食水系感染症病原体の検査法－7

サルモネラ

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I. 病原体

1. 病原体

サルモネラは1885年、SalmonとSmithによって豚コレラ罹患豚（豚コレラの病原体は*Flaviviridae Pestivirus*）から新しい種菌として分離され、*Bacillus choleraesuis*と命名された。その菌は後にSalmonの名に由来した*Salmonella*となった。現在*Salmonella*は*S. enterica*, *S. bongori*, *S. subterrane*[1]の3菌種で*S. enterica*は6亜種（subsp. *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *indica*）に分類されている。

サルモネラはOおよびH抗原の組み合わせで、2000種類以上の血清型が存在する。*Salmonella enterica* subsp. *enterica* serovar Typhimuriumは短縮してS. Typhimuriumとなる。学名はイタリック体で、血清型は通常の字体で書く。

感染症・食中毒として重要となるのは*S. enterica* subsp. *enterica*の多くの血清型である。S. TyphiおよびS. Paratyphi Aは「感染症の予防及び感染症の患者に対する医療に関する法律」における三類感染症の腸チフスならびにパラチフスに各々該当する。家畜では鶏、あひる、七面鳥、うずらを対象動物としたS. Gallinarum-Pullorum感染症は「家きんサルモネラ感染症」となり、家畜伝染病予防法の法定伝染病として、牛、水牛、しか、豚、いのしし、鶏、あひる、七面鳥、うずらを対象動物としたS. Dublin、S. Enteritidis、S. Typhimurium、S. Choleraesuis感染症は「サルモネラ症」となり、届出伝染病と定義されている。

2. 疫学

サルモネラは細菌性食中毒の原因菌の中で常に上位であり、2009年（平成21年）の食中毒統計では、細菌性食中毒のうちサルモネラ食中毒は67件（12.5%）、1,518人（22.7%）で、件数では第2位、患者数は第3位である。

牛の保菌率は一般的に低値であり、成牛ではほとんど分離されることはない[2,3]。しかし子牛のサルモネラ症はまれに発生する。

豚の保菌率は約4～7%であり[3-5]、多くの血清型が分離される。近年、S. Choleraesuisによる全身感染例が散見され、これらからはH_2S非産生株（バイオタイプCholeraesuis）も分離される[6]。

鶏の保菌率は高率で、53%（17/32）の鶏盲腸内容から本菌は分離される[7]。ブロイラー処理場の18～21%、成鶏処理場の36～48%の食鳥肉[8]、12%（7/60）の市販鶏ひき肉からも検出される[7]。1998年（平成10年）、卵によるサルモネラ食中毒の発生防止に関するリスクアセスメントが食品衛生調査会で実施され、殻付き卵の0.03%（6/24,000）からS. Enteritidisが検出されること（平成4年調査）。未殺菌液卵（全卵）の4.0%（55/1,370）（平成2年調査）や、12.0%（18/150）（平成2年調査）からS. Enteritidisが検出されること等のデータの分析から、卵の消費期限の設定、液卵の衛生対策が講じられた。

爬虫類も保菌しており、米国では約74,000名が爬虫類に関連したサルモネラ症に罹患し、本症全体の6%、特に子供では11%が爬虫類関与である[9]。2005年、わが国でも厚生労働省は「ミドリガメ等のハ虫類を原因とするサルモネラ症発生事例に係る注意喚起について」（健感発第1222002号）を通知している。また、壁谷ら[10]はグリーンイグアナの17%（17/98）の糞便からサルモネラを分離し、分離株の多くは*S. enterica* subsp. *houtenae*であることを報告している。

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3. 臨床症状

サルモネラは感染型食中毒を引き起こし、臨床症状は多様であるが、主に急性胃腸炎を示す。潜伏期間は8～48時間であり、最近の *S.* Enteritidis 感染症では少量でも発症することから、潜伏期間が3～4日と長いこともある。症状は、悪心・嘔吐とともに高熱が起こり、腹痛・下痢となる。下痢は1日数回から十数回で3～4日持続するが、1週間以上に及ぶこともある。小児では意識障害、痙攣および菌血症、高齢者では急性脱水症および菌血症を起こすなど重症化しやすく、回復も遅れる。また、サルモネラは胆汁に耐性を示すことから、胆のう中に本菌が侵入した場合は、難治療性となり、長期間便中に排菌する健康保菌者となることもある。

Ⅱ. 検査法

1. 培養法

サルモネラの培養法は多くの報告があるが、前増菌培地としてはBPWやEEMブイヨン、選択増菌培地としてはRV培地とTetrathionate (TT) 培地。選択培地としては日本では H_2S 産生 (SS, DHL, MLCB 等)、諸外国ではリシン脱炭酸性 (dmLIA, BGA 等) を確認する培地を用いることが多い。また、酵素基質培地 (ESサルモネラ寒天培地、ESサルモネラ寒天培地Ⅱ；栄研、CHROMagar™ Salmonella；CHROMagar 社、X-SAL 寒天培地；日水、Salmonella chromogenic agar；Oxoid 等) も数多く市販されている。図1に米国農務省 (USDA) 食品安全検査局 (FSIS) のサルモネラ分離法を示す。なお、著者らは H_2S 非産生株も念頭におき、選択培地はdmLIA培地 (脚注) と酵素基質培地を併用している。写真1に各種選択培地上のサルモネラ集落を示す。

国立医薬品食品衛生研究所・食品からの微生物標準試験法検討委員会の示したサルモネラ検査プロトコールは http://www.nihs.go.jp/fhm/kennsahou-index.html から、AOAC 検査法は http://www.scribd.com/doc/17367740/Aoac-Official-Method-Salmonella) から、WHO の推奨する検査法は http://www.antimicrobialresistance.dk/232-169-215-protocols.htm#Salmonella から入手できる。

2. 遺伝子検査法

通常の PCR 法としては、上皮細胞侵入性関連遺伝子 (*invA*)[11]、エンテロトキシン産生遺伝子 (*stn*)[12]、病原性プラスミド関連遺伝子 (*SpvC*)[11]、*S.* Typhimurium 株についてはファージタイプDT104関連遺伝子[13] 等が報告されており、その用途に応じて検査が実施される。*invA* はほとんどすべてのサルモネラ属菌が保有しているので、本属の同定試験として用いられている。Real-Time PCR 法も多くの報告があるが、サルモネラを含む食中毒菌発生時の迅速スクリーニングでは福島ら[14] の方法が有効と思われる。LAMP 法 (Loopamp® サルモネラ検出試薬キット：栄研)、DNA プローブ法 (核さんテスト サルモネラ：ファスマック) 等も市販されている。以下に、サルモネラ同定のための *invA* 遺伝子検出の方法を記述する。

