, Japan). After washing with phosphate buffered saline containing 0.05% of tween-20 (PBST), the wells were coated with 5% skim milk in PBST for 1 hr at room temperature and then incubated with 100 μl of serum samples at a dilution of 1:200 in 1% skim milk in PBST for 1 hr at room temperature. The wells were washed with PBST 3 times, and the bound IgG antibodies were probed with peroxidase-labeled goat anti-swine IgG antibodies (heavy plus light chain; Kirkegaard and Perry Laboratories, Gaithersburg, MD, U.S.A.). After washing 3 times with PBST, 100 μl of substrate, for wild boar samples, TMB HRP Microwell substrate, Bio FX Laboratories, MD, USA; for swine samples, 200 μM of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), Sigma, St. Louis, MO, U.S.A.) was added, and the plates were incubated for 10 min (for wild boar samples) or 30 min (for swine samples) at room temperature. Following the incubation period, 100 μl of stop solutions was added to the plates. The density at 450 nm (wild boar samples) or 415 nm (swine samples) was measured using an automatic ELISA reader (Benchmark Plus, BioRad, U.S.A.). A sample was considered positive for anti-HEV IgG when the average of OD value was greater than the cut-off value. To determine the cut-off value of the IgG, each of the 10 samples that had the lowest OD values and were negative in the western blot analysis were used as negative sera. In the present study, ODs of 2.597 and 0.197, which were calculated as three standard deviations above the mean values for the wild boar and swine negative controls, respectively, were used as the tentative cut-off values for each sample.

Extraction of RNA and reverse transcription polymerase chain reaction: Frozen serum samples were thawed at room temperature and then centrifuged at 3,000 × *g* at 4°C for 30 min, and the supernatants were then used for RT-PCR and sequence analysis. Total RNA was extracted from 140 μl of the re-centrifuged serum using a QIAamp Viral RNA Mini kit (Qiagen, MD, U.S.A.). The extracted RNA was then suspended in 60 μl of DNase/RNase-free water and treated with 5 U of DNase I (Takara, Tokyo, Japan). To amplify the 326- nucleotide region from open reading frame 1 (ORF1) by RT-PCR, we used genotype-specific primers as previously described [21]. The amplified DNA fragment was separated by electrophoresis on a 3% agarose gel, and the DNA fragments were purified using a QIAquick PCR Purification kit (Qiagen). The nucleotide sequence was determined using an automated DNA sequencer (ABI PRISM™ 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, U.S.A.) using a Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems). Nucleotide sequences of the partial ORF1 of HEV (positions 124 to 449: 326 bp) were analyzed phylogenetically using CLUSTALW on the DNA database of Japan (DDBJ) homepage (http://hypernig.nig.ac.jp/homology/clustalw-e.shtml) and TreeExplorer (Version 2.12; http://evolgen.biol.metro-u.ac.jp/TE/). Evolutionary distances were estimated using Kimura's two-parameter method, and phylogenic trees were constructed using the neighbor-joining (NJ) method [16]. The reliability of the trees was estimated using 1000 bootstrap replications.

Statistical analysis: The chi-square test with Yates' continuity correction was used to compare the positive detection rates of anti-HEV IgG between male and female wild boars. Differences were considered significant when the p value was less than 0.05.

RESULTS

Prevalence of HEV infection in wild boars in Gunma Prefecture: Anti-HEV IgG was detected in 4 (4.5%) of the 89 wild boars (Table 1). No significant difference was found between for the male (2.9%; 1/35) and female (6.7%; 3/45) wild boars (chi-square test with Yates' continuity correction, p=0.7960). HEV was detected in only 1 wild boar (WBG06–01), giving a 1.1% (1/89) positive rate.

Prevalence of HEV infection in slaughtered pigs in Gunma Prefecture: Anti-HEV IgG was detected in 126 (74.6%) of the 169 slaughtered pigs (Table 2). The positive rates among the individual 17 pig farms varied from 20% to 100%. HEV RNA was detected in 1.8% (3/169) of the pigs, 1 from farm M(PG05–03) and 2 from farm H (PG05–01 and PG02–02), and the positive rates of anti-HEV IgG for these farms were 60% and 89%, respectively.

Information on HEV RNA-positive animals: We detected HEV RNA in one wild boar and three pigs. The wild boar (WBG06–01; male; body weight of about 80 kg) was estimated to be approximately 5 years of age by the hunters and was negative for anti-HEV IgG. Of the 3 pigs, 2 (PG05–01 and PG05–02) were from farm H, and the other (PG05–03) was from farm M. Farms E and L are located in the center of Gunma Prefecture and have no history of contact with wild boars. Of these 3 pigs, 2 (PG05–01 and PG05–03) were negative and 1 (PG05–02) was positive for anti-HEV IgG.

Phylogenetic analysis of the HEV isolates based on the sequences of open reading frame 1: The phylogenetic tree based on the ORF1 gene in HEV detected in Japan and other countries is shown in Fig. 1. The strains were divided into 4 genotypes as described in a previous report [17]. All 4

Table 1. Detection of anti HEV-IgG and HEV RNA in wild boars

Age (months)	Sex	Number of samples	IgG positive samples (%)	HEV RNA detection (%)
< 12	Male	4	0 (0)	0 (0)
	Female	7	0 (0)	0 (0)
13–24	Male	8	0 (0)	0 (0)
	Female	3	0 (0)	0 (0)
25–36	Male	4	1 (25.0)	0 (0)
	Female	10	0 (0)	0 (0)
37–48	Male	6	0 (0)	0 (0)
	Female	7	0 (0)	0 (0)
49–62	Male	1	0 (0)	1[a](100)
	Female	9	1 (11.1)	0 (0)
>62	Male	5	0 (0)	0 (0)
	Female	6	1 (16.7)	0 (0)
Unknown	Male	7	0 (0)	0 (0)
	Female	3	1 (33.3)	0 (0)
	No record	9	0 (0)	0 (0)
Subtotal	Male	35	1 (2.9)	1 (2.9)
	Female	45	3 (6.7)	0 (0)
	No record	9	0 (0)	0 (0)
Total		89	4 (4.5)	1 (1.1)

a) Sample number: WBG06–01.

Table 2. Detection of anti HEV-IgG and HEV RNA in 17 pig farms

Farm	Number of samples	IgG positive samples (%)	HEV RNA detection (%)
A	10	10 (100)	0 (0)
B	10	10 (100)	0 (0)
C	10	10 (100)	0 (0)
D	10	10 (100)	0 (0)
E	10	10 (100)	0 (0)
F	10	9 (90)	0 (0)
G	10	9 (90)	0 (0)
H	9	8 (88.9)	2[a](22.2)
I	10	8 (80)	0 (0)
J	10	7 (80)	0 (0)
K	10	7 (70)	0 (0)
L	10	6 (70)	0 (0)
M	10	6 (60)	1[b](10)
N	10	5 (50)	0 (0)
O	10	5 (50)	0 (0)
P	10	4 (40)	0 (0)
Q	10	2 (20)	0 (0)
Total	169	126 (74.6)	3 (1.8)

a) Sample number: PG05–01 and PG05–02.
b) Sample number: PG05–03.

strains detected in the present study were classified into genotype III, which includes several genotypes of Japanese domestic animals previously reported [8, 11, 12, 20, 23, 28, 31]. The sequences of the 2 pigs (PG05–01 and PG05–02) from farm H were identical (AB362371 and AB362372) but were different from that for farm M by approximately 0.11, while the distances of the wild boar sequence (AB362374) from the sequences of boars from farms H and M were 0.1 and 0.07, respectively.

