各国のリステリアの管理体制

各国のリステリアの管理体制において規制対象として考えられる食品は、非加熱で喫食する食品（いわゆるready-to-eat食品）である。しかし、その規格・基準の基本的な考え方は、米国と欧州では大きく異なる。

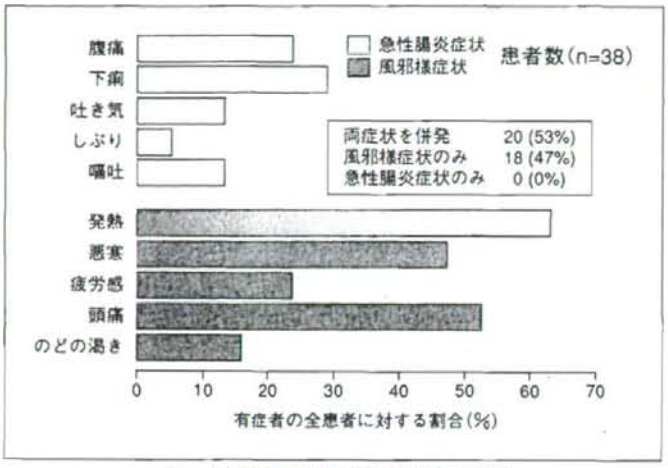

図2　当該事例における発症者の臨床症状

米国の場合

米国では「食品中からリステリアが検出されてはならない」という、いわゆるゼロ・トレランスの方針である。このような厳しい基準が規定されている背景には、米国特有の事情がある。

米国では1996年前後のアクティブ・サーベイランスによって、リステリアによる年間の患者数は約2500人、死者数は約500人と推測された。また、実際にリステリアによる集団事例もたびたび経験している。リステリアにひどく苦しめられている国である。また、リステリアは低温でも増殖できるので、米国では「食品流通時に増菌する可能性があるので、消費者が摂取する直前の最終的な菌数を担保することはできない」と考えている。

そのため、米国政府は、食品業界に対して厳しいリステリア管理を要求しており、結果としてゼロ・トレランスが規定されている。現在も、パルスネットの整備等を通じて、広域的な発生の掌握や感染拡大の阻止に努めている。

欧州やカナダの場合

欧州やカナダでは、いわゆるゼロ・トレランスではなく、一定菌数までの菌の存在を許容している。これは国際的な一般的な流れでもある（下記「国際機関における取り組み」参照）。

市販食品の多くがリステリアに汚染されていることは事実である。しかし、消費者が低い菌数のリステリアを摂取したところで、食中毒のような健康被害が発生する可能性は低い。また「ゼロ・トレランスは現実的ではない」という業界の認識もある。そのため、一部の食品を除いて、ゼロ・トレランス規定にはなっていない（例えば、EUでも「乳製品の場合は、25g中に検出不可」とされている）。

日本の場合

日本の食品衛生法ではリステリアに関する具体的な規格・基準は規定されていないが、法の「不衛生食品等の販売等の禁止」の項目を根拠に輸入禁止や回収が行われることがある。

輸入食品の場合は、検疫所の抜き取り検査でリステリアが検出されれば輸入禁止となる。また、輸入後に輸出国から汚染情報が得られた場合、回収・検査命令が出される。リステリアに関連して命令回収や自主回収が行われた事例としては、デンマーク産生ハム（2000年）、イタリア産サラミ、米国産ウインナー（2001年）、国内メーカー製生ハム、イタリア産チーズ（2003年）、輸入チーズ（2004年）、フランス産チーズ（2006年）等がある。

ちなみに、わが国の一般消費者のリステリアに関する認知度は、どの程度のものだろうか。2000年に兵庫県の食肉衛生検査所が、一般消費者を対象に「知っている食中毒菌」についてアンケート調査を実施した。その結果「サルモネラ」については回答者の95%が知っていたのに対し「リステ