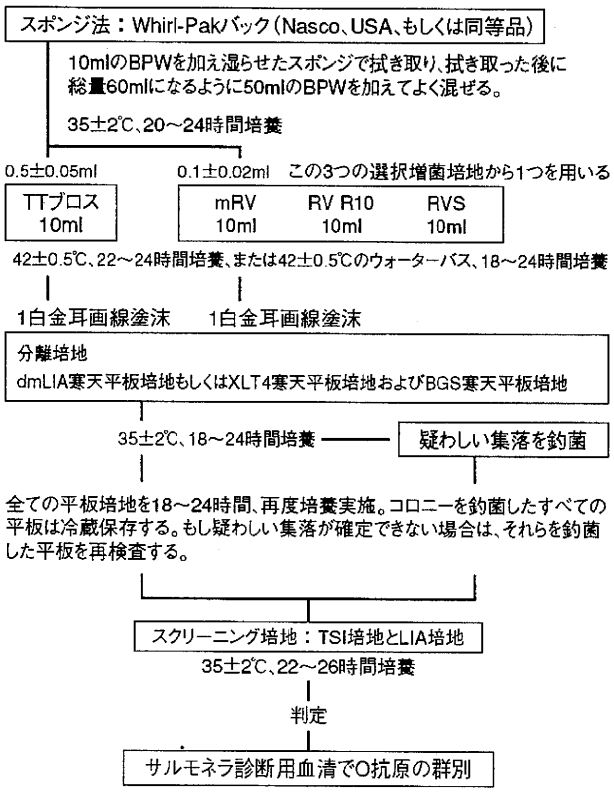

図1　USDA FSISの食品のふきとり検体からのサルモネラの検査法

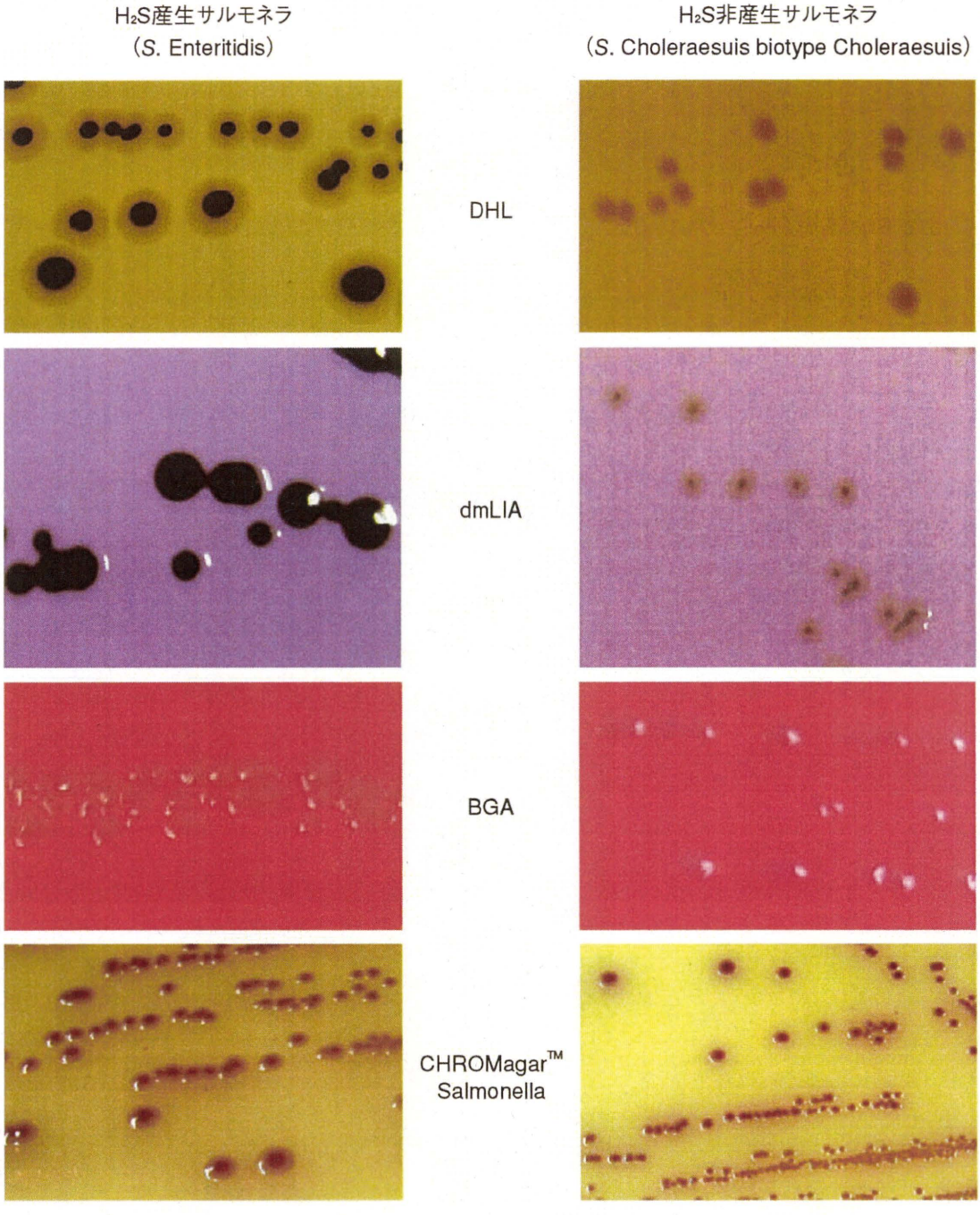

写真1　各種選択培地上のH_2S産生・非産生サルモネラ集落

Double Modified Lysin Iron Agar（dmLIA）

組 成（1000mL）

リシン鉄寒天培地（OXOID）	34.0g
Bile salt No.3（OXOID）	1.5g
乳糖	10.0g
白糖	10.0g
チオ硫酸ナトリウム	6.76g
クエン酸鉄アンモニウム	0.3g
ノボビオシンナトリウム	0.015g

作製方法

- ノボビオシンナトリウム以外のすべての成分を蒸留水1000mLで混合し、約30分間煮沸して完全に溶解させる（禁オートクレーブ）。
- ウォーターバスなどで50℃に冷却し、ノボビオシンナトリウム〔0.0075g（力価）/1mL（DDW）：濾過滅菌済み〕*を加えてよく混合する。
- 滅菌シャーレに分注する（20mL程度）
- 冷蔵で3週間保存可能。

*：ノボビオシンナトリウムについては、1本（1mL）/500mL（培地）となるように前もって調整して−80℃保存し、それを解凍して使用。

食水系感染症病原体の検査法－7

1）DNA抽出

増菌培養液や培地上の集落をかきとった菌浮遊水溶液に95～100℃、5分間熱処理を実施する。または市販DNA抽出キット（InstaGene™ Matrix：BIO RAD等）を用いる。

2）PCR増幅

Chiu CH and Ou JT[11]が報告している*invA*を増幅するPrimerを用いて、PCRを実施する。なお、試薬や検査時間を考慮して、反応液の組成、反応時間等は変更して実施している。

①Primer

INVA-1：5'-ACAGTGCTCGTTTACGACCTGAAT-3'

INVA-2：5'-AGACGACTGGTACTGATCGATAAT-3'

②反応液

試薬はGoTaq® Green Master Mix（Promega）を用い、反応組成は次のとおりとしている。

Green Master Mix、2X	12.5μl
Primer INVA-1（10pmol）	1.0μl
Primer INVA-2（10pmol）	1.0μl
Nuclease-Free Water	9.5μl
DNA Template	1.0μl
Total	25.0μl

③PCR条件

Initial denaturation	95℃	2分間	
Denaturation	95℃	30秒間	30 cycles
Annealing	56℃	30秒間	
Extension	72℃	1分間	
Final extension	72℃	5分間	

3）電気泳動

2～3%アガロースを用いて電気泳動し、244bpの増幅産物を確認する。

3. その他の簡易・迅速診断法等

サルモネラは微生物学的危害として重要視されているため各種簡易・迅速診断法が市販されている。ラテックス凝集法（サルモネラLA：デンカ生研、Salmonella Latex test：Oxoid,等）、イムノクロマト法（VIP for Salmonella：BioControl Systems, Reveal Salmonella：Neogen, Singlepath salmonella：Merck, 等）、EIA（酵素抗体）法（バイダス アッセイキット サルモネラ：シスメックス・ビオメリュー）、免疫拡散法（salmonella 1-2test：BioControl Systems）等がある。また、少数菌を効率的に検出するための免疫磁気ビーズ法も市販されている。

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Research Note

Detection of Human Enteric Viruses in Japanese Clams

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ABSTRACT

A total of 57 clam packages that were collected from supermarkets and fish markets from 11 different sites in western Japan between 8 December 2005 and 6 September 2006 were examined for human enteric viruses (i.e., norovirus, Aichi virus, rotavirus, adenovirus, hepatitis A virus, and astrovirus), using PCR and reverse transcription PCR. Sixty-one percent of the packages were contaminated with one type of virus, 9% had two different types of viruses, 28% had three different types of viruses, and 9% had at least four different types of viruses. Thirty-one (54%) of 57 packages were contaminated with noroviruses. Norovirus genogroup I and genogroup II sequences were detected in 24 and 23 packages, respectively, and these sequences belonged to nine genogroup I and eight genogroup II genotypes. Aichi viruses were found in 19 (33%) of 57 packages, and these belonged to genogroup A. Rotaviruses (group A) were detected in 14 (42%) of 33 of packages and 9 of 14 rotavirus-positive packages contained two or more rotavirus genogroup types. Adenoviruses (Ad40 and Ad41) were detected in 17 (52%) of 33 packages. One of the 57 (2%) packages was positive with hepatitis A virus (subtype IA). Astrovirus was not detected in any of the packages. This is the first study to detect such a high level of contamination in Japanese clams. These results represent an important finding because the Japanese clams were considered suitable for human consumption. Further studies are needed to determine the health risks associated with eating these highly contaminated clams.

Gastroenteritis is one of the leading causes of death by an infectious disease *(19)*, with more than 700 million cases of acute diarrheal disease occurring annually. The main viral agents that cause gastroenteritis are norovirus, rotavirus, sapovirus, astrovirus, and enteric adenoviruses. These viruses have been detected in environmental samples (e.g., lakes and sewage) as well as in foods such as oysters, clams, sandwiches, and raspberries. Other important viral agents that can accumulate in oysters and clams are hepatitis A virus (HAV) and hepatitis E virus (HEV) *(4, 17)*. The impact of viral contamination in the environment is evident in Japan, where outbreaks of norovirus oyster–associated gastroenteritis increases in winter, and this coincides with the oyster-harvesting season in winter *(21)*. The detection methods for these viruses in environmental samples and clinical specimens have greatly improved over the past 10 years and have provided a better understanding and distribution of these viruses.

The purpose of this study was to detect norovirus, Aichi virus, rotavirus, adenovirus, HAV, and astrovirus in clam packages sold at supermarkets and fish markets, which were destined for human consumption in Japan, using PCR and reverse transcription PCR, and then describe the genetic diversity of the positive noroviruses.

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MATERALS AND METHODS

Clam samples. A total of 57 clam (*Corbicula japonica*) packages (30 to 60 clams per package) were collected from supermarkets or fish markets (nonexport) from 11 different geographically distinct sites in western Japan between 8 December 2005 and 6 September 2006. The clam packages were screened for norovirus, Aichi virus, rotavirus, adenovirus, HAV, and astrovirus using PCR and reverse transcription PCR. These 57 packages were previously screened for sapovirus *(9)*, and 46 of 57 packages were screened for HEV *(17)*.

Viral extraction. The clams were shucked, the digestive diverticulum removed by dissection on the day of harvest *(16)*, and then weighed and homogenized in nine times their weight of phosphate-buffered saline without magnesium or calcium. One gram of digestive diverticulum (10 to 15 clams per package) was homogenized with an Omni mixer (Omni International, Marietta, Ga.) in 10 ml of phosphate-buffered saline (pH 7.2). After centrifugation at 10,000 × *g* for 30 min at 4°C, the supernatant was layered onto 1 ml of 30% sucrose solution and ultracentrifuged at 154,000 × *g* for 3 h at 4°C. The pellet was resuspended in 140 μl of distilled water and stored at −80°C until use.

DNA and RNA extraction and reverse transcription. Viral DNA (for detection of adenovirus) was extracted from resuspended pellet, using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Viral RNA (for detection of norovirus, Aichi virus, rotavirus, and HAV) was extracted from the resuspended pellet, using QIAmp Viral RNA Mini Kit (Qiagen.). For reverse transcription, the RNA solution was treated with 2 U of RNase-free DNase I (Takara, Tokyo, Japan) for 30 min at 37°C, and was

