TABLE 6. Amino acid substitutions at six positions in the P domain among Melksham-like strains

Strain description			Amino acid and codon at the indicated position of the capsid protein[a]											
			245		342		344		345		364		440	
Name[b]	Country[c]	Detection date (mo/day/yr)	Residue	Codon	Residue	Codon	Residue	Codon	Residue	Codon	Residue	Codon	Residue	Codon
Melksham	UK	1994	P	CCC	Q	CAG	A	GCC	A	GCT	A	GCA	L	CTC
CF434	US	1997	—	CCC	—	CAG	—	GCT	—	GCT	—	GCA	—	CTT
S99-191	NL	1/1999	S	TCT	—	CAA	—	GCC	T	ACT	—	GCA	I	ATC
OB0037	NL	9/2000	S	TCT	—	CAA	—	GCC	T	ACT	—	GCA	I	ATC
OB0048	NL	12/2000	S	TCT	—	CAA	—	GCC	T	ACT	—	GCA	I	ATC
OB0115	NL	2/2001	S	TCT	H	CAC	—	GCC	S	TCT	—	GCA	I	ATC
EP0125	NL	11/2001	S	TCC	H	CAC	—	GCC	S	TCT	—	GCA	I	ATC
02012	JP	1/2002	S	TCC	H	CAC	—	GCC	S	TCT	—	GCA	I	ATC
EP0207	NL	1/2002	S	TCC	H	CAC	—	GCC	S	TCT	—	GCA	I	ATC
EP0239	NL	2/2002	S	TCC	H	CAC	—	GCC	S	TCT	—	GCA	I	ATC
OB0371	NL	10/2003	S	TCC	H	CAC	—	GCC	S	TCT	—	GCA	I	ATC
04038	JP	3/11/2004	S	TCC	H	CAC	S	TCC	P	CCT	S	TCA	I	ATC
04059	JP	4/18–30/2004	S	TCC	H	CAC	S	TCC	P	CCT	S	TCA	I	ATC
S040035	JP	4/21/2004	S	TCC	H	CAC	S	TCC	P	CCT	S	TCA	I	ATC
04071	JP	5/16–23/2004	S	TCC	H	CAC	S	TCC	P	CCT	P	CCA	I	ATC
04073	JP	5/22/2004	S	TCC	H	CAC	S	TCC	P	CCT	S	TCA	I	ATC
04075	JP	5/25/2004	S	TCC	H	CAC	S	TCC	P	CCT	S	TCA	I	ATC
S040100	JP	5/31/2004	S	TCC	H	CAC	S	TCC	P	CCT	S	TCA	I	ATC
OB0528	NL	1/2005	S	TCC	H	CAC	S	AGC	P	CCT	—	GCA	I	ATT
OB0587	NL	4/2005	S	TCC	H	CAC	S	AGC	P	CCT	—	GCA	I	ATT

[a] The position number corresponds to the capsid sequence of the Melksham/94/UK strain; a dash indicates sequence identity with this strain. Residues are indicated by their single-letter codes.
[b] For strain abbreviations, see Table 1.
[c] UK, United Kingdom; US, United States; NL, The Netherlands; JP, Japan.

City, and strains from Japan and The Netherlands could be consistently grouped in the same alignment, suggesting continuous widespread circulation and an ability to cause disease. The recombinant genomes with GII.2 capsids, however, occur sporadically and did not show evidence for evolution, suggesting that their circulation is limited (1, 4, 17). The finding that strains from such widely separated geographic regions were similar shows that evolution of GII.2 NoVs is a global phe-