DISCUSSION

Epidemiological studies have reported that HEV infection is prevalent among wild boars [5, 12, 26, 30] and pigs [10, 25] and have suggested that consumption of the meat and liver of these animals is a risk in terms of HEV infection in Japan [5, 26, 30]. In the present study, the positive rates of anti-HEV IgG and HEV RNA (genotype III) in the wild boars were 4.5% (4/89) and 1.1% (1/89), respectively. The positive detection rates showed no relationship with the age of the animals. Sonoda *et al.* [18] reported that anti-HEV IgG is present in 8.6% (3/35) of wild boars and that HEV RNA genotype III has been detected in a 60-kg male wild boar (2.9%, 1/35) that was negative for anti-HEV IgG (presumed to be approximately 2 years of age). In other study in Japan, Michitaka *et al.* [7] reported a positive rate of anti-HEV IgG in wild boars of 25.5% (100/392), and 3.1% (12/392) of the wild boars in their study were positive for the HEV RNA genotype III. In the present study, although the seroprevalence of HEV infection in the wild boar was considerably lower than in previous reports, some of the animals in Japan are infected with GIII and may potentially serve as a source of infection in humans.

The prevalence of anti-HEV IgG in pigs depends on the age of the animals, and HEV RNA has been detected in 2- to 4-month-old pigs and less commonly in older pigs [10, 14, 23, 25]. Takahashi *et al.* [23, 25] reported detection rates of anti-HEV IgG in 6-month-old pigs that ranged from 73.5% (100/136) to 90.4% (226/250), with no HEV RNA detection from any prefecture examined in Japan to date. Although the positive rates of anti-HEV IgG in the present study were similar to those in previous reports, HEV RNA (genotype III) was detected in 1.8% (3/169) of the pig serum samples, and this suggests that HEV genotype III is highly prevalent and widely distributed in pigs. Thus, it is highly possible that pigs are a source of HEV infection in humans. A nationwide campaign prohibiting consumption of uncooked liver and meat from wild boars and pigs should be implemented to prevent HEV infection in humans.

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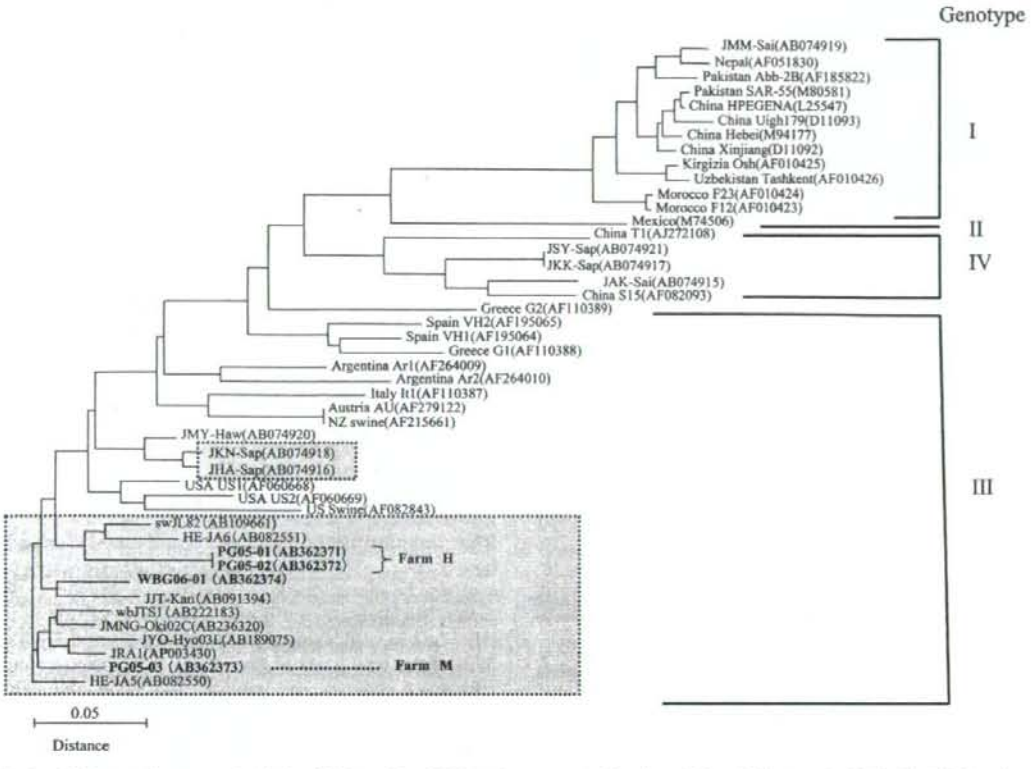

Fig. 1. Phylogenetic trees on the basis of 326 nt of the ORF1 region constructed by the neighbor-joining method [16]. The HEV strains from one wild boar and three pigs from farms are shown in bold type. In addition, genotype III strains reported in previous studies in Japan are indicated by gray boxes. The GenBank accession numbers of the identified strains are enclosed in parentheses.