TABLE 1. *Details of the clam samples*

Package no.	Site	Mo/day/yr	No. of norovirus genotypes	Norovirus strain name (genogroup/genotype)	Aichi virus	Rotavirus: G types	Adeno-virus	HAV	HEV (from *(17)*)	Sapovirus (from *(9)*)
1	A	12/08/05	2	GI-Shijimi1 (GI/4), GII-Shijimi1 (GII/3)	Aic-1	G1/G4	−	−	−	−
2	D	12/10/05	−		−	−	+	−	−	−
3	A	12/22/05	2	GI-Shijimi2 (GI/1), GII-Shijimi2 (GII/2)	Aic-2	G9	+	−	−	−
4	D	12/17/05	−		−	G8	+	−	−	−
5	K	01/10/06	1	GII-Shijimi3 (GII/3)	Aic-3	NTa	NT	−	−	−
6	A	01/14/06	3	GI-Shijimi4 (GI/11), GII-Shijimi4a (GII/4), GII-Shijimi4b (GII/3)	Aic-4	G4	+	−	−	−
7	J	01/16/06	1	GI-Shijimi5 (GI/8)	Aic-5	NT	NT	−	−	−
8	A	01/20/06	2	GI-Shijimi6 (GI/14), GII-Shijimi6 (GII/3)	−	NT	NT	−	−	−
9	B	01/22/06	3	GI-Shijimi7 (GI/12), GII-Shijimi7a (GII/New), GII-Shijimi7b (GII/3)	Aic-6	G?	+	−	−	−
10	B	01/22/06	−		−	−	+	−	−	−
11	C	01/21/06	3	GI-Shijimi8 (GI/11), GII-Shijimi8a (GII/3), GII-Shijimi8b (GII/3), GII-Shijimi8c (GII/4), GII-Shijimi8d (GII/3)	−	−	−	−	−	−
12	D	01/24/06	3	GI-Shijimi9 (GI/8), GII-Shijimi9a (GII/3), GII-Shijimi9b (GII/4)	Aic-7	G?	+	−	−	−
13	C	01/26/06	3	GII-Shijimi10a (GII/3), GII-Shijimi10b (GII/3), GII-Shijimi10c (GII/3)	Aic-8	−	+	−	−	SaV-3
14	D	02/07/06	1	GI-Shijimi11 (GI/11)	Aic-18	NT	NT	−	+	−
15	B	02/05/06	4	GI-Shijimi12a (GI/1), GI-Shijimi12b (GI/1), GI-Shijimi12c (GI/8), GI-Shijimi12d (GI/11)	Aic-9	−	+	−	−	−
16	D	02/19/06	2	GI-Shijimi13 (GI/1), GII-Shijimi13 (GII/3)	Aic-10	G1/G8/G9	+	−	−	−
17	B	02/17/06	1	GII-Shijimi14 (GII/New)	−	G4	+	−	−	−
18	C	02/25/06	1	GII-Shijimi15 (GII/3)	Aic-11	G2/G4	−	−	−	−
19	D	03/01/06	7	GI-Shijimi16a (GI/11), GI-Shijimi16b (GI/8), GI-Shijimi16c (GI/4), GI-Shijimi16d (GI/8), GII-Shijimi16a (GII/3), GII-Shijimi16b (GII/4), GII-Shijimi16c (GII/4)	Aic-12	NT	NT	−	+	−
20	B	03/02/06	3	GI-Shijimi17 (GI/1), GII-Shijimi17a (GII/5), GII-Shijimi17b (GII/4)	Aic-19	−	+	−	−	−
21	C	03/10/06	2	GI-Shijimi18 (GI/8), GII-Shijimi18 (GII/3)	−	−	+	−	−	SaV-1
22	B	03/14/06	3	GI-Shijimi19a (GI/1), GI-Shijimi19b (GI/1), GII-Shijimi19 (GII/3)	Aic-13	−	+	−	−	−
23	A	03/14/06	−		−	NT	NT	−	−	−
24	E	03/13/06	−		−	NT	NT	−	−	−
25	E	03/14/06	−		−	NT	NT	−	−	−
26	B	03/15/06	2	GI-Shijimi20a (GI/5), GI-Shijimi20b (GI/14)	Aic-14	−	+	−	−	−
27	D	03/17/06	1	GI-Shijimi21 (GI/1)	−	G1/G2/G8	+	−	−	−
28	F	03/18/06	2	GI-Shijimi22 (GI/8), GII-Shijimi22 (GII/New)	−	G1/G2/G3/G8/G9	+	−	−	−
29	E	03/18/06	−		−	NT	NT	−	−	−
30	E	03/18/06	−		−	NT	NT	−	−	−

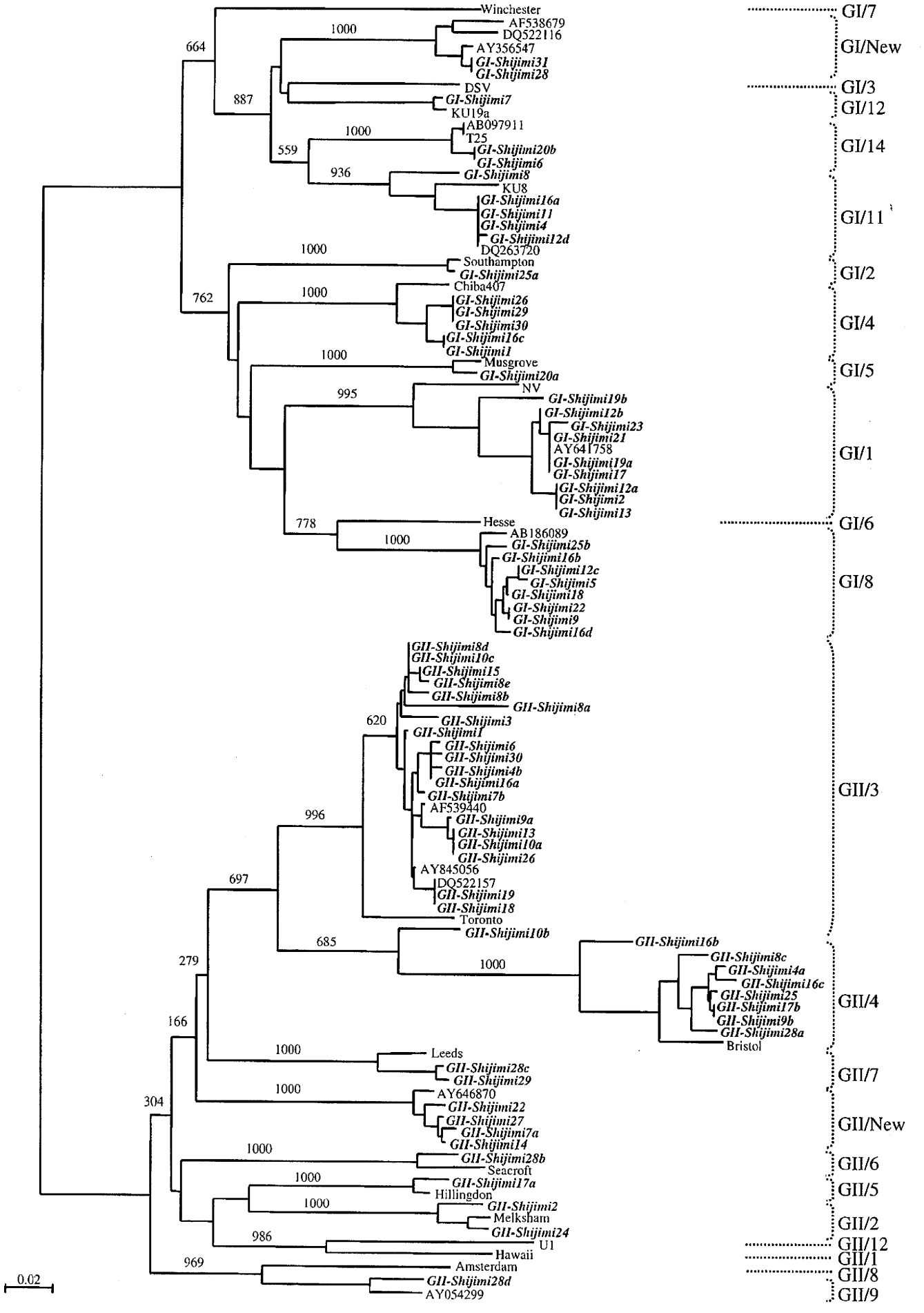
TABLE 1. *Continued*

Package no.	Site	Mo/day/yr	No. of norovirus genotypes	Norovirus strain name (genogroup/genotype)	Aichi virus	Rotavirus: G types	Adeno-virus	HAV	HEV (from *(17)*)	Sapovirus (from *(9)*)
31	G	03/18/06	1	GI-Shijimi23 (GI/1)	–	NT	NT	–	–	–
32	H	03/18/06	–		–	NT	NT	–	–	–
33	D	03/30/06	1	GII-Shijimi24 (GII/2)	Aic-15	G1/G8	–	–	–	–
34	B	04/07/06	3	GI-Shijimi25a (GI/2), GI-Shijimi25b (GI/8), GII-Shijimi25 (GII/4)	–	–	–	–	–	–
35	D	04/13/06	2	GI-Shijimi26 (GI/4), GII-Shijimi26 (GII/3)	Aic-16	G1/G2/G8	–	–	–	SaV-2
36	B	04/26/06	1	GII-Shijimi27 (GII/New)	–	G2/G4	–	–	–	–
37	A	05/16/06	5	GI-Shijimi28 (GI/New), GII-Shijimi28a (GII/4), GII-Shijimi28b (GII/6), GII-Shijimi28c (GII/7), GII-Shijimi28d (GII/9)	Aic-17	–	–	–	–	SaV-4
38	D	05/13/06	2	GI-Shijimi29 (GI/4), GII-Shijimi29 (GII/7)	–	NT	NT	1A	–	–
39	D	05/27/06	–		–	G8/G9	–	–	–	–
40	A	05/29/06	2	GI-Shijimi30 (GI/4), GII-Shijimi30 (GII/3)	–	–	–	–	–	–
41	I	06/14/06	–		–	–	–	–	–	–
42	D	06/16/06	–		–	G3	–	–	–	–
43	A	06/16/06	–		–	–	–	–	–	–
44	D	06/23/06	–		–	G?	–	–	–	–
45	B	07/05/06	–		–	–	–	–	–	–
46	D	07/06/06	–		–	–	–	–	–	–
47	A	07/13/06	–		–	NT	NT	–	–[b]	–
48	D	07/21/06	–		–	NT	NT	–	–[b]	–
49	B	07/21/06	–		–	NT	NT	–	–[b]	–
50	A	07/27/06	–		–	NT	NT	–	–[b]	–
51	A	08/06/06	–		–	NT	NT	–	–[b]	–
52	D	08/07/06	–		–	NT	NT	–	–[b]	–
53	B	08/10/06	–		–	NT	NT	–	–[b]	–
54	D	08/23/06	–		–	NT	NT	–	–[b]	–
55	I	09/04/06	–		–	NT	NT	–	–[b]	–
56	B	09/04/06	1	GI-Shijimi31 (GI/New)	–	NT	NT	–	–[b]	–
57	D	09/06/06	–		–	NT	NT	–	–[b]	–
Total				31/57	19/57	17/33	17/33	1/57	2/46	4/57

[a] NT, not tested.
[b] Tested in this study.

followed by the inactivation of the enzyme at 75°C for 5 min. Reverse transcription was performed with 15 μl of RNA solution and 15 μl of reverse transcription mixture that contained 1 mM dNTP mixture, 10 mM dithiothreitol, 0.75 μg of random hexamers (Takara), 33 U of RNase inhibitor (Takara), 300 U of reverse transcriptase Superscript II (Invitrogen, San Diego, Calif.), and 4.5 μl of Superscript II buffer (Invitrogen).