リア」は6％しか知らなかった。今後、リステリアに関する啓蒙活動を（特に高齢者や妊産婦を中心に）積極的に展開し、ハイリスク食品の喫食回避を促すよう、さまざまな媒体を通じて正しい知識を普及させていく必要があるのではないか。米国では1996～2000年のアクティブ・サーベイランスにおいて、リステリア症がヒスパニック系に多く、乳幼児や子供を持つ年齢層に多いことが確認されたので、これらの人々を対象にした教育の強化に着手した。

国際機関における取り組み

コーデックス委員会では、2005年に開催された食品衛生部会においてready-to-eat食品におけるリステリアのコントロールについて、食品衛生上の取り扱いに関するガイドラインを提案した（現在もその議論は継続されている）。このガイドラインでは（先に述べた欧州やカナダの規格・基準と同じように）いわゆるゼロ・トレランスではなく、一定菌数までの存在は許容し（例えば1g当たり100個までの汚染を許容する等）、さらにプラスアルファとして衛生管理に関する要件を盛り込んだ内容になると思われる。

なお、FAO/WHOが2004年に採択したリステリアのリスクアセスメント（最終版）では、リステリア制御の考え方について、①リステリア症はスタンダードを大きく逸脱した多数の病原体を摂取したことにより引き起こされる、②消費段階での高レベルの汚染を阻止することが、リステリア症発生率低下に最も効果がある、③小売段階での高レベル汚染は比較的稀（まれ）であるが、製造～小売を通して菌の増殖が抑制されれば、リスクはさらに減る、④菌の増殖が可能な食品では、適切な温度コントロールと保存期間の制限が重要である――といった内容が記載されている。これが国際的に共通するリステリア制御の基本的な考え方である。（以上）

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Note

Separation of Viable Histamine-Producing Bacteria from Yellowtail Meat Components by Density Gradient Centrifugation

EMIKO ARAKI[1,2], KODAI TAKAYAMA[2], MIKAKO SAITO[2], AND HIDEAKI MATSUOKA[2]*

[1]*Japan Food Research Laboratories, 52-1, Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062, Japan*
[2]*Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2-24-16, Nakacho, Koganei-shi, Tokyo 184-8588, Japan*

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Quantitative separation of live cells from food samples is essential for non-culture methods to be validated. In this viewpoint, the feasibility of density gradient centrifugation (DGC) was demonstrated for the first time using samples of yellowtail meat to which *Morganella morganii*, a histamine producing bacterium had been added. Using a Ficoll density gradient from 50 to 10 w/v % with 10 w/v % steps, meat-free fractions of *M. morganii* cells were collected in 20-50 w/v % layers. The total cell collection rate ranged from 73-86 % irrespective of the cell density in the range 10^2-10^6 cells/200 μl.

Key words : Density gradient centrifugation/Viable cell separation/Histamine-producing bacteria/Histamine-producing fish/*Morganella morganii*.

The detection of live bacteria in foods is essential to ensure food safety and food security. In particular, to find the cause of bacterial foodborne diseases, the detection of live bacteria from the contaminated food must be carried out quickly. Conventionally, living bacteria have been detected by means of culture, but in recent years non-culture methods such as flow cytometry (Steen, 2000) and microscopic fluorescent imaging have been used as an alternative to the culture method for foods, pharmaceuticals, and cosmetics (Matsuoka et al., 2003; Shimakita et al., 2006). However, these non-culture methods are influenced by various components of food matrices, and therefore those components need to be removed by a proper pretreatment method. Filtration is thought to be a simple and efficient method. In fact, filtration was shown to be effective for various types of food (Shimakita et al., 2006). However, there are many kinds of food that can hardly be filtered. Raw milk, fish meat, and shellfish are typical examples.

*Corresponding author. Tel : +81-42-388-7029, Fax : +81-42-387-1503, E-mail: mhide@cc.tuat.ac.jp

In this study, we investigated the applicability of the density gradient centrifugation (DGC) method. This method has been well applied to spermatozoa and intracellular particles. There are also reports of its application to bacteria (Makinoshima et al., 2002; Nayak et al., 2005). However DGC has never been applied to food samples in practical food safety control, because it is too tedious to use DGC in daily routine operations. Therefore it is necessary to find the optimum DGC conditions for a specific food matrix as well as for the future development of a high throughput system for the fractionation of post-DGC samples. This paper describes the feasibility of using DGC from this viewpoint. The first challenge involved the use of yellowtail meat to which a histamine producing bacterium was added.