REFERENCES

1. Abe, T., Aikawa, T., Akahane, Y., Arai, M., Asahina, Y., Atarashi, Y., Chayama, K., Harada, H., Hashimoto, N., Hori, A., Ichida, T., Ikeda, H., Ishikawa, A., Ito, T., Kang, J. H., Karino, Y., Kato, H., Kato, M., Kawakami, M., Kitajima, N., Kitamura, T., Masaki, N., Matsubayashi, K., Matsuda, H., Matsui, A., Michitaka, K., Mihara, H., Miyaji, K., Miyakawa, H., Mizuo, H., Mochida, S., Moriyama, M., Nishiguchi, S., Okada, K., Saito, H., Sakugawa, H., Shibata, M., Suzuki, K., Takahashi, K., Yamada, G., Yamamoto, K., Yamanaka, T., Yamato, H., Yano K. and Mishiro, S. 2006. Demographic, epidemiological, and virological characteristics of hepatitis E virus infections in Japan based on 254 human cases collected nationwide. *Kanzo* **47**: 384–391.
2. Koizumi, Y., Isoda, N., Sato, Y., Iwaki, T., Ono, K., Ido, K., Sugano, K., Takahashi, M., Nishizawa, T. and Okamoto, H. 2004. Infection of a Japanese patient by genotype 4 hepatitis e virus while traveling in Vietnam. *J. Clin. Microbiol.* **42**: 3883–3885.
3. Li, T.C., Yamakawa, Y., Suzuki, K., Tatsumi, M., Razak, M.A., Uchida, T., Takeda, N. and Miyamura, T. 1997. Expression and self-assembly of empty virus-like particles of hepatitis E virus. *J. Virol.* **71**: 7207–7213.
4. Li, T.C., Zhang, J., Shinzawa, H., Ishibashi, M., Sata, M., Mast, E.E., Kim, K., Miyamura, T. and Takeda, N. 2000. Empty virus-like particle-based enzyme-linked immunosorbent assay for antibodies to hepatitis E virus. *J. Med. Virol.* **62**: 327–333.
5. Masuda, J., Yano, K., Tamada, Y., Takii, Y., Ito, M., Omagari, K. and Kohno, S. 2005. Acute hepatitis E of a man who consumed wild boar meat prior to the onset of illness in Nagasaki, Japan. *Hepatol. Res.* **31**: 178–183.
6. Matsubayashi, K., Nagaoka, Y., Sakata, H., Sato, S., Fukai, K., Kato, T., Takahashi, K., Mishiro, S., Imai, M., Takeda, N. and Ikeda, H. 2004. Transfusion-transmitted hepatitis E caused by apparently indigenous hepatitis E virus strain in Hokkaido, Japan. *Transfusion* **44**: 934–940.
7. Michitaka, K., Takahashi, K., Furukawa, S., Inoue, G., Hiasa, Y., Horiike, N., Onji, M., Abe, N. and Mishiro, S. 2007. Prevalence of hepatitis E virus among wild boar in the Ehime area of western Japan. *Hepatol. Res.* **37**: 214–220.
8. Mizuo, H., Suzuki, K., Takikawa, Y., Sugai, Y., Tokita, H., Akahane, Y., Itoh, K., Gotanda, Y., Takahashi, M., Nishizawa, T. and Okamoto, H. 2002. Polyphyletic strains of hepatitis E

virus are responsible for sporadic cases of acute hepatitis in Japan. *J. Clin. Microbiol.* **40**:3209–3218.

9. Mizuo, H., Yazaki, Y., Sugawara, K., Tsuda, F., Takahashi, M., Nishizawa, T. and Okamoto, H. 2005. Possible risk factors for the transmission of hepatitis E virus and for the severe form of hepatitis E acquired locally in Hokkaido, Japan. *J. Med. Virol.* **76**: 341–349.
10. Nakai, I., Kato, K., Miyazaki, A., Yoshii, M., Li, T.C., Takeda, N., Tsunemitsu, H. and Ikeda, H. 2006. Different fecal shedding patterns of two common strains of hepatitis e virus at three Japanese Swine farms. *Am. J. Trop. Med. Hyg.* **75**: 1171–1177.
11. Nakamura, M., Takahashi, K., Taira, K., Taira, M., Ohno, A., Sakugawa, H., Arai, M. and Mishiro, S. 2006. Hepatitis E virus infection in wild mongooses of Okinawa, Japan: Demonstration of anti-HEV antibodies and a full-genome nucleotide sequence. *Hepatol. Res.* **34**: 137–140.
12. Nishizawa, T., Takahashi, M., Endo, K., Fujiwara, S., Sakuma, N., Kawazuma, F., Sakamoto, H., Sato, Y., Bando, M. and Okamoto, H. 2005. Analysis of the full-length genome of hepatitis E virus isolates obtained from wild boars in Japan. *J. Gen. Virol.* **86** (PT 12): 3321–3326.
13. Ohnishi, S., Kang, J.H., Maekubo, H., Takahashi, K. and Mishiro, S. 2003. A case report: two patients with fulminant hepatitis E in Hokkaido, Japan. *Hepatol. Res.* **25**: 213–218.
14. Okamoto, H., Takahashi, M., Nishizawa, T., Fukai, K., Muramatsu, U. and Yoshikawa, A. 2001. Analysis of the complete genome of indigenous swine hepatitis E virus isolated in Japan. *Biochem. Biophys. Res. Commun.* **289**: 929–936.
15. Purcell, R. H. and Emerson, S. U. 2001. Hepatitis E virus. pp. 3051–3061. *In*: Fields Virology, 4th ed. (Knipe, D. M., Howley, P. M., Griffin, D. E., Martin, M. A., Lamb, R. A., Roizman, B. and Straus, S. E. eds.), Lippincott Williams and Wilkins, Philadelphia.
16. Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406–425.
17. Schlauder, G. G. and Mushahwar, I. K. 2001. Genetic heterogeneity of hepatitis E virus. *J. Med. Virol.* **65**: 282–292.
18. Sonoda, H., Abe, M., Sugimoto, T., Sato, Y., Bando, M., Fukui, E., Mizuo, H., Takahashi, M., Nishizawa, T. and Okamoto, H. 2004. Prevalence of hepatitis E virus (HEV) infection in wild boars and deer and genetic identification of a genotype 3 HEV from a boar in Japan. *J. Clin. Microbiol.* **42**: 5371–5374.
19. Suzuki, K., Aikawa, T. and Okamoto, H. 2002. Fulminant hepatitis E in Japan. *New Engl. J. Med.* **347**: 1456.
20. Takahashi, K., Iwata, K., Watanabe, N., Hatahara, T., Ohta, Y., Baba, K. and Mishiro, S. 2001. Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. *Virology* **287**: 9–12.
21. Takahashi, K., Kang, J.H., Ohnishi, S., Hino, K. and Mishiro, S. 2002. Genetic heterogeneity of hepatitis E virus recovered from Japanese patients with acute sporadic hepatitis. *J. Infect. Dis.* **185**: 1342–1345.
22. Takahashi, K., Kang, J.H., Ohnishi, S., Hino, K., Miyakawa, H., Miyakawa, Y., Maekubo, H. and Mishiro, S. 2003. Full-length sequences of six hepatitis E virus isolates of genotypes III and IV from patients with sporadic acute or fulminant hepatitis in Japan. *Intervirology* **46**: 308–318.
23. Takahashi, M., Nishizawa, T., Miyajima, H., Gotanda, Y., Iita, T., Tsuda, F. and Okamoto, H. 2003. Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. *J. Gen. Virol.* **84**: 851–862.
24. Takahashi, M., Nishizawa, T., Yoshikawa, A., Sato, S., Isoda, N., Ido, K., Sugano, K. and Okamoto, H. 2002. Identification of two distinct genotypes of hepatitis E virus in a Japanese patient with acute hepatitis who had not travelled abroad. *J. Gen. Virol.* **83**: 1931–1940.
25. Takahashi, M., Nishizawa, T., Tanaka, T., Tsatsralt-Od, B., Inoue, J. and Okamoto, H. 2005. Correlation between positivity for immunoglobulin A antibodies and viraemia of swine hepatitis E virus observed among farm pigs in Japan. *J. Gen. Virol.* **86** (PT 6): 1807–1813.
26. Tamada, Y., Yano, K., Yatsuhashi, H., Inoue, O., Mawatari, F. and Ishibashi, H. 2004. Consumption of wild boar linked to cases of hepatitis E. *J. Hepatol.* **40**: 869–870.
27. Tei, S., Kitajima, N., Ohara, S., Inoue, Y., Miki, M., Yamatani, T., Yamabe, H., Mishiro, S. and Kinoshita, Y. 2004. Consumption of uncooked deer meat as a risk factor for hepatitis E virus infection: an age- and sex-matched case-control study. *J. Med. Virol.* **74**: 67–70.
28. Tei, S., Kitajima, N., Takahashi, K. and Mishiro, S. 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. *Lancet* **362** (9381): 371–373.
29. Yamamoto, T., Suzuki, H., Toyota, T., Takahashi, M. and Okamoto, H. 2004. Three male patients with sporadic acute hepatitis E in Sendai, Japan, who were domestically infected with hepatitis E virus of genotype III or IV. *J. Gastroenterol.* **39**: 292–398.
30. Yano, K. 2007. Wild boar as an important reservoir of hepatitis E virus in western Japan. *Hepatol. Res.* **37**: 167–169.
31. Yazaki, Y., Mizuo, H., Takahashi, M., Nishizawa, T., Sasaki, N., Gotanda, Y. and Okamoto, H. 2003. Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. *J. Gen. Virol.* **84**: 2351–2357.