PCR. For the norovirus PCR, the primers were designed to amplify the 5′ end of the capsid gene *(10, 14)*. For norovirus genogroup I (GI), COG1F and G1SKR primers were used for the first PCR, and then G1SKF and G1SKR primers were used for the nested PCR. For norovirus genogroup II (GII), COG2F and G2SKR were used for the first PCR, and then G2SKF and G2SKR primers were used for the nested PCR. For the Aichi virus, C94b and 264K primers were used, and these were designed to amplify the 3C–D junction (protease-polymerase) *(26)*. For the rotavirus (group A), primers were designed to amplify the major outer capsid glycoprotein VP7, and the rotavirus type was determined by PCR size *(7)*. For the adenovirus, primers were designed to detect the E1B region of enteric adenoviruses, i.e., Ad40 and Ad41, and determined by PCR size *(1)*. For HAV, we used a set of nested in-house primers designed to amplify the capsid gene. For the first HAV PCR, we used sense HAV+2799 primer (5′-ATTCAGAT TAGACTGCCTTGGTA-3′) and antisense HAV-3273 primer (5′-CCAAGAAACCTTCATTATTTCATG-3′). For HAV nested PCR, we used sense HAV+2907 primer (5′-GCAAATTACAAT CATTCTGATGA-3′) and antisense HAV-3162 primer (5′-CTTC YTGAGCATACTTKARTCTTG-3′). The HAV PCR conditions were the same as those for the norovirus *(14)*. For the astrovirus, PreCAP1 and 12GR primers were used to amplify the first PCR product, and then Mon244 and 82b primers were used for nested PCR, which were designed to amply the 5′ end of the capsid gene *(18)*. Two types of positive controls and a virus-free negative control per five assays for norovirus PCR were used. All PCR prod-


Winchester
AF538679
DQ522116
AY356547
GI-Shijimi31
GI-Shijimi28
DSV
GI-Shijimi7
KU19a
AB097911
T25
GI-Shijimi20b
GI-Shijimi6
GI-Shijimi8
KU8
GI-Shijimi16a
GI-Shijimi11
GI-Shijimi4
GI-Shijimi12d
DQ263720
Southampton
GI-Shijimi25a
Chiba407
GI-Shijimi26
GI-Shijimi29
GI-Shijimi30
GI-Shijimi16c
GI-Shijimi1
Musgrove
GI-Shijimi20a
NV
GI-Shijimi19b
GI-Shijimi12b
GI-Shijimi23
GI-Shijimi21
AY641758
GI-Shijimi19a
GI-Shijimi17
GI-Shijimi12a
GI-Shijimi2
GI-Shijimi13
Hesse
AB186089
GI-Shijimi25b
GI-Shijimi16b
GI-Shijimi12c
GI-Shijimi5
GI-Shijimi18
GI-Shijimi22
GI-Shijimi9
GI-Shijimi16d
GII-Shijimi8d
GII-Shijimi10c
GII-Shijimi15
GII-Shijimi8e
GII-Shijimi8b
GII-Shijimi8a
GII-Shijimi3
GII-Shijimi1
GII-Shijimi6
GII-Shijimi30
GII-Shijimi4b
GII-Shijimi16a
GII-Shijimi7b
AF539440
GII-Shijimi9a
GII-Shijimi13
GII-Shijimi10a
GII-Shijimi26
AY845056
DQ522157
GII-Shijimi19
GII-Shijimi18
Toronto
GII-Shijimi10b
GII-Shijimi16b
GII-Shijimi8c
GII-Shijimi4a
GII-Shijimi16c
GII-Shijimi25
GII-Shijimi17b
GII-Shijimi9b
GII-Shijimi28a
Bristol
Leeds
GII-Shijimi28c
GII-Shijimi29
AY646870
GII-Shijimi22
GII-Shijimi27
GII-Shijimi7a
GII-Shijimi14
GII-Shijimi28b
Seacroft
GII-Shijimi17a
Hillingdon
GII-Shijimi2
Melksham
GII-Shijimi24
U1
Hawaii
Amsterdam
GII-Shijimi28d
AY054299
0.02
GI/7
GI/New
GI/3
GI/12
GI/14
GI/11
GI/2
GI/4
GI/5
GI/1
GI/6
GI/8
GII/3
GII/4
GII/7
GII/New
GII/6
GII/5
GII/2
GII/12
GII/1
GII/8
GII/9

ucts were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Norovirus, Aichi virus, and HAV nucleotide sequences were prepared as previously described *(11)*.

Sequence analysis. Norovirus, Aichi virus and HAV nucleotide sequences were prepared with the terminator cycle sequence kit (version 3.1, Applied Biosystems, Warrington, UK) and determined with the ABI 3130 sequencer (ABI, Boston, Mass.). In order to determine the norovirus genotypes in the packages with multiple genotypes, we cloned the reverse transcription PCR products into pCR2.1 (Invitrogen), and at least four clones from each sample were sequenced. The genetic diversity of the adenoviruses was not determined in this study. Norovirus nucleotide sequences were aligned with ClustalX, and the distances were calculated by Kimura's two-parameter method. The norovirus nucleotide sequence data determined in this study has been deposited in GenBank under accession no. EF424485 through EF424557.

RESULTS

Thirty-five (61%) of 57 packages were contaminated with one type of virus, 5 (9%) of 57 packages were contaminated with two different types of viruses, 16 (28%) of 57 packages were contaminated with three different types of viruses, and 5 (9%) of 57 packages were contaminated with at least four different types of viruses (Table 1). Astrovirus was not detected in any of the packages.

Noroviruses. Thirty-one (54%) of 57 packages were contaminated with noroviruses (Table 1). Norovirus GI and GII sequences were detected in 24 and 23 packages, respectively (Fig. 1). A total of 24 norovirus GI sequences were detected, and these clustered into nine different GI genotypes (Fig. 1), including one unpublished GI genotype (GI/1, GI/2, GI/4, GI/5, GI/8, GI/11, GI/12, GI/14, and GI/New). A total of 23 norovirus GII sequences were detected, and these clustered into eight different GII genotypes (Fig. 1), including one unpublished GII genotype (GII/2, GII/3, GII/4, GII/5, GII/6, GII/7, GII/9, and GII/New). More than half of the norovirus-positive packages, 20 (65%) of 31, contained two or more norovirus genotypes. Twenty-three (74%) of 31 norovirus-positive packages were co-contaminated with two or more other types of viruses (Table 1).

Aichi virus. We found that 19 (33%) of 57 packages were contaminated with Aichi viruses. The 19 Aichi virus sequences shared over 95% nucleotide homology, suggesting that the same strain contaminated the clams. These 19 sequences closely matched (approximately 95% nucleotide homology) genogroup A sequences found on the database (data not shown). All of the Aichi virus–positive packages were co-contaminated with other viruses (Table 1).

Rotavirus. Fourteen (42%) of 33 packages were contaminated with rotavirus (24 packages were unavailable for screening). Six different rotavirus G types were detected, i.e., G1, G2, G3, G4, G8, and G9. Of the 14 rotavirus-positive packages nine (53%) contained two or more rotavirus G types (Table 1).

Adenovirus. Seventeen (52%) of 33 packages were contaminated with adenoviruses, using primers designed to detect the two enteric adenoviruses, i.e., Ad40 and Ad41. Fourteen (82%) of 17 adenovirus-positive packages were co-contaminated with other viruses (Table 1).

HAV and HEV. One (2%) of 57 packages was contaminated with HAV. Sequence analysis of the capsid gene indicated that it belonged to subtype IA. HEV was previously detected in 2 of 46 packages *(17)*. An additional 11 packages were screened for HEV; however these were all negative (Table 1).

DISCUSSION

The current study has shown that Japanese clams (*C. japonica*) purchased in supermarkets and fish markets were highly contaminated with human enteric viruses from the natural environment. Similarly, a 3-year study in France found that mussel samples (*Mytilus galloprovincialis*) were highly contaminated with enteric viruses *(15)*. However, an important difference between the study conducted in France and the current study was that the French mussels were collected in areas where sewage was discharged and were prohibited for human consumption, whereas the Japanese clams were sold in supermarkets and fish markets and were considered suitable for human consumption.

Noroviruses are the dominant cause of outbreaks of gastroenteritis worldwide. In this study, the noroviruses were the dominant virus detected the clam packages (found in 54% of the packages). In a comparative study, noroviruses were detected in only approximately 5 to 9% of Japanese oysters (*Crassostrea gigas* or *Crassostrea nippona*) *(20, 21)*. These results suggested that the Japanese clams were more highly contaminated with noroviruses than were the Japanese oysters, or alternatively, it was just a reflection on the different collection sites, i.e., the clams were collected from brackish waters, whereas the oysters were collected from the sea. Alternatively, the different detection rates in clams and oysters were a result of the different sample preparations. Nevertheless, all of the norovirus sequences detected in the clam packages closely matched other sequences detected in patients with gastroenteritis in Japan (using GenBank BLAST searches), suggesting that the contaminated Japanese clams could cause gastroenteritis in humans, although direct evidence is lacking.