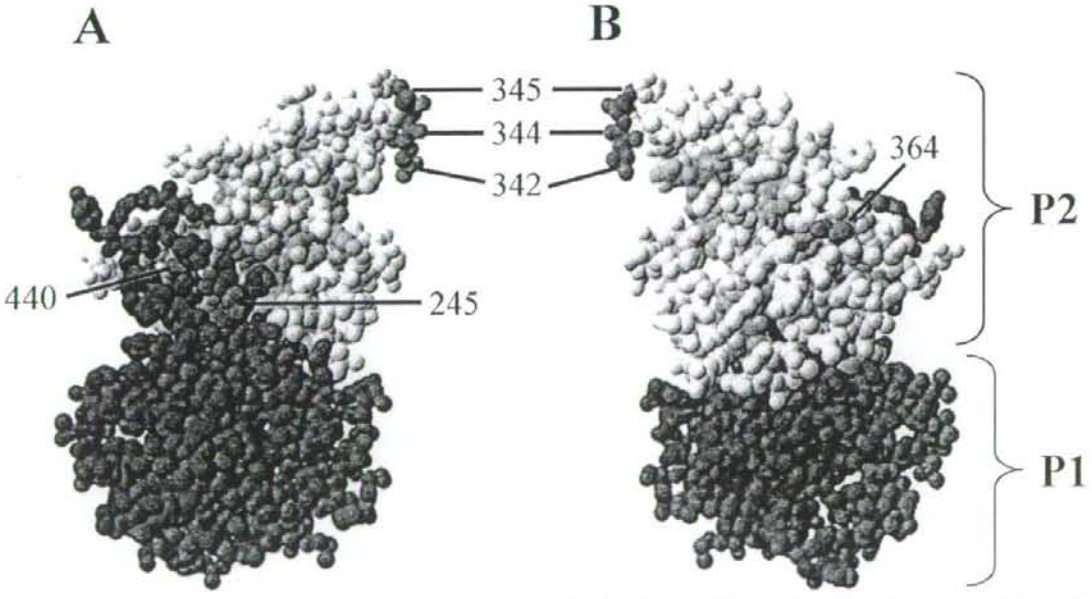

FIG. 4. Location of six fixed amino acid residues (positions 245, 342, 344, 345, 364, and 440, shown in red) on the monomer of the capsid protein. This 3D structure for the monomer P domain of the GII.2 NoV capsid protein was made by WHAT IF. The P1 and P2 subdomains are shown in blue and gray, respectively. The S domain is not shown. The KGE motif in the P2 domain is shown in green. (A) Predicted location of amino acid residues 342, 344, 345, and 364 in the P2 subdomain. (B) A view identical to panel A rotated 180° horizontally showing the location of amino acid residues 245 and 440 in the P1 subdomain.

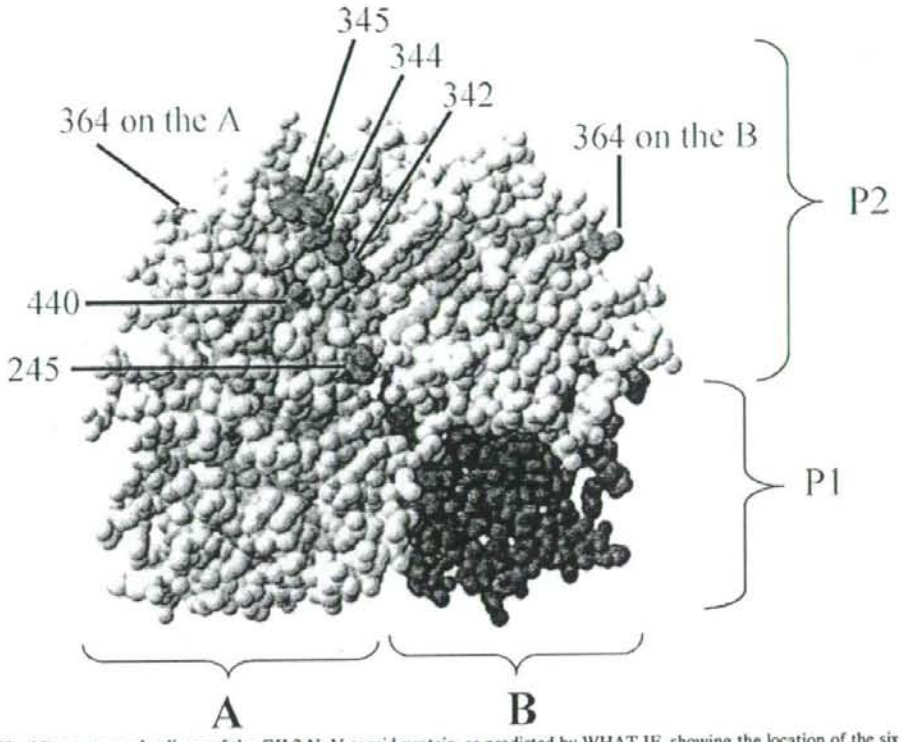

FIG. 5. The 3D structure of a dimer of the GII.2 NoV capsid protein, as predicted by WHAT IF, showing the location of the six informative amino acid residues (red). Monomer A is shown in yellow, while the monomer B is shown in blue (P1) and gray (P2). The S domains are not shown. The KGE motif (green) in the P2 subdomain on monomer B is also shown.

nomenon, similar to what has been described for GII.4 NoVs (5, 27, 33).