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Epidemic of Genotype GII.2 Noroviruses during Spring 2004 in Osaka City, Japan▽

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Between March and May 2004, a GII.2 genotype norovirus strain caused an epidemic of acute gastroenteritis in Osaka, Japan. Phylogenetic analysis showed that this strain was distinct from all other GII.2 strains detected in Osaka City between April 1996 and March 2005.

Noroviruses (NoVs) are a major cause of acute gastroenteritis worldwide. Their transmission modes are food, person-to-person contact, and environmental contamination (5). In many countries, cold weather seasonality of NoV infections has been observed (9, 13, 14). The human NoVs are divided into three genogroups (GI, GII, and GIV), of which GI and GII strains are the most commonly found (2, 21). Within a genogroup, strains can be further divided into genotypes based on >80%

TABLE 1. Description of outbreaks in which NoVs were detected in Osaka City, Japan, between March and May 2004[a]

Outbreak no.	Day(s)/mo	Place	Source	Age group	Attack rate (no. ill/no. at risk)	No. of specimens		Genotype(s) (capsid)
						Total	NoV positive	
04032	3/March	Restaurant	Oysters	Adults	9/12	7	5	GI.1, GII.5
04034[b]	8/March	Restaurant	Oysters	Adults	3/3	2	1	GI.1, GII.8
04037[b]	6/March	Restaurant	Oysters	Adults	2/2	2	1	GII.12[c]
04038	**11/March**	**Restaurant**	**UK[d]**	**Children**	**29/60**	**29**	**22**	**GII.2**
04039[b]	**15/March**	**Home**	**UK**	**Children**	**2/UK**	**2**	**1**	**GII.2**
04041	14/May	Restaurant	Food	Adults	2/UK	2	2	GII.5
04042	**14/March**	**Restaurant**	**Food**	**Adults**	**40/71**	**10**	**9**	**GII.2**
04043	**17/March**	**Kindergarten**	**PP[e]**	**Children**	**20/UK**	**2**	**2**	**GII.2**
04047[f]	3/April	Hotel	Food	Adults	162/565	3	3	GII.4
04048[f]	7/April	Restaurant	Oysters	Adults	6/14	1	1	GII.8
04056	**12–13/April**	**Kindergarten**	**PP**	**Children**	**114/UK**	**60**	**50**	**GII.2**
04057[f]	23/April	Hotel	UK	Adults	325/796	1	1	GII.4
04059	**18–30/April**	**School**	**PP**	**Children**	**268/UK**	**84**	**74**	**GII.2**
04062[f]	1/May	Restaurant	UK	Adults	72/176	2	2	GII.6
04067	**10–15/May**	**School**	**PP**	**Children**	**154/UK**	**41**	**26**	**GII.2**
04071	**16–23/May**	**Kindergarten**	**PP**	**Children**	**95/UK**	**56**	**49**	**GII.2**
04073	**22/May**	**Restaurant**	**UK**	**Adults**	**4/5**	**2**	**2**	**GII.2**
04075	**25/May**	**School**	**PP**	**Children**	**41/UK**	**22**	**19**	**GII.2**
04076	**25–26/May**	**School**	**PP**	**Children**	**11/UK**	**9**	**9**	**GII.2**

[a] GII.2-cap NoV-associated outbreaks are indicated in boldface.
[b] Only one sample tested positive for NoV, but the outbreak was confirmed based on epidemiological data.
[c] Kageyama et al. (10).
[d] UK, unknown route.
[e] PP, person-to-person contact.
[f] The outbreaks occurred in other cities and had other NoV-positive patients.

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sequence identity in the complete capsid protein VP1 (5, 23). However, for molecular epidemiological investigations, tentative genotyping methods based on partial genomic sequencing of the RNA-dependent RNA polymerase (RdRp) and capsid genes are commonly used (3, 10, 19, 20). Between March and May 2004, an unusual increase in NoV-associated outbreaks was observed in Osaka City, Japan.

In Osaka City, with a population of approximately 2.6 million, NoV surveillance is conducted by collecting a basic set of epidemiological data (age range of patients, setting of outbreak, mode of transmission, date of onset, and attack rate) and testing stool specimens. An outbreak of acute gastroenteritis is defined as two or more patients with diarrhea and or vomiting who are linked by place and time. Patients with acute gastroenteritis attending sentinel pediatric clinics in Osaka are included as sporadic cases (8). Stool specimens were tested for NoV by reverse transcription-PCR (RT-PCR) using primers targeting the RdRp region until April 2001 (9) and by real-time RT-PCR since that time (18). All GII.2 strains were characterized by both partial RdRp and capsid gene sequencing as follows. RT-PCR assays were developed to amplify long genomic fragments using different sets of primers: (i) primer pair LV4282-99F (5′-YCAY TATGATGCWGAYTA-3′)/N235Rex (5′-GCWANRAAAGCT CCWGCCAT-3′) for the partial RdRp and the complete capsid genes (2,451 bp) and (ii) LV4282-99F/G2SKR (12) for the partial RdRp and the capsid N-terminal/shell (N/S) genes (1,108 bp). The amplified fragments were sequenced in both orientations with the primers. Phylogenetic analysis and genotyping based on the capsid N/S domain were performed as described by Katayama et al. (11). Assignment of genotype was based on the complete VP1 sequence according to Zheng et al. (23) and expressed as "genotype number-cap" (for example, "GII.2-cap"). Genotyping based on the RdRp region was performed using the criteria described by Vinjé et al. (19). The RdRp genotype was expressed as "genotype number-pol" (for example, "GII.2-pol").