Over the past 10 years, the norovirus GII/4 strains have become the dominant cause of outbreaks of gastroenteritis

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FIGURE 1. *Phylogenetic analysis of norovirus capsid sequences (approximately 300 nucleotides) showing the different genogroups and genotypes. The numbers on each branch indicate the bootstrap values for the genotype. Bootstrap values of 950 or higher were considered statistically significant for the grouping. The scale represents nucleotide substitutions per site. The frequency of each norovirus genotype was 9, 1, 5, 1, 8, 5, 1, 2, 2, 1, 20, 8, 1, 1, 2, 1, and 4 for GI/1, GI/2, GI/4, GI/5, GI/8, GI/11, GI/12, GI/14, GI/New, GII/2, GII/3, GII/4, GII/5, GII/6, GII/7, GII/9, and GII/New, respectively.*

worldwide. In a recent study, we also found that the GII/4 strains were the dominant cause of outbreaks of gastroenteritis in food-catering settings in Japan *(22)*. In the current study, the norovirus GII/3 sequences were detected more frequently than were the norovirus GII/4 sequences, i.e., 20 versus 8 sequences, respectively (Fig. 1). This result may only reflect that the GII/3 strains were more dominant in this area of Japan; however, the norovirus GII/3 strains were the second most dominant cause of gastroenteritis in Japan, Australia, and Vietnam *(2, 8, 22)*, indicating that this genotype is indeed a major cause of gastroenteritis. Noteworthy were two new norovirus genotypes (GI/New and GII/New; Fig. 1) detected in the clam packages, at three different sites, and several months apart. Similar norovirus sequences were recently reported in patients in Thailand, Taiwan, Hong Kong, and from an outbreak on a U.S. navy ship (data not shown), indicating that there may be a widespread distribution of these two newly identified genotypes.

We found that more than half (65%) of the norovirus-positive packages contained two or more norovirus genotypes (Table 1 and Fig. 1). Multiple norovirus genotypes have also been found in oyster-associated outbreaks of gastroenteritis *(10)*, and in a recent study, we found multiple norovirus genotypes in outbreaks of gastroenteritis at various food-catering settings throughout Japan *(22)*. These findings indicate that like oyster-associated outbreaks, clam-associated outbreaks may also be caused by multiple norovirus genotypes, although further studies are needed.

The Aichi virus was found in 33% of the clam packages, and all of these packages were co-contaminated with other viruses. The Aichi virus sequences detected in the packages closely matched other Aichi virus sequences (genogroup A) that were detected in patient stool specimens from oyster-associated gastroenteritis *(26)*. To the best of our knowledge, these results have shown for the first time that the Aichi virus can also accumulated in these Japanese clams. The importance of Aichi virus in human gastroenteritis is still poorly understood, and very few studies have reported Aichi virus infections since its first discovery in 1989 *(25)*. One recent study detected Aichi virus in only 3% (28 of 912) of stool specimens from infants with sporadic cases of gastroenteritis (collected in Japan, Bangladesh, Thailand, and Vietnam), which were negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus *(23)*. Further studies are clearly needed in order to determine the importance of this virus in humans.

Rotavirus was detected in 14 of 33 available clam packages. A similar study in France found rotavirus in 52% of mussel samples and 27% of oyster samples *(15)*. Rotavirus infections usually causes sporadic cases of gastroenteritis in children in the winter season, but our results suggest that rotavirus may persist longer in the environment, at least up to June (Table 1). A great genetic diversity of rotavirus G types was detected in the packages, and we also found that 9 of 17 rotavirus-positive packages contained two or more rotavirus G types. Likewise, a study in Egypt and Spain also found a great genetic diversity of rotavirus G types as well as unusual genotypes in sewage samples *(24)*.

Enteric adenoviruses (Ad40 and Ad41) were detected in 17 of 33 available clam packages. Adenovirus infections in the western part of Japan were reported to be low, with one study reporting adenovirus serotype 41 in only approximately 3% of stool specimens from infants with sporadic cases of gastroenteritis *(6)*. The high detection rate of adenoviruses in these packages may indicate that adenovirus prevalence is variable, although further studies are needed.

One (2%) of 57 packages was contaminated with HAV, and sequence analysis indicated that it belonged to subtype IA. The low detection rate of HAV was also observed in an oyster study that found only 2 of 112 samples positive in Japan *(12)*. The low detection rate of HAV in the clams and oysters was not unusual, because the prevalence of HAV infections is low in Japan, although this may be increasing *(13)*. More surveillance is clearly needed in order to locate other contaminated areas and help control the spread of HAV contamination.

Astroviruses were not detected in any of the Japanese clam packages. This result is surprising because astroviruses were detected in more than half (61%) of African clam samples *(5)*, 50% of French mussel samples *(15)*, and 17% of French oyster samples *(15)*. This result suggested that the astrovirus may not concentrate to detectable levels in certain species of shellfish or the level of contamination differs in each place, which was similarly observed in two other studies *(3, 21)*.

In conclusion, this study has shown that the Japanese clams were highly contaminated with many types of human enteric viruses capable of causing gastroenteritis and/or acute viral hepatitis. At present, the Enforcement Regulation of Food Sanitation Law mainly focuses on bacterial contamination in Japan *(21)*. Clearly, regulations and standards need to be revised in order to address this problem of viral contamination in the Japanese clams. The health risks associated with eating contaminated oysters have been well documented, but further studies are clearly needed in order to determine the health risks associated with eating these contaminated Japanese clams.

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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

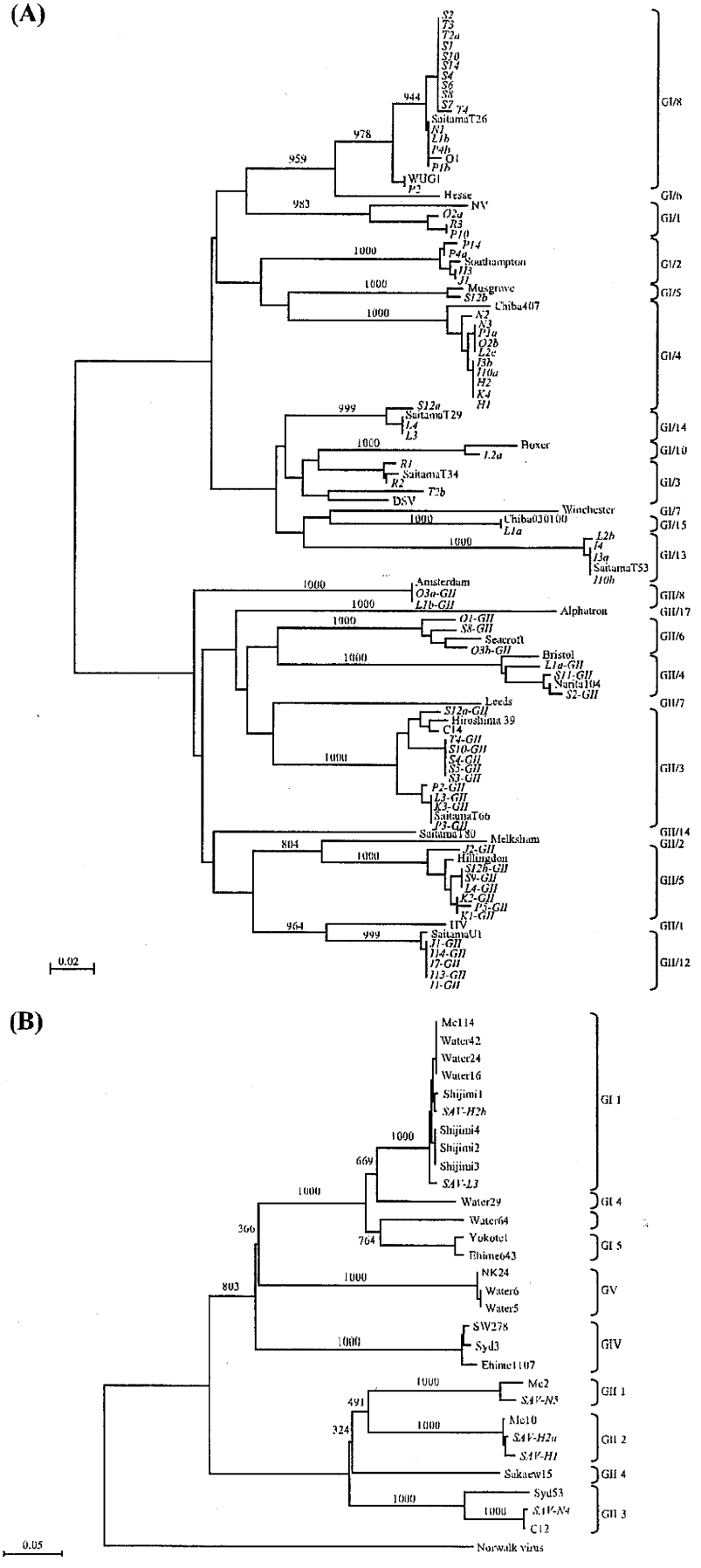

Fig. 1. Phylogenetic tree of the noroviruses (A) and sapoviruses (B) detected in this study. The trees were constructed with the partial N-terminal capsid region. The numbers on the branches indicate the bootstrap values for the clusters. Sequences and accession numbers from references (8) and (26), 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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Divergent Evolution of Norovirus GII/4 by Genome Recombination from May 2006 to February 2009 in Japan▽‡

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Norovirus GII/4 is a leading cause of acute viral gastroenteritis in humans. We examined here how the GII/4 virus evolves to generate and sustain new epidemics in humans, using 199 near-full-length GII/4 genome sequences and 11 genome segment clones from human stool specimens collected at 19 sites in Japan between May 2006 and February 2009. Phylogenetic studies demonstrated outbreaks of 7 monophyletic GII/4 subtypes, among which a single subtype, termed 2006b, had continually predominated. Phylogenetic-tree, bootscanning-plot, and informative-site analyses revealed that 4 of the 7 GII/4 subtypes were mosaics of recently prevalent GII/4 subtypes and 1 was made up of the GII/4 and GII/12 genotypes. Notably, single putative recombination breakpoints with the highest statistical significance were constantly located around the border of open reading frame 1 (ORF1) and ORF2 ($P \leq 0.000001$), suggesting outgrowth of specific recombinant viruses in the outbreaks. The GII/4 subtypes had many unique amino acids at the time of their outbreaks, especially in the N-term, 3A-like, and capsid proteins. Unique amino acids in the capsids were preferentially positioned on the outer surface loops of the protruding P2 domain and more abundant in the dominant subtypes. These findings suggest that intersubtype genome recombination at the ORF1/2 boundary region is a common mechanism that realizes independent and concurrent changes on the virion surface and in viral replication proteins for the persistence of norovirus GII/4 in human populations.