The molecular characterization of Melksham-like strains showed accumulation of mutations over a 12-year period, segregating them into five genetic groups. Three of these were supported in phylogenetic analysis with high bootstrap values. Two others (Fig. 2, 1999 to 2000 and 2001 to 2003) were not distinguishable in phylogenetic analysis, possibly because of the small number of isolates in the comparison. The present collection, however, contains all GII.2 strains detected in the two countries in the past 12 years of surveillance. Amino acid position 345 was the most variable in all GII.2 strains. This is suggestive of immune pressure, particularly because the amino acid is predicted to be located on a surface-exposed part of the capsid. The fixed amino acid changes were topologically clustered, judging from the 3D structure prediction, indicating that combined they may form an epitope. Immunity to NoVs has not been studied very systematically, but recent work on GII.4 NoVs has suggested that these viruses evolved under immune pressure (9, 30, 33). The lack of a tissue culture model makes it difficult to corroborate these findings by using cross-neutralization tests with hyperimmune serum directed against specific variants. Evolution of strains in an immunocompromised patient with chronic shedding of NoVs has been demonstrated by Nilsson et al. (28), who suggested that the cumulative amino acid substitutions appearing in the P2 subdomain were immune response driven. To clarify the relations between these amino acid mutations and receptor or antibody binding further immunological studies are needed.

Recently, new variants of the predominant NoV, the GII.4 genotype, appeared in 2002 (27), 2004 to 2005 (5), and 2006 (33) with global distribution. These new GII.4 variants were characterized into different clusters by the phylogenetic analysis based on the capsid gene (5, 9, 30). Okada et al. (30) reported that GII.4 strains had cumulative amino acid changes in the P domain and 5% to 18% amino acid diversity in the P2 subdomain between GII.4 subtypes for 5 years study. Bull et al. (5) also described more than 5% amino acid diversity in the complete capsid gene between past GII.4 strains and variants. Siebenga et al. (33) found 9% fixed amino acid mutations across the capsid gene over a 12-year period, but only five of these consistently delineated subsequent epidemic strains. In contrast, here we found only 2.6% amino acid diversity across the complete capsid gene of Melksham-like strains in a 12-year period, showing a clearly lower rate of change. The difference between these genotypes is intriguing and suggests clear differences in epidemiology that are not easy to explain. This study has been done using a limited number of strains in a rare genotype from a limited number of locations. It is necessary to

continue molecular surveillance of NoV infections to further the evolutional analysis of each genotype.

In conclusion, the present study showed that NoVs belonging to a rare genotype evolved by the accumulation of mutations in the surface-exposed parts of the capsid, suggesting immune response-driven evolution. The pattern of change similar to what has been observed for GII.4 is a global one, suggesting that these viruses circulate much more than is apparent from their presence in surveillance data. The data also showed for the first time that recombinant genomes of NoVs behave differently, even when they share the same capsid genes as nonrecombinant genomes. Understanding how NoVs evolve is necessary for finding more effective ways to control this disease and particularly its impact in healthcare settings.

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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