A total of 238 NoV-positive outbreaks and 300 positive sporadic cases were detected between April 1996 and March 2005. Most (91.6%) of the NoV-positive outbreaks occurred between November and March of each year, whereas 85.0% of the NoV-positive sporadic cases occurred between October and February of each year. Between March and May 2004, 11 GII.2-cap NoV-associated outbreaks were observed (Table 1). In other years, a total of eight genetically different GII.2-cap strains, found on a separate branch on the phylogenetic tree (Fig. 1A), were detected. Thus, the number of the GII.2-cap NoV-associated outbreaks in the spring of 2004 was unusual compared with those for other seasons and higher than in all previous years (Poisson distribution, $P < 0.0001$). No NoV-associated outbreaks were observed between June and October 2004.

Of the 11 GII.2-cap NoV-associated outbreaks in the spring of 2004, nine occurred in children (81.8%), whose most common transmission mode was person-to-person contact (63.6%) (Table 1). In both children and adults, symptoms in GII.2-cap NoV-associated outbreaks were similar to those in outbreaks caused by other NoV genotypes. No epidemiological links were found among the outbreaks that could explain their spring emergence. In contrast, the eight genetically different GII.2-cap strains observed during our

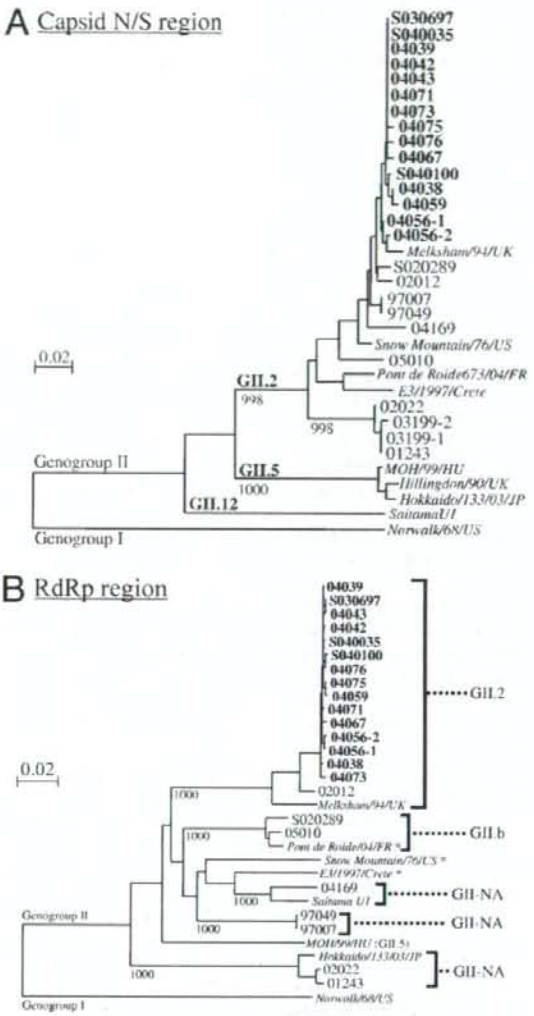

FIG. 1. Phylogenetic analysis of the capsid N/S (278 nucleotides) (A) and the partial RdRp (B) regions of the GII.2-cap strains detected in Osaka City. The GII.2-cap strains detected between March and May 2004 (04spring strains) are represented in boldface. Reference strains of NoV used in this study are represented in italics. The bootstrap values are indicated on each branch. The scale indicates the number of substitutions per site. (A) In outbreaks 03199 and 04056, there were two kinds of sequences, whereas all other outbreaks had only one type of sequence. (B) The tree was constructed with 720 nucleotides of the 3′ end of ORF1. Strains 03199-1 and -2 could not be amplified in the RdRp gene. The asterisks indicate the GII.2-cap NoVs, which have been reported as the GII.2-capsid sequences associated with other RdRp sequences (1, 3, 7). The genotypes at the RdRp region, which are not assigned numbers, are represented as GII-NA. The GenBank accession numbers for the reference strains of NoV used in this study are as follows. E3/97/Crete, AY682552; Hillingdon/90/UK, AJ277607; Hokkaido/133/03/JP, AB212306; Melksham/94/UK, X81879; MOH/99/HU, AF397256; Norwalk/68/US, M87661; Pont de Roide 673/04/FR, AY682549; Saitama U1, AB039775; Snow Mountain/76/US, AY134748.

9-year NoV surveillance were found mainly in December or January, mostly in adults, with transmission by the consumption of contaminated foods. Among sporadic cases, three GII.2-cap strains were detected in the spring of 2004. These cases seemed to be epidemiologically unrelated to the 11 outbreaks of the same period. From the genetic analysis, all GII.2-cap strains detected during the spring of 2004 (04spring strains) were classified into the GII.2 genotype at the RdRp region and were closely related to one another (≥99.1% nucleotide and ≥98.5% nucleotide identities in RdRp and capsid N/S regions, respectively). The eight genetically distinct GII.2-cap strains from other seasons were segregated into GII.2 (strain 02012) and other four other genotypes (one GII.b and three GII-NA) at the RdRp region, suggesting that these four were recombinant strains (Fig. 1B). Comparison of the amino acid sequences of the complete capsid genes revealed no common difference between the 04spring strains and the other GII2-cap strains (data not shown).

In this study, we focused on an unusual cluster of GII.2 NoV-associated outbreaks in spring 2004 in Osaka City. These GII.2-cap strains were rare in Osaka City in the previous 9 years of our surveillance. The spring 2004 outbreaks were distinct from the other GII.2-cap NoV-associated outbreaks in seasonality (spring versus winter), age of patients (children versus adults), and transmission mode (contact versus food). These occurrences could be explained by the rarity of GII.2 strains in the population. Since the strains were rare, children in Osaka City most likely did not have antibodies to the 04spring strains. The genetic characterization of these strains showed that they formed a distinct cluster that suddenly appeared, spread in Osaka City for a few months, and disappeared. Their disappearance may reflect acquisition of immunity to the 04spring strains in the population. Previous reports described the sudden emergence and disappearance of certain genotypes of NoV (6, 8, 9, 17) in a limited region. For GII.4 strains, this phenomenon has been observed globally (13, 15, 16, 22). It is unclear why differences in behavior exist among NoVs belonging to different genotypes. The emergence of a GII.2 strain with matching RdRp and capsid genotypes as the dominant cause of a cluster of outbreaks suggests that recombination may affect the behavior of NoV strains. Most other GII.2 viruses found throughout the surveillance period were recombinant strains detected in isolated outbreaks. Gallimore et al. (4) likewise suggest that variants differ in their impact on public health according to the accumulation of point mutations and recombinants. Future studies using structured surveillance are needed to address this hypothesis and improve our understanding of NoV epidemiology. Such insight is essential to design evidence-based strategies for NoV control and prevention.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study were deposited in DDBJ with the following accession numbers: AB089882 and AB279553 to AB279576.