Norovirus (NoV) is a nonenveloped RNA virus that belongs to the family *Caliciviridae* and can cause acute gastroenteritis in humans. The NoV genome is a single-stranded, positive-sense, polyadenylated RNA that encodes three open reading frames, ORF1, ORF2, and ORF3 (68). ORF1 encodes a long polypeptide (~200 kDa) that is cleaved in the cells by the viral proteinase ($3C^{pro}$) into six proteins (4). These proteins function in NoV replication in host cells (19). ORF2 encodes a viral capsid protein, VP1. The capsid gene evolved at a rate of 4.3×10^{-3} nucleotide substitutions/site/year (7), which is comparable to the substitution rates of the envelope and capsid genes of human immunodeficiency virus (30). The capsid protein of NoV consists of a shell (S) and two protruding (P) domains: P1 and P2 (47). The S domain is relatively conserved within the same genetic lineages of NoVs (38) and is responsible for the assembly of VP1 (6). The P1 subdomain is also relatively conserved (38) and has a role in enhancing the stability of virus particles (6). The P2 domain is positioned at the most exposed surface of the virus particle (47) and forms binding clefts for putative infection receptors, such as human histo-blood group antigens (HBGA) (8, 13, 14, 60). The P2 domain also contains epitopes for neutralizing antibodies (27, 33) and is consistently highly variable even within the same genetic lineage of NoVs (38). ORF3 encodes a VP2 protein that is suggested to be a

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minor structural component of virus particles (18) and to be responsible for the expression and stabilization of VP1 (5).

Thus far, the NoVs found in nature are classified into five genogroups (GI to GV) and multiple genotypes on the basis of the phylogeny of capsid sequences (71). Among them, genogroup II genotype 4 (GII/4), which was present in humans in the mid-1970s (7), is now the leading cause of NoV-associated acute gastroenteritis in humans (54). The GII/4 is further subclassifiable into phylogenetically distinct subtypes (32, 38, 53). Notably, the emergence and spread of a new GII/4 subtype with multiple amino acid substitutions on the capsid surface are often associated with greater magnitudes of NoV epidemics (53, 54). In 2006 and 2007, a GII/4 subtype, termed 2006b, prevailed globally over preexisting GII/4 subtypes in association with increased numbers of nonbacterial acute gastroenteritis cases in many countries, including Japan (32, 38, 53). The 2006b subtype has multiple unique amino acid substitutions that occur most preferentially in the protruding subdomain of the capsid, the P2 subdomain (32, 38, 53). Together with information on human population immunity against NoV GII/4 subtypes (12, 32), it has been postulated that the accumulation of P2 mutations gives rise to antigenic drift and plays a key role in new epidemics of NoV GII/4 in humans (32, 38, 53).

Genetic recombination is common in RNA viruses (67). In NoV, recombination was first suggested by the phylogenetic analysis of an NoV genome segment clone: a discordant branching order was noted with the trees of the $3D^{pol}$ and capsid coding regions (21). Subsequently, many studies have reported the phylogenetic discordance using sequences from various epidemic sites in different study periods (1, 10, 11, 16, 17, 22, 25, 40, 41, 44–46, 49, 51, 57, 63, 64, 66). These results suggest that genome recombination frequently occurs among distinct lineages of NoV variants *in vivo*. However, the studies were done primarily with direct sequencing data of the short genome portion, and information on the cloned genome segment or full-length genome sequences is very limited (21, 25). Therefore, we lack an overview of the structural and temporal dynamics of viral genomes during NoV epidemics, and it remains unclear whether NoV mosaicism plays a role in these events.

To clarify these issues, we collected 199 near-full-length genome sequences of GII/4 from NoV outbreaks over three recent years in Japan, divided them into monophyletic subtypes, analyzed the temporal and geographical distribution of the subtypes, collected phylogenetic evidence for the viral genome mosaicism of the subtypes, identified putative recombination breakpoints in the genomes, and isolated mosaic genome segments from the stool specimens. We also performed computer-assisted sequence and structural analyses with the identified subtypes to address the relationship between the numbers of P2 domain mutations at the times of the outbreaks and the magnitudes of the epidemics. The obtained data suggest that intersubtype genome recombination at the ORF1/2 boundary region is common in the new GII/4 outbreaks and promotes the effective acquisition of mutation sets of heterogeneous capsid surface and viral replication proteins.

MATERIALS AND METHODS

Stool specimens. The Norovirus Surveillance Group of Japan collected stool specimens from NoV-GII- or GII/4-positive individuals with acute gastroenteritis ($n = 247$). Most of the specimens were from NoV outbreaks around the collection sites. The group collected the specimens in spring, summer, autumn, and winter for 3 years: the 2006/2007 season (May 2006 to January 2007), 2007/2008 season (March 2007 to February 2008), and 2008/2009 season (May 2008 to February 2009). The collection sites were located at 20 different regional public health institutes in Japan (five samples from each institute per year). The genogroup of NoVs was evaluated by real-time reverse transcription-PCR (RT-PCR) (23). In some cases, the genotype of NoVs was evaluated by sequencing of the reverse transcription-PCR products of the ORF1 and ORF2 bordering region (29). Near-full-length genome sequences were obtained with 199 of the 247 specimens. Epidemiological information on 37 of the 199 samples from the 2006/2007 season was described previously (38). Information on the rest ($n = 162$) is described in Tables S1 and S2 in the supplemental material. Briefly, the 162 specimens were from outbreaks ($n = 90$), sporadic infection cases ($n = 15$), and undescribed cases ($n = 57$) during December 2006 to February 2009 in Japan. The major sites of the incidences were a nursing care center ($n = 19$), restaurant ($n = 17$), kindergarten ($n = 15$), hotel ($n = 8$), hospital ($n = 7$), sports event ($n = 1$), self-defense force ($n = 1$), family home ($n = 1$), elementary school ($n = 1$), and bank ($n = 1$), and one was undescribed ($n = 91$). The viral RNA copy numbers in the specimens ranged from 5.0×10^4 to 1.9×10^{11} copies/g stool (average, 6.1×10^9 copies/g stool) as judged by the real-time quantitative reverse transcription-PCR assay (23). All stool specimens were stored at −80°C until use.

Viral genome sequencing. NoV GII/4 genome sequencing was done as described previously (38). Briefly, two overlapping fragments (approximately 5.2 and 2.5 kb) were amplified by RT-PCR from stool specimens. The PCR products were purified and used as a template for sequencing in a 96-well scale using an ABI 3730 xl DNA analyzer (Applied Biosystems, Foster City, CA). The sequences of 5.2-kb and 2.5-kb segments from the same individual were used to reconstruct near-full-length genome sequences (about 7.5 kb) by alignment at an overlapping region using the Staden Package (http://staden.sourceforge.net). The 5.2-kb fragment covers the complete ORF1 and the 5′ end of ORF2. The 2.5-kb fragment covers the 3′ end of ORF1, complete ORF2 and ORF3, and 3′-end noncoding region of the genome. The primers used for reverse transcription and nested PCR for the 5.2-kb fragment were GII4-1F/GII4r5412 (outer primer pair) and GII4-2F/GII4r5295 (inner primer pair) (38). Those for the 2.5-kb fragment were COG-2F/Tx30SXN (outer primer pair) and G2SKF/Tx30SXN (inner primer pair) (38). The initial 22 nucleotides at the 5′ ends of the reconstructed genomes were from PCR primers. The final 45 nucleotides at the 3′ ends of the genome were excluded from analysis because of the low levels of sequence accuracy. We obtained 199 near-full-length genome sequences from 247 GII-positive specimens. The 199 sequences included 37 GII/4 sequences previously reported between May 2006 and January 2007 (38) and 162 sequences newly obtained between December 2006 and February 2009.

Molecular cloning and sequencing of genome segments. The 5.2-kb, 1.0-kb, and 2.8-kb genome segments were amplified by RT-PCR products as described above and cloned into pPCR-XL-TOPO vectors (Invitrogen, Carlsbad, CA). Each of the segments covers a junction of putative recombination breakpoints around the 5′ end of ORF2: the 5.2-kb segment contains the near-full-length ORF1 and 5′-end portion of ORF2, the 2.8-kb segment contains the 3′-end portion of ORF1, complete ORF2, and complete ORF3, and the 1.0 kb segment contains the 3′-end portion of ORF1 and 5′-end portion of ORF2. The primers used for the nested PCR of the 5.2-kb segment were the same ones described above: GII4-1F/GIIr5412 (outer primer pair) and GII4-2F/GIIr5295 (inner primer pair) (38). The primers used for the nested PCR of the 2.8-kb fragment were GII4f4117 (5′-CTGACAAAATTTATGGTAAGATCAAGAAGAGG-3′)/Tx30 SXN (outer primer pair) and GII4f4762 (5′-GACCCAGCTGGTTGGTTTGGAAAA-3′)/GII4r7516 (5′-ATAGTTTAGCGGCCGCATTCTTATCACATTACACCCGTGACTCCCCTCG-3′) (inner primer pair). The primers used for the nested PCR of the 1.0-kb fragment were GII4f4117/GII4r5412 (outer primer pair) and GII4f4223 (5′-GGTATGAATATGAATGAGGATG-3′)/GII4r5295 (inner primer pair). The single clones of the 5.2-kb, 2.8-kb, and 1.0-kb genome segments of the GII/4 subtypes were randomly chosen and sequenced in 96-well plates using an ABI 3730 xl DNA analyzer as described above.

Phylogenetic analysis. Phylogenetic trees were constructed using the neighbor-joining method and maximum-likelihood method. Briefly, the near-full-length genome sequences from this study were aligned with the available GII/4 genome sequences from past NoV epidemics occurring over the past 3 decades, using CLUSTAL W software included in the MEGA software package, version 4.0

(58) (http://evolgen.biol.metro-u.ac.jp/MEGA/) and the MAFFT multiple sequence alignment software program, version 6.0 (26) (http://align.bmr.kyushu-u.ac.jp/mafft/software/). The neighbor-joining trees were constructed with the nucleotide substitution values estimated with the maximum composite likelihood model (59) using MEGA. The maximum-likelihood trees were inferred on the basis of the general time reversible models (31) using the PHYML software program included in the RDP3 software package (35) (http://darwin.uvigo.es/rdp/rdp.html). The reliability of interior branches in the phylogenetic tree was assessed by the bootstrap method with 1,000 resamplings. The GII/4 genome reference sequences were from samples taken before 1990 (<1990) (6 sequences, CHDC591-1974, CHDC2490-1974, CHDC4871-1977, CHDC4108-1987, Lordsdale, MD145-12/US/1987, and CHDC3967-1988), before 2000 (<2000) (2 sequences, and Dresden174/US/1997), in 2002/2003 (6 sequences, Farmington Hill, B2S16/2002/UK, B5S22/2002/UK, Langen1061/2002/DE, YUR132073/2002/JPN, and MD-2004/2004/US), and in 2004/2005 (4 sequences, Guangzhou/NVgz01/CHN/2006, Chiba/04-1050/2005/JP, Sakai/04-179/2005/JP, and Ehime/05-30/2005/JP). Accession numbers for the reference genome sequences are given elsewhere (7, 38).