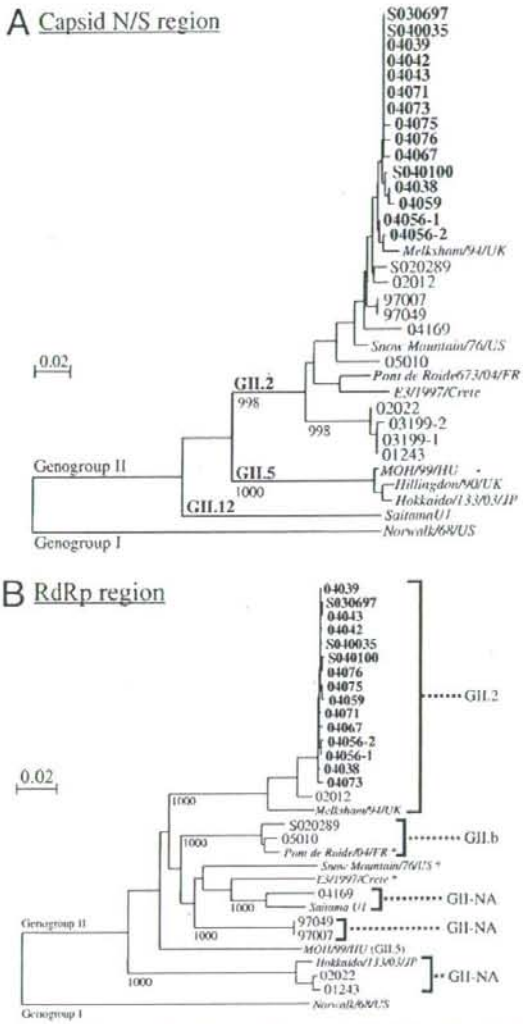

FIG. 1. Phylogenetic analysis of the capsid N/S (278 nucleotides) (A) and the partial RdRp (B) regions of the GII.2-cap strains detected in Osaka City. The GII.2-cap strains detected between March and May 2004 (04spring strains) are represented in boldface. Reference strains of NoV used in this study 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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ノート

細菌添加培養処理によるカキなどからのノロウイルス検出率の向上

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Enhancement of Norovirus Detection Rates in Oysters and Other Food Samples by Using Bacterial Treatment

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Factors such as low recovery rate and food contaminants may be responsible for the difficulty of detecting Norovirus (NV) by PCR in foodborne outbreaks. To detect NV more efficiently, we introduced a bacterial treatment, in which concentrated samples were incubated overnight with *Klebsiella oxytoca* at 35℃ before RNA extraction using the standard protocol. Recovery rates of NVs (G I/8 or G II/13) added to food suspensions in the modified method were compared with those in the standard method by quantification of NV RNAs using real-time PCR. Recovery rates in the modified method were 8.6% for G I/8 and 11.6% for G II/13 in 18 oyster samples and 13.9% for G I/8 and 19.6% for G II/13 in 15 other food samples, while those in the standard method were 0.3% for G I/8 and 0.5% for G II/13 in the oyster samples and 1.9% for G I/8 and 7.9% for G II/13 in the other food samples. These results indicate that the bacterial treatment increase the recovery of NV from foods such as oysters, suggesting that the modified method will be useful for NV detection in food samples.

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Key words: ノロウイルス Norovirus; 食品 food sample; 細菌 bacteria; 回収率 recovery rate

緒 言

食品からのノロウイルス(NV)検出は，食中毒原因物質の特定および感染経路の究明を行い，新たな感染の拡大や発生を未然に防止する上で重要な検査である．しかし，実際に食品からNVが検出される事例は非常に少なく，東京都では2006年に1,108件の食中毒関連食品の検査を行ったが，NVは全く検出されなかった．これは食品成分由来の夾雑物が検査に影響することや，食品中に含まれる微量なNVを効率よく回収することが難しいためと考えられる．NVの検出は平成15年11月5日付けの厚生労働省医薬食品局食品安全部監視安全課長通知，食安監発第1105001号[*1)]に記載された方法（以下，通知法とする）により実施されているが，その後もカキなどからの検出感度を高めるための検討が行われている[1),2)]．筆者らは食品由来夾雑物の除去法に焦点を絞って検討を行い，細菌を利用してカキやその他の食品中に含まれる食品由来夾雑物を分解あるいは消化させる前処理法を考案した．今回NV添加回収実験において回収率が向上する結果を得たので，以下にその概要を報告する．

材料および方法

1. 供試食品

2007年10月から12月に東京都内で市販された生食用マガキ18検体，同時期に東京都内で市販または調理され

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た食品15検体を用いた．カキは1検体につき2～4個体から取り出した中腸腺にPBS (－) (pH 7.4: 日水製薬) を加えてホモジナイズし10%乳剤を作製した[2,4]．他の食品は4～5 gを秤量後，5～10倍量のPBS (－) を加えたフィルター付き細菌検査用ポリ袋中で1分間ストマッカー処理を行った．