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REFERENCES

1. **Ambert-Balay, K., F. Bon, F. L. Guyader, P. Pothier, and E. Kohli.** 2005. Characterization of new recombinant noroviruses. J. Clin. Microbiol. **43:** 5179–5186.
2. **Ando, T., J. S. Noel, and R. L. Fankhauser.** 2000. Genetic classification of "Norwalk-like viruses." J. Infect. Dis. **181**(Suppl. 2):S336–S348.
3. **Bull, R. A., G. S. Hansman, L. E. Clancy, M. M. Tanaka, W. D. Rawlinson, and P. A. White.** 2005. Norovirus recombination in ORF1/ORF2 overlap. Emerg. Infect. Dis. **11:**1079–1085.
4. **Gallimore, C. I., M. Iturriza-Gomara, J. Xerry, J. Adigwe, and J. J. Gray.** 2007. Inter-seasonal diversity of norovirus genotypes: emergence and selection of virus variants. Arch. Virol. **152:**1295–1303.
5. **Green, K. Y.** 2007. Caliciviridae: the noroviruses, p. 949–979. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
6. **Hansman, G. S., L. T. P. Doan, T. A. Kguyen, S. Okitsu, K. Katayama, S. Ogawa, K. Natori, N. Takeda, Y. Kato, O. Nishio, M. Noda, and H. Ushijima.** 2004. Detection of norovirus and sapovirus infection among children with gastroenteritis in Ho Chi Minh City, Vietnam. Arch. Virol. **149:**1673–1688.
7. **Hardy, M. E., S. F. Kramer, J. J. Treanor, and M. K. Estes.** 1997. Human calicivirus genogroup II capsid sequence diversity revealed by analyses of the prototype Snow Mountain agent. Arch. Virol. **142:**1469–1479.
8. **Iritani, N., Y. Seto, H. Kubo, T. Murakami, K. Haruki, M. Ayata, and H. Ogura.** 2003. Prevalence of Norwalk-like virus infections in cases of viral gastroenteritis among children in Osaka City, Japan. J. Clin. Microbiol. **41:**1756–1759.
9. **Iritani, N., Y. Seto, K. Haruki, M. Kimura, M. Ayata, and H. Ogura.** 2000. Major change in the predominant type of "Norwalk-like viruses" in outbreaks of acute nonbacterial gastroenteritis in Osaka City, Japan, between April 1996 and March 1999. J. Clin. Microbiol. **38:**2649–2654.
10. **Kageyama, T., M. Shinohara, U. Uchida, S. Fukushi, F. B. Hoshino, S. Kojima, R. Takai, T. Oka, N. Takeda, and K. Katayama.** 2004. Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to norovirus in Japan. J. Clin. Microbiol. **42:**2988–2995.
11. **Katayama, K., H. Shirato-Horikoshi, S. Kojima, T. Kageyama, T. Oka, F. B. Hoshino, S. Fukushi, M. Shinohara, K. Uchida, Y. Suzuki, T. Gojobori, and N. Takeda.** 2002. Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. Virology **299:**225–239.
12. **Kojima, S., T. Kageyama, S. Fukushi, F. B. Hoshino, M. Shinohara, K. Uchida, K. Natori, N. Takeda, and K. Katayama.** 2002. Genogroup-specific PCR primers for detection of Norwalk-like viruses. J. Virol. Methods **100:** 107–114.
13. **Lopman, B., H. Vennema, E. Kohli, P. Pothier, A. Sanchez, A. Negredo, J. Buesa, E. Schreier, M. Reacher, D. Brown, J. Gray, M. Iturriza, C. Gallimore, B. Bottiger, K. O. Hedlund, M. Torven, C. H. Von Bondsdorf, L. Maunula, M. Poljsak-Prijatelj, J. Zimsek, G. Reuter, G. Szucs, B. Melegh, L. Svennson, Y. van Duijnhoven, and M. Koopmans.** 2004. Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. Lancet **363:**682–688.
14. **Mounts, A. W., T. Ando, M. Koopmans, J. S. Bresee, J. Noel, and R. I. Glass.** 2000. Cold weather seasonality of gastroenteritis associated with Norwalk-like viruses. J. Infect. Dis. **181**(Suppl. 2):S284–S287.
15. **Noel, J. S., R. L. Fankhauser, T. Ando, S. S. Monroe, and R. I. Glass.** 1999. Identification of a distinct common strain of "Norwalk-like viruses" having a global distribution. J. Infect. Dis. **179:**1334–1344.
16. **Okada, M., T. Ogawa, I. Kaiho, and K. Shinozaki.** 2005. Genetic analysis of noroviruses in Chiba Prefecture, Japan, between 1999 and 2004. J. Clin. Microbiol. **43:**4391–4401.
17. **Phan, T. G., T. Kuroiwa, K. Kaneshi, Y. Ueda, S. Nakaya, S. Nishimura, A. Yamamoto, K. Sugita, T. Nishimura, F. Yagyu, S. Okitsu, W. E. G. Muller, N. Maneekarn, and H. Ushijima.** 2006. Changing distribution of norovirus genotypes and genetic analysis of recombinant GIIb among infants and children with diarrhea in Japan. J. Med. Virol. **78:**971–978.
18. **Seto, Y., N. Iritani, H. Kubo, A Kaida, T. Murakami, K. Haruki, O. Nishio, M. Ayata, and H. Ogura.** 2005. Genotyping of *Norovirus* strains detected in outbreaks between April 2002 and March 2003 in Osaka City, Japan. Microbiol. Immunol. **49:**275–283.
19. **Vinjé, J., J. Green, D. C. Lewis, C. I. Gallimore, D. W. Brown, and M. P. G. Koopmans.** 2000. Genetic polymorphism across regions of the three open reading frames of "Norwalk-like viruses." Arch. Virol. **145:**223–241.
20. **Vinjé, J., R. A. Hamidjaja, and M. D. Sobsey.** 2004. Development and

application of a capsid VP1 (region D) based reverse transcription PCR assay for genotyping of genogroup I and II noroviruses. J. Virol. Methods **116:**109–117.

21. **Wang, J., X. Jiang, H. P. Madore, J. Gray, U. Desselberger, T. Ando, Y. Seto, I. Oishi, J. F. Lew, K. Y. Green, and M. Estes.** 1994. Sequence diversity of small, round-structured viruses in the Norwalk virus group. J. Virol. **68:**5982–5990.

22. **White, P. A., G. S. Hansman, A. Li, J. Dable, M. Isaacs, M. Ferson, C. J. Melver, and W. D. Rawlinson.** 2002. Norwalk-like virus 95/96-US strain is a major cause of gastroenteritis outbreaks in Australia. J. Med. Virol. **68:**113–118.

23. **Zheng, D. P., T. Ando, R. L. Fankhauser, R. S. Beard, R. I. Glass, and S. S. Monroe.** 2006. Norovirus classification and proposed strain nomenclature. Virology **346:**312–323.