We initially constructed the phylogenetic trees with 199 genome sequences from the Japanese variants from 2006 to 2009 and 6 representative sequences of GII genotypes whose complete genome sequences were available in GenBank in October 2009 (GII/1, GII/3, GII/4, GII/6, GII/10, and GII/12; accession no. U07611, AB067542, X86557, AB039776, AY237415, and AB039775, respectively). The trees showed that the 199 genome sequences reproducibly grouped with the GII/4 reference sequences outside other GII references. The GII/4 cluster was positioned most closely to the GII/12 reference (Saitama U1/JP). Therefore, we used GII/12 as an outgroup in the present study for a better grasp of the relationship of the phylogeny among the Japanese GII/4 variant subgroups and between GII/4 and GII/12 variants.

Bootscanning-plot analysis. Bootscanning-plot analysis was performed as described previously (69). Briefly, each query sequence was aligned with three NoV reference sequences using CLUSTAL W software, version 1.4 (62). The bootstrap values were plotted for a window of 300 bp, moving in increments of 10 bp along the alignment using the software program Simplot (48) (version 3.5.1; http://sray.med.som.jhmi.edu/SCRoftware/simplot/). Thus far, 19 genotypes of the NoV GII variants have been reported on the basis of complete capsid sequences (65, 71). Among them, only 7 genotypes have been fully sequenced at the genome level (GII/1, GII/3, GII/4, GII/6, GII/8, GII/10, and GII/12; accession numbers U07611, AB067542, X86557, AB039776, AB067543, AY237415, and AB039775, respectively). To search for sequences that are phylogenetically relevant to the query sequences, we constructed phylogenetic trees of the complete ORF1, ORF2, and ORF3 sequences using all available representatives of the 19 genotypes in the GenBank database. We also used the automated exploratory analysis tool included in the RDP3 software package (35). The genome sequence set used for the analysis consisted of 7 query sequences (2004/05, 2006a, 2006b, 2007a, 2007b, 2008a, and 2008b), all available GII genotype representatives, and all available GII/4 variant subgroups which caused epidemics over the past 34 years (7, 31). Two putative parent sequences with the best confidence values and a single distantly related sequence were used for the bootscanning plots with MEGA. The confidence values of the recombination events were also assessed with tools included in the RDP3 software package, such as RDP, GENECONV, Maxchi, Chimera, 3seq, and Siscan. The query sequences used in this study were Sakai2/2006/JP for the 2004/05 subtype (accession no. AB447448) and representative genomes of the 2007a (Osaka1/2007/JP), 2007b (Iwate5/2007/JP), 2008a (Hokkaido5/2008/JP), and 2008b (Hokkaido4/2008/JP) subtypes obtained in this study. The reference sequences were Saitama_U1/JP (GII/12 genotype [25], accession no. AB039775), B2S16/2002/UK (2002/03 subtype [38], accession no. AY587989), Saitama_U3/JP (GII/6 genotype [25], accession no. AB039776), Sakai2/2006/JP (2004/05 subtype [38], accession no. AB447448), Aomori1/2006/JP (2006a subtype [38], accession no. AB447432), Aichi3/2006/JP (2006b subtype [38], accession no. AB447446), and Hokkaido5/2008/JP and Hokkaido4/2008/JP (2008a and 2008b subtypes, respectively, obtained in this study).

Informative-site analysis. The informative-site analysis was performed as described previously (50). Briefly, each query sequence was aligned with two putative parental sequences and an outgroup sequence. The alignments were used to identify informative sites that support alternative tree topologies between downstream and upstream regions using the Simplot software program (48), version 3.5.1. This information allowed identification of genome regions that were assigned as chimeras of heterologous sequences of distinct evolutionary origins. The statistical significance of the resultant division by the informative sites was evaluated by the maximal χ^2 test using in-house programs. The programs were designed to execute the calculation algorithms described by Robertson et al. (50, 55).

Molecular modeling. Three-dimensional (3-D) structural models of the capsid P-domain dimers were constructed by homology modeling as described previously (38). Briefly, the P-domain monomer models were first constructed using the crystal structure of the NoV capsid P domain of the GII/4 VA387 strain at a resolution of 2.00 Å (PDB code 2OBS [13]) as the template. The P domains of the GII/4 subtypes described in this study have sequence similarities of greater than 90% to that of VA387, high enough to construct models with a root mean square distance (RMSD) of ~1 Å for the main chain between the predicted and actual structures (3). The P-domain monomer models were used to construct the P-domain dimer models by superimposing the chains A and B using the crystal structure of the NoV capsid dimer (PDB code 1IHM [47]).

Nucleotide sequence accession numbers. The DDBJ database accession numbers for the nucleotide sequences of NoV genomes for the 2006/2007 season (n = 37) have been reported elsewhere (38). The DDBJ database accession numbers for the nucleotide sequences of NoV genomes for the 2007/2008 and 2008/2009 seasons (n = 162) are AB541201 to AB541362. The DDBJ database accession numbers for the nucleotide sequences of NoV genome segment clones (n = 11) are AB541190 to AB541200.

RESULTS

Phylogenetic classification of NoV GII/4 subtypes in Japan during 2006 and 2009. First, we investigated the phylogeny of the NoV near-full-length genome sequences (about 7.5 kb). For this study, we used sequences obtained in this study from 19 sites in Japan between May 2006 and February 2009 (n = 199), various reported GII/4 reference sequences of past global or Japanese epidemics, and various reported outgroup sequences of other NoV genotypes. Figure 1 shows a maximum-likelihood tree constructed with the 199 Japanese genome sequences and the 19 GII/4 reference sequences from past NoV epidemics throughout the world during the 1970s and 1980s (7), <2000, in 2002/2003, and in 2004/2005 (38). The tree shows that the 199 Japanese sequences are divisible into 7 distinct lineage groups within a GII/4 cluster with a high bootstrap value (the 7 colored ovals in Fig. 1). The monophyly of the 7 GII/4 groups was reproducible independently of the algorithms to infer the phylogeny and reference sequences used. We tentatively named the 7 monophyletic subtypes of GII/4 variants 2004/05, 2006a, 2006b, 2007a, 2007b, 2008a, and 2008b.

The 2004/05 genome sequences were first obtained in Japan in the winter of 2004-2005 (accession no. AB220921 to AB220923 [42]). The geographic distribution of the 2004/05 sequences seemed to be restricted to East Asia (54). The 2006a and 2006b genome sequences were first obtained in Japan during the winter of 2006-2007 (accession no. AB447427 to AB447463 [38]). The 2006a and 2006b sequences were detected in many countries in Europe, North America, and East Asia during 2006-2007, wherein the 2006b subtype was generally more dominant than the 2006a subtype (54). The 2007a, 2007b, 2008a, and 2008b genome sequences were newly obtained in this study. Phylogenetic tree analyses showed that the nucleotide sequences of ORF2 of the 2008a subtype were genetically closely related to the ORF2 sequence obtained in the Netherlands in 2008 (accession no. AB445395), and together these sequences formed a single monophyletic group with a high bootstrap value (data not shown). These results suggest that at least 4 of the 7 GII/4 subtypes identified in Japan during 2006 and 2009, i.e., the 2004/05, 2006a, 2006b, and 2008a subtypes, caused NoV infections outside Japan.

We estimated the genetic divergence within and between the 7 monophyletic groups on the basis of the maximum composite

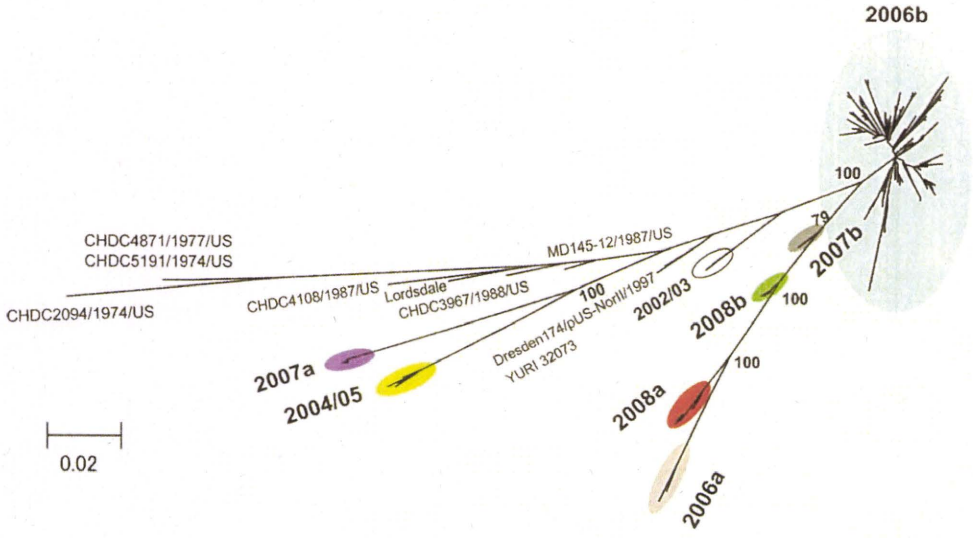

FIG. 1. Phylogenetic classification of the NoV GII/4 subtypes in Japan during 2006 and 2009. The maximum-likelihood tree was constructed with the near-full-length genome sequences (about 7.5 kb) obtained from stool specimens collected at 19 sites in Japan between May 2006 and February 2009 in this study (n = 199) and GII/4 reference genome sequences from past epidemics in Japan and other countries in the <2000, 2002/2003, and 2004/2005 winter seasons (7, 38) (n = 18). The sequence clusters enclosed by colored ovals indicate the 7 monophyletic GII/4 subtypes identified in Japan in previous (38) and present studies.

likelihood model using MEGA software. The intragroup divergence was comparably high in the 2006b subtype among the 7 groups (see Table S3, diagonal lines, in the supplemental material), suggesting that the diversity of the 2006b genome is higher than that of the other subtypes. This is consistent with the epidemiological data that 2006b had predominated for 3 years in Japan whereas the others emerged only temporally. The intergroup divergence was comparably high between 2004/05 and the other groups and between 2007a and the other groups, and about 12 to 15% sequence divergence existed in the genomes (see Table S3, bottom left portion, in the supplemental material).