2. 供試材料の作製

過去に東京都健康安全研究センターで食中毒患者の糞便から検出され，Kageyamaらの方法[5]により遺伝子型を決定したNV遺伝子型G I/8株（以下，G I/8とする），G II/13株（以下，G II/13とする）を添加用NVとした．それぞれのNVが検出された糞便乳剤をPBS (－) で1,000倍に希釈後等量混合し，孔径0.22 μmのフィルターでろ過して添加用ウイルス浮遊液を作製した．この添加用ウイルス浮遊液140 μLを各食品乳剤6 mLに添加したものを供試材料，PBS (－) 6 mLに添加したものを対照材料とした．

3. 前処理用菌浮遊液の作製

東京都健康安全研究センターにおいて，食品から分離同定された*Klebsiella oxytoca*を35℃，20時間培養した普通斜面培地から，PBS (－) を用いてMcFarland 4 (1.2×10^9/mL) の菌浮遊液を作製した．

4. 通知法による前処理

通知法に従い，供試材料および対照材料を4℃，10,000 rpm，20分間遠心後，上清を30%ショ糖溶液1 mLに重層し，40,000 rpm，2時間 (HITACHI himac CP80 WX) 超遠心した（以下，標準法とする）．得られた沈渣を滅菌蒸留水140 μLで再浮遊し，全量をRNA抽出に用いた．

5. 細菌を用いた前処理

供試材料および対照材料に，上記の前処理用菌浮遊液10 μLを添加後35℃で一晩培養した．培養後の材料は標準法と同様に遠心処理した（以下，A3T法とする）後，沈渣を滅菌蒸留水140 μLで再浮遊した全量をRNA抽出に用いた．

6. NV検出方法

RNA抽出，DNase処理，cDNA合成およびリアルタイムPCRによるNVの検出は，通知法に準拠して行った．RNAの抽出はQIAamp Viral RNA Mini Kit (QIAGEN) を用い，DNase処理にはDNase I (TaKaRa) を用いた．逆転写酵素はMMLV (invitrogen)，プライマーはRandom hexamer (Amersham Biosciences) を用いた．合成したcDNA 5 μLを鋳型として，ABIPRISM 7900 (Applied Biosystems) によるリアルタイムPCRを行い，添加したNVを検出，定量した．プライマーおよびプローブはG I検出用にCOG1F/COG1R, RING1-TP (a), G II用としてCOG2F/COG2R, RING2-TPを用い，50℃ 2分，95℃ 10分を1回，95℃ 15秒，56℃ 1分を45回繰り返した．

7. 定量用標準曲線

国立感染症研究所より分与されたNVコントロールDNAを10倍段階希釈した標準液を作製し，各濃度の標準液より得られたリアルタイムPCRのthreshold cycle (Ct値) から定量用標準曲線を作製した．

結　果

標準法およびA3T法による前処理後に得られたリアルタイムPCRのCt値，Ct値と定量用標準曲線から求めたウイルスRNAコピー数をTable 1に示した．標準法では29検体中G I/8で4検体，G II/13で1検体がNV不検出となった．検出できなかった検体はいずれもカキであった．NV不検出の検体を除いてCt値を比較した場合，A3T法は標準法に比べ，カキではG I/8で2.9～10.5（平均6.8）サイクル，G II/13で2.6～8.8（平均6.4）サイクルの短縮が見られた．得られたCt値を用いてt検定を行った結果，両前処理法には$p<0.001$（両側）で有意差が認められた．他の食品においてもG I/8で0.5～6.7（平均3.4）サイクル，G II/13で0～4.4（平均1.9）サイクルCt値は短縮（$p=0.002$）したが，対照材料では有意差は見られなかった（$p=0.796$）．

添加用ウイルス浮遊液140 μLを直接RNA抽出した場合のCt値はG I/8が28.6サイクル，G II/13が25.1サイクルであり，このNV RNA定量値は，定量用標準曲線からG I/8では201,071 copies/test, G II/13では504,045 copies/testと算出された．この濃度を100%とし，供試材料に添加したNVの回収率 (x) をx＝(供試材料中のNV定量値/添加用ウイルス浮遊液中のNV定量値)×100により求めた (Fig. 1)．標準法の回収率の平均は，カキではG I/8で0.3%，G II/13で0.5%，他の食品ではG I/8で1.9%, G II/13で7.9%であった．これに対しA3T法の回収率の平均は，カキではG I/8で8.6%, G II/13で11.6%，他の食品ではG I/8で13.9%，G II/13で19.6%であった．標準法による対照材料の回収率はG I/8で21.9%，G II/13で31.2%であった．なお，菌浮遊液のみから核酸抽出し，DNase処理を行わずにリアルタイムPCRで検査した結果，添加した*K. oxytoca*のDNAによると思われる偽陽性反応は見られなかった．また，カキ乳剤を用いた事前実験で，菌浮遊液を添加せずに35℃で一晩培養した場合のNV回収率は，標準法との間に大きな差は認められなかった．