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Genetic Analysis of the Capsid Gene of Genotype GII.2 Noroviruses▽

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Noroviruses (NoVs) are considered to be a major cause of acute nonbacterial gastroenteritis in humans. The NoV genus is genetically diverse, and genotype GII.4 has been most commonly identified worldwide in recent years. In this study we analyzed the complete capsid gene of NoV strains belonging to the less prevalent genotype GII.2. We compared a total of 36 complete capsid sequences of GII.2 sequences obtained from the GenBank ($n = 5$) and from outbreaks or sporadic cases that occurred in The Netherlands ($n = 10$) and in Osaka City, Japan ($n = 21$), between 1976 and 2005. Alignment of all capsid sequences did not show fixation of amino acid substitutions over time as an indication for genetic drift. In contrast, when strains previously recognized as recombinants were excluded from the alignment, genetic drift was observed. Substitutions were found at five informative sites (two in the P1 subdomain and three in the P2 subdomain), segregating strains into five genetic groups (1994 to 1997, 1999 to 2000, 2001 to 2003, 2004, and 2005). Only one amino acid position changed consistently between each group (position 345). Homology modeling of the GII.2 capsid protein showed that the five amino acids were located on the surface of the capsid and close to each other at the interface of two monomers. The data suggest that these changes were induced by selective pressure, driving virus evolution. Remarkably, this was observed only for nonrecombinant genomes, suggesting differences in behavior with recombinant strains.

Noroviruses (NoVs) are an important cause of acute nonbacterial gastroenteritis in adults and children worldwide (13). NoVs are members of the family *Caliciviridae*, having a positive-sense single-stranded RNA genome. Their genome is organized into three open reading frames (ORFs). ORF1 encodes nonstructural proteins including the RNA-dependent RNA polymerase (RdRp), ORF2 encodes a major structural capsid protein including a shell (S) domain and a protruding (P) domain, and ORF3 encodes a minor structural protein (13, 18, 41). The S domain forms the inner part (shell) of the viral capsid, and the P domain forms the arch-like structures that protrude from the virion. The P domain is further divided into P1 and P2 subdomains that correspond to the sides and the top of the arch-like capsomeres, respectively (13, 31).

Based on the genetic analysis of the RdRp and capsid regions, human NoVs can be divided into three genogroups (Gs), GI, GII, and GIV (2, 14, 39), which further segregate into distinct lineages called genotypes (2, 20, 36, 37). Recently, Kageyama et al. (20) proposed that at least 31 genotypes could be distinguished within GI and GII. The GII.4 genotype, which is represented by the Lordsdale/93/UK strain, has been the most commonly identified genotype worldwide in recent years. Genetic characterization of strains belonging to this genotype have shown a sequence of variants that have arisen over time, suggesting that rapid genetic evolution of GII.4 NoVs may in part explain their successful spread and impact on people of all ages (5, 9, 27, 29, 30, 33, 40).

Viruses belonging to other NoV genotypes are found less consistently, causing sporadic outbreaks or temporary epidemics in a limited geographic region or time period (5, 17, 23, 26). As a result, far less is known about the population structure of these variants (28). The genetic analysis of other genotype NoVs excluding GII.4 will improve our understanding of genetic evolution and its relevance for the epidemiology of NoVs.

During the spring of 2004, an epidemic of GII.2 NoV (which is represented by the Melksham/94/UK strain [Melksham]) occurred in Osaka City, Japan. Our previous study of this regional epidemic described the molecular epidemiology of these GII.2 strains (17). Here, we describe the genetic characterization of GII.2 strains from those outbreaks in comparison with viruses detected over a 12-year period in the GenBank, The Netherlands, and Japan.

MATERIALS AND METHODS

GII.2 strains. A total of 36 NoV strains that had been characterized as GII.2 genotype were used for this study (Table 1). The capsid sequence data for five GII.2 strains were obtained from the GenBank. Of these, the Melksham strain and the Chesterfield/434/1997/US strain (11, 29) have been characterized as belonging to the GII.2 genotype on the basis of RdRp as well as capsid regions. The Snow Mountain/76/US strain has been characterized as a recombinant NoV, with a distinct (non-GII.2) RdRp region and a GII.2 capsid region (4, 16). The other two strains from the GenBank (Ina/02/JP and Buds/02/US) were characterized as GII.2 genotype on the basis of the capsid region, but their sequences of the RdRp region were unknown. The capsid sequence data of Buds/02/US strain lacked the first 6 nucleotides (nt) from the 5′ end of the capsid gene. Twenty-one GII.2 strains were obtained from outbreaks or sporadic cases detected in Osaka City, Japan, between April 1996 and March 2005. These were 21

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TABLE 1. GII.2 NoV strains used in this study

Source	Strain (abbreviation)[a]	RdRp sequence type[b]	Accession no.	Note[c]
GenBank	Snow Mountain/76/US (SM)	GII-NA	AY134748	
	Melksham/94/UK (Melksham)	GII.2	X81879	
	Chesterfield/434/97/US (CF434)	GII.2	AY054300	
	Ina/02/JP (Ina)	Unknown	AB195225	
	Buds/02/US (Buds)	Unknown	AY660568	
Osaka City, Japan	OC97049/97/JP (97049)	GII-NA	AB279553	O
	OC01243/01/JP (01243)	GII-NA	AB279554	O
	OC02012/02/JP (02012)	GII.2	AB279555	O
	OC02022/02/JP (02022)	GII-NA	AB279556	O
	OCS020289/02/JP (S020289)	GII.b	AB279570	S
	OC04038/04/JP (04038)[d]	GII.2	AB279557	O[e]
	OC04042/04/JP (04042)[d]	GII.2	AB279558	O[e]
	OC04043/04/JP (04043)[d]	GII.2	AB279559	O[e]
	OCS030697/04/JP (S030697)[d]	GII.2	AB279571	S[e]
	OC04056-1/04/JP (04056-1)[d]	GII.2	AB279560	O[e]
	OC04056-2/04/JP (04056-2)[d]	GII.2	AB279561	O[e]
	OC04059/04/JP (04059)	GII.2	AB279562	O[e]
	OCS040035/04/JP (S040035)	GII.2	AB279572	S[e]
	OC04067/04/JP (04067)[d]	GII.2	AB279563	O[e]
	OC04071/04/JP (04071)	GII.2	AB279564	O[e]
	OC04073/04/JP (04073)	GII.2	AB279565	O[e]
	OC04075/04/JP (04075)	GII.2	AB279566	O[e]
	OC04076/04/JP (04076)[d]	GII.2	AB279567	O[e]
	OCS040100/04/JP (S040100)	GII.2	AB279573	S[e]
	OC04169/04/JP (04169)	GII-NA	AB279568	O
	OC05010/05/JP (05010)	GII.b	AB279569	O
The Netherlands	Sensor99-191/99/NL (S99-191)	GII.2	AB281081	S
	OB0037-246/00/NL (OB0037)	GII.2	AB281082	O
	OB0048-318/00/NL (OB0048)	GII.2	AB281083	S
	OB0115-195/01/NL (OB0115)	GII.2	AB281084	O
	EP0125-006/01/NL (EP0125)	GII.2	AB281085	O
	EP0207-001/02/NL (EP0207)	GII.2	AB281086	O
	EP0239-001/02/NL (EP0239)	GII.2	AB281087	O
	OB0371-459/03/NL (OB0371)	GII.2	AB281088	O
	OB0528-158/05NL (OB0528)	GII.2	AB281089	O
	OB0587-470/05/NL (OB0587)	GII.2	AB281090	O