Temporal and geographical distribution of Nov GII/4 subtypes in Japan. We then analyzed the temporal and geographical distribution of the 7 GII/4 subtypes in Japan. The 199 near-full-length genome sequences were divided into 3 groups according to the collection periods: the 2006/2007 (May 2006 to January 2007) (n = 39), 2007/2008 (March 2007 to February 2008) (n = 78), and 2008/2009 (May 2008 to February 2009) (n = 82) seasons. The frequencies of detection of particular NoV subtypes were obtained for each of the three seasons. We also used published subtyping data for the analysis of the previous winter season in Japan (November 2005 to March 2006) (n = 38) (38, 43).

The 2004/05 and 2006a sequences were detected at multiple collection sites and were prevalent in the 2005/2006 season (38, 43) (Fig. 2A and B, 2004/05 and 2006a). However, they became minor in the 2006/2007 season and were hardly detected thereafter. The 2006b sequences were minor in the 2005/2006 season (38) (Fig. 2A, 2006b). However, they rapidly became dominant in the 2006/2007 season and continually predominated in most of the collection sites in Japan, representing 176 of the 199 genome sequences (88.4%) during the study period. This result is consistent with the data of partial capsid sequences obtained during December 2007 to January 2008 in Japan (28). The 2007a and 2007b sequences were detected only at single collection sites in the 2007/2008 season (Fig. 2A and B, 2007a and 2007b). The 2008a and 2008b sequences were detected most recently at multiple collection sites in the 2008/2009 season (Fig. 2A and B, 2008a and 2008b). These data indicate that the 2006b subtype displaced the 2004/05 subtype in the 2006/2007 season and continued to predominate for the next 2 years in Japan. During the period of the 2006b predominance, however, several GII/4 subtypes caused NoV outbreaks in Japan, and the frequencies and sites of non-2006b outbreaks increased slightly in the 2008/2009 season.

Phylogenetic evidence for NoV genome mosaicism. Next, we investigated the possibility of genome mosaicism of the 7 GII/4 subtypes. For this purpose, we first compared the branching orders of the subtype clusters in the maximum-likelihood and neighbor-joining trees of the ORF1, ORF2, and ORF3 sequences using representative sequences of the 19 GII genotypes (GII/1 to GII/19) reported to date in the GenBank database. Figure 3A shows the maximum-likelihood trees, in which most of the non-GII/4 sequences were positioned far from the GII/4 cluster and were therefore excluded for a better grasp of the relationship of the phylogeny among the GII/4 variant subgroups. The exception was the ORF1 tree, in which the GII/12 sequence branched inside the GII/4 cluster. The comparisons of the three trees revealed that there was marked inconsistency in the branching orders of the GII/4 subgroups. The inconsistency was reproducible independently of the algorithms to infer the phylogeny and reference sequences used. First, the ORF1 sequences of the 2006b, 2007a, 2007b, and 2008b subtypes formed independent monophyletic clusters, whereas the ORF2 sequences of the 2006b, 2007a, and 2008b subtypes formed a single cluster and the ORF3 sequences of these four subtypes formed the same cluster (Fig. 3A, light blue circles). Second, the ORF1 sequences of the 2004/05 subtype were clustered near the ORF1 sequence of a GII/12

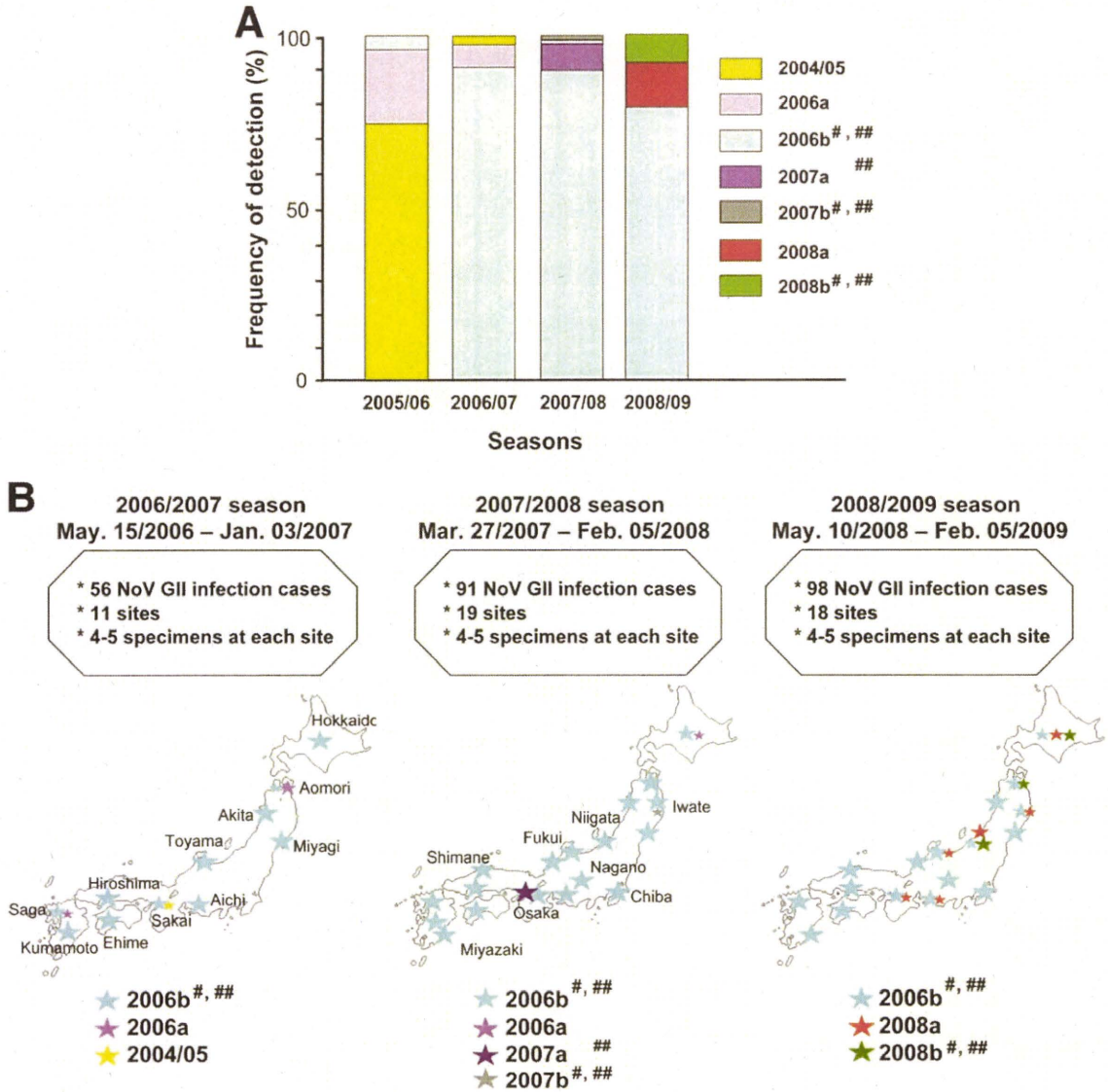

FIG. 2. Temporal and geographical distribution of the NoV GII/4 subtypes in Japan. The 199 near-full-length genome sequences were divided into 3 subgroups according to the collection periods: the 2006/2007 (May 2006 to January 2007) (n = 39), 2007/2008 (March 2007 to February 2008) (n = 78), and 2008/2009 (May 2008 to February 2009) (n = 82) seasons. For the analysis of the 2005/2006 season, published subtyping data (38, 43) were used (n = 38). (A) Frequencies of detection of particular NoV GII/4 subtypes in each season in Japan. (B) Geographic locations of the GII/4 subtype outbreaks. Colored stars indicate the locations of sample collection sites. Larger stars indicate the collection sites with greater frequencies of detection. #, ORF2s were classified as the same phylogenetic group (see Fig. 3A, ORF2). ##, ORF3s were classified as the same phylogenetic group (see Fig. 3A, ORF3).

strain (Saitama_U1/JP [25]) and relatively distant from the reported GII/4 reference sequences, whereas the ORF2 and ORF3 sequences of the 2004/05 subtype were very distantly related to the Saitama_U1/JP sequence and closely related to the GII/4 reference sequences (Fig. 3A, yellow circles). Third, the branching orders of the 2008a sequences were also different in the ORF1, ORF2, and ORF3 trees (Fig. 3A, red circles). These results suggested that most subtypes identified in this study had mosaic genomes.

To further assess this possibility, we performed bootscanning-plot analyses as described previously (69). For each bootscanning plot, we used a query genome sequence of a given subtype, two to three reference sequences that were positioned relatively closely to the query sequence in the neighbor-joining trees, and a distantly related outgroup sequence. The analyses showed that the genomes of the 2004/05, 2007a, 2007b, 2008a, and 2008b subtypes were indeed composed of multiple segments from recently prevalent or as-yet-undefined genogroups, genotypes, and subtypes of NoVs in this and previous reports (2, 7, 25, 38, 53, 65, 71) (Fig. 3B; see also Fig. 3A). The 2004/05 genome (Sakai2/2006/JP) was comprised of the ORF1 related to GII/12 (Saitama_U1/JP) and the ORF2/3 related to GII/4 2002/03 (B2S16/2002/UK). The 2007a genome (Osaka1/2007/JP) was made up of the ORF1 related to GII/12, the ORF2 of as-yet-undefined classes of GII/4, and the ORF3 related to GII/4 2006b (Aichi3/2006/JP). The 2007b genome (Iwate5/2007/JP) was made up of the ORF1 related to GII/4 2006b and 2006a (Aomori1/2006/JP) and the ORF2 and