考　察

今回筆者は，食品からのNV検出率の改善を目的に細菌を利用した前処理A3T法を試み，一定の効果が認められる結果を得た．これまでの遠心分離による方法では，遠心濃縮後の沈渣，沈澱物中に残存する食品由来夾雑物の除去が不十分であり，その後の核酸抽出からPCR反応に至る過程に影響を与えると考えられた．A3T法は，遠心濃縮前に細菌を添加し培養処理を行うことで食品由来夾雑物

Table 1. Threshold cycles and NV RNA copies (/test) of standard method and modified method with bacterial treatment

Samples	G I/8					G II/13				
	Standard		A3T			Standard		A3T		
	Ct	Copies	Ct	Copies		Ct	Copies	Ct	Copies	
Oyster 1	ud	0	33.8	9,259	(−)*	ud	0	29.6	49,632	(−)
Oyster 2	41.2	116	34.7	5,435	(46.9)	35.9	1,934	30.4	32,869	(17.0)
Oyster 3	37.7	921	34.8	5,123	(5.6)	33.8	5,704	31.0	24,130	(4.2)
Oyster 4	ud	0	34.3	6,887	(−)	37.8	727	30.3	34,607	(47.6)
Oyster 5	ud	0	33.1	14,013	(−)	36.6	1,348	28.8	74,943	(55.6)
Oyster 6	44.4	17	33.9	8,727	(513.4)	37.8	727	29.4	55,018	(75.7)
Oyster 7	ud	0	36.4	1,987	(−)	39.0	392	30.9	25,406	(64.8)
Oyster 8	42.5	54	34.4	6,492	(120.2)	36.3	1,574	29.0	67,606	(43.0)
Oyster 9	40.6	165	36.9	1,478	(9.0)	35.8	2,036	31.3	20,675	(10.2)
Oyster 10	42.9	42	33.6	10,423	(248.2)	36.5	1,420	29.9	42,525	(29.9)
Oyster 11	40.5	176	34.9	4,828	(27.4)	33.9	5,417	31.3	20,675	(3.8)
Oyster 12	41.4	103	32.6	18,839	(182.9)	37.9	690	29.1	64,212	(93.1)
Oyster 13	41.2	116	32.2	23,872	(205.8)	35.9	1,934	29.6	49,632	(25.7)
Oyster 14	40.7	156	30.8	54,676	(350.5)	36.4	1,495	27.6	139,055	(93.0)
Oyster 15	34.8	5,123	30.6	61,547	(12.0)	32.1	13,692	27.9	119,144	(8.7)
Oyster 16	36.0	2,518	31.0	48,571	(19.3)	33.7	6,005	28.1	107,480	(17.9)
Oyster 17	39.2	379	32.3	22,500	(59.4)	35.9	1,934	28.9	71,180	(36.8)
Oyster 18	41.8	81	34.9	4,828	(59.6)	36.2	1,657	29.3	57,926	(35.0)
Mean	40.4	554	33.6	17,194	(31.1)	36.0	2,705	29.6	58,706	(21.7)
Sashimi	36.8	1,568	33.2	13,208	(8.4)	31.2	21,768	28.2	102,084	(4.7)
Steamed chicken	34.7	5,435	30.6	61,547	(11.3)	29.6	49,632	27.0	189,415	(3.8)
Rice	35.2	4,043	30.7	58,010	(14.3)	29.3	57,926	27.5	146,405	(2.5)
Pear	38.6	540	34.2	7,307	(13.5)	33.3	7,379	31.6	17,715	(2.4)
Tofu	36.9	1,478	32.4	21,207	(14.3)	32.7	10,052	28.3	96,959	(9.6)
Tsukemono	35.7	3,007	34.7	5,435	(1.8)	31.6	17,715	31.6	17,715	(1.0)
Bread	33.1	14,013	31.3	40,669	(2.9)	29.1	64,212	28.5	87,467	(1.4)
Fried chicken	34.0	8,226	31.3	40,669	(4.9)	29.2	60,988	27.6	139,055	(2.3)
Salad	40.8	147	40.3	198	(1.3)	34.5	3,977	32.1	13,692	(3.4)
Cake	35.0	4,551	31.4	38,331	(8.4)	29.4	55,018	26.9	199,428	(3.6)
Stew	40.5	176	33.8	9,259	(52.6)	34.1	4,887	30.7	28,163	(5.8)
Gyoza	35.7	3,007	32.0	26,873	(8.9)	29.3	57,926	27.4	154,145	(2.7)
Scrambled eggs	34.5	6,118	30.9	51,533	(8.4)	28.1	107,480	27.5	146,405	(1.4)
Cookie	35.9	2,671	31.3	40,669	(15.2)	29.2	60,988	27.9	119,144	(2.0)
Hamburger	37.2	1,238	35.5	3,385	(2.7)	32.1	13,692	30.8	26,749	(2.0)
Mean	36.3	3,748	32.9	27,887	(7.4)	30.8	39,576	28.9	98,969	(2.5)
PBS 1	31.2	43,147	30.6	61,547	(1.4)	27.1	179,905	27.0	189,415	(1.1)
PBS 2	30.9	51,533	30.7	58,010	(1.1)	27.3	162,293	27.0	189,415	(1.2)
PBS 3	31.3	40,668	30.1	82,745	(2.0)	27.4	154,145	27.2	170,872	(1.1)
PBS 4	31.3	40,668	30.6	61,547	(1.5)	27.7	132,073	27.6	139,055	(1.1)
Mean	31.2	44,004	30.5	65,962	(1.5)	27.4	157,104	27.2	172,189	(1.1)
Viral fluid	28.6	201,071				25.1	504,045			