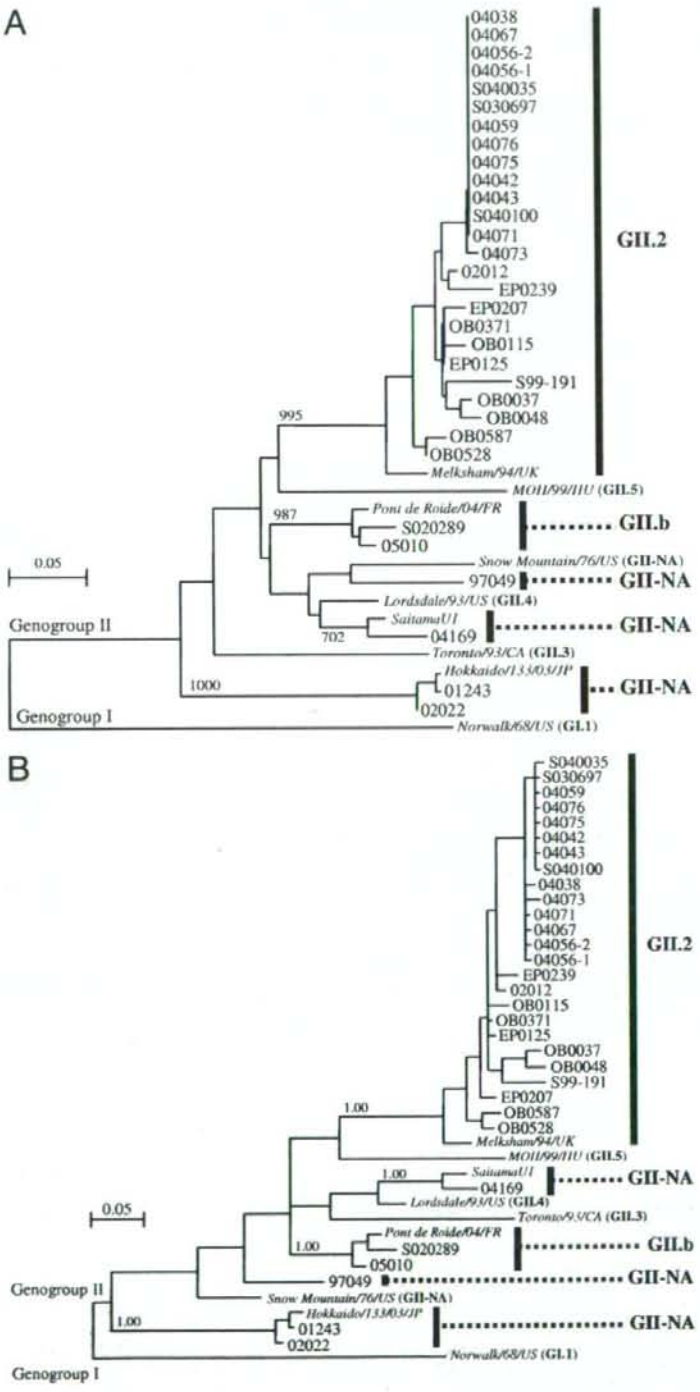
[a] NoV strains are arranged in chronology of detection from top (oldest) to bottom (most recent) for each source.
[b] NA, not assigned.
[c] O, outbreak; S, sporadic case; [e], spring epidemic in 2004.
[d] These strains have identical amino acid sequences in the complete capsid gene and only one strain (OC04038/04/JP) has been used for long-term genetic analysis.

of the 23 GII.2 strains identified during a 9-year study period out of a total of 238 outbreaks and 200 sporadic cases of NoV infection. From the genetic analysis across the junction between the RdRp and the capsid regions, 6 of these 21 GII.2 strains have been characterized as recombinants, which have non-GII.2 RdRp regions and GII.2 capsid regions (Fig. 1) (17).

The strains from The Netherlands were collected from a 12-year study period. Between 1994 and 2005, GII.2 NoVs were detected in 13 (1.7%) out of 745 NoV-associated outbreaks and three sporadic cases in The Netherlands. Initially, these GII.2 NoVs were characterized by the comparison of sequences in the RdRp region (Fig. 1). The detection method and criteria for genotyping at the RdRp region have been previously described (8, 36). The complete capsid genes of 10 strains from eight outbreaks and two sporadic cases were amplified by reverse transcription-PCR (RT-PCR) and were used for this analysis.

Amplification and sequencing for the complete capsid gene of GII.2 strains. Viral RNA was extracted from stool suspensions by using a QIAamp viral RNA Mini kit (Qiagen, Hilden, Germany). RT-PCR was carried out with the reaction mixtures and enzymes as previously described (8). RT was performed at 42°C for 2 to 3 h with reverse primer, N235Rec (17), and enzyme was inactivated at 95°C for 5 min. PCR was performed using several pairs of PCR primers (Table 2) with a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) under the following conditions: denaturation at 95°C for 1 min; 40 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 1 min; and a final cycle of incubation at 72°C for 5 min. When a PCR failed to produce strong products, we performed nested PCR. The amplified fragments were sequenced directly with a Big Dye terminator cycle sequencing kit and ABI 3700 sequencer (Applied Biosystems, Foster City, CA). The nucleotide sequences were determined in both orientations using the PCR primers. DNA sequences were edited using SeqManII (DNAStar Inc., Konstanz, Germany).

Sequence analysis. Nucleotide or amino acid sequence alignments were performed with BioEdit (version 7.052) (15), Clustal X (version 1.81) (35), or MUSCLE (version 3.51) (10). The extraction of the informative sites from nucleotide or amino acid sequence alignments was performed with ProSeq (version 2.91) (12). The rate of change for different domains was compared using chi-square statistics. In this analysis, a site was designated as an informative site when at least two strains had an identical amino acid in the alignment that differed from the other sequences. A phylogenetic tree with 1,000 bootstrap replications was constructed by the neighbor-joining method, and the genetic distances were calculated according to the Kimura two-parameter method (21). We performed additional phylogenetic analysis by the Bayesian method using MrBayes (version 3.1.2) (32). Location of specific domains of the GII.2 NoV capsid gene was done according to Chen et al. (7). For computational predictions of the structure of the GII.2 NoV capsid protein, we used the X-ray crystal structures of the capsid protein of Norwalk/68/US (GI.1 genotype, Protein Data Bank identifier [PDB ID] 1IHM, consisting of a complete trimer) (31) and VA387/98/US (GII.4 genotype, PDB ID 2OBR, consisting of only a monomeric P domain) (6) as templates to build homology models. The sequence alignments for the structure and the three-dimensional (3D) models for GII.2 NoV capsid proteins were made by using the WHAT IF program (38) and the 3D-Jigsaw (3)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Melksham/94/UK
MOH/99/IIU (GII.5)
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Pont de Roide/04/FR
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GII.b
Snow Mountain/76/US (GII-NA)
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GII-NA
Lordsdale/93/US (GII.4)
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GII-NA
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Toronto/93/CA (GII.3)
Hokkaido/133/03/JP
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Genogroup I
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