ud: undetected

* Ratio of copy numbers in the case of A3T treatment to copy numbers in the case of standard treatment

の分解，消化を図るものである．通知法では，超遠心後の浮遊液に不純物が多いと判断された場合のみ 10,000 rpm, 20 分間遠心処理し，不純物との分離を行うとされている．しかし，この遠心処理は NV 回収率が一定程度向上する場合と逆に低下する場合の両方が見られ，不純物が多いと判断する基準，あるいは行ったほうが良いと判断する基準が明確でない．このことから，今回の検討では標準法，A3T 法とも超遠心後の遠心処理は省略することとした．

Escherichia coli など 10 種の細菌とカキ乳剤を用いた事前実験の結果において，最も高い回収率を示した *K. oxytoca* を今回の前処理用細菌として用いた．高い回収率を示した理由として，*K. oxytoca* は比較的多くの糖類やアミノ酸類に対して分解能を有しているためと推察された．標準法と比較して A3T 法の有用性が認められなかった食品では，*K. oxytoca* による処理がこれらの食品由来夾雑物には不足，または不適当であったものと考えられた．添加する菌数を 10 倍量あるいは 1/10 量とした場合や，培養時間を 48 時間まで延長した場合の回収率にはほとんど変化は見られなかった．したがって，今後はより分解能の高い細菌や複数種の細菌を添加する，あるいは食品群別に適当

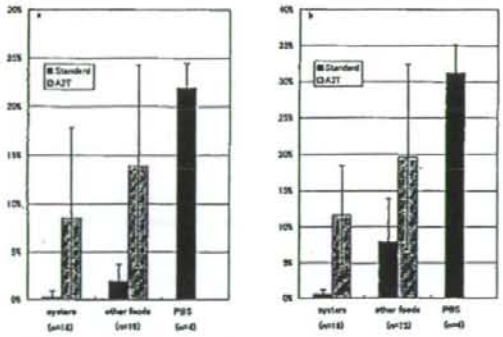

Fig. 1. Reccovery of NV from oysters, other foods, and PBS. a: G I/8, b: G II/13. Data indicates % recovery rate with standard deviation

な細菌を選択して使用することなどを検討することで，さらに回収率が高まることに期待する．PCR反応を用いるための夾雑物の除去法として，酵素や化学反応，フィルターを利用した処理方法[2), 3), 6)]が試みられているが，細菌を用いた方法は特殊な器具や試薬類を必要とせず，添加した細菌とNVを分離することが比較的に容易であることから，食品からのNV検出法に1つの方向性を示唆するものであると考えられた．

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