Histamine poisoning often occurs in fish in the families of *Carangidae* and *Scombridae* including yellowtail. Histamine poisoning is understood to be caused by specific bacteria that contaminate fish meat and produce histidine decarboxylase. Free histidine in fish meat is then converted to histamine, resulting in the accumulation of histamine in the fish

meat to a level that causes food poisoning. *M. morganii*, *Photobacterium damselae* and *P. phosphoreum* are typical examples of histamine-producing bacteria (Allen et al., 2005; Ben-Gigrey et al., 1999; Kanaki et al., 2004; Kim et al., 2001; Morii et al., 2004; Tsai et al., 2005; Fujii, 2006). Among them, *M. morganii* was selected as the target bacterium in this study.

Ficoll PM400 (GE Healthcare Bio-Sciences) was sterilized (121℃, 15 min) and dissolved in 50 mM phosphate buffer solution (PB) (pH 7.0) to prepare a 50 w/v % solution. The concentrate was diluted with PB to prepare 10, 20, 30, and 40 w/v % Ficoll solutions. Two hundred microliters of each solution, beginning with the highest concentration, were put into centrifugation tubes (Thinwall Polyallomer, 11 × 34 mm) by using a micropipette to prepare a stepwise gradient. In preparing the gradient, the tube was held upright and the solution was put into the tube carefully not to cause disturbance between Ficoll layers. Each Ficoll solution was refrigerated for storage and the density gradient was prepared before use.

The strain of *M. morganii* was provided by T. Fujii, Tokyo University of Marine Science and Technology, and maintained on a TSA plate at 4°C. Before use, *M. morganii* was inoculated into 10 ml of the LB medium using a platinum loop, and then cultured in a shaking incubator (130 rpm, 24 h, 37°C). Then 100 μl of the cultured cell suspension was transferred in 10 ml of the LB medium and incubated again in the same manner. The resulting cell suspension was diluted with PB to prepare a test cell suspension containing ca. 10^2 cells per 100 μl.

Two hundred microliters of the test cell suspension were laid onto the Ficoll density gradient prepared as described above. The test tube was centrifuged using an ultra centrifugation system (BECKMAN Optima™ TLM and TLS-55 Swing bucket rotor) at 30,000 rpm for 20 min at 4°C. After the centrifugation, each layer in the test tube was collected by careful manipulation using a micropipette. A hundred microliter aliquot of each fraction was plated on the plate count agar (Nissui Pharmaceutical Co., Ltd.), and then incubated at 37°C for 18 h. The number of colonies was counted to calculate the number of bacteria in each fraction. As shown in Fig. 1, *M. morganii* cells were distributed principally in the 20 w/v % and 30 w/v % Ficoll fractions. The total of 20-50 w/v % layers reached 100 %.

A five gram portion of farmed yellowtail fillet was placed in a stomacher bag, and PB (10 times the volume of the fillet) was added to the bag, and then stomached for 60 s to obtain a fish meat preparation. To the fish meat was added the test cell suspension

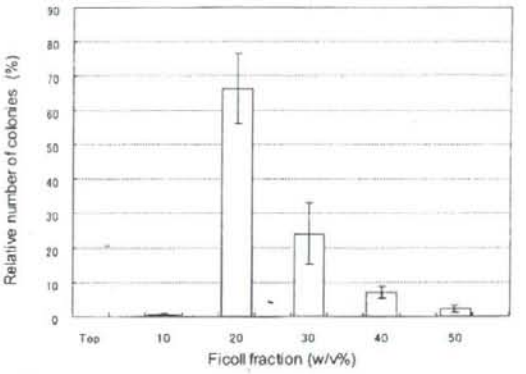

FIG. 1. Distribution of *M. morganii* collected after DGC. Sample: 200 μl of *M. morganii* suspended in PB (100 cells/100 μl). N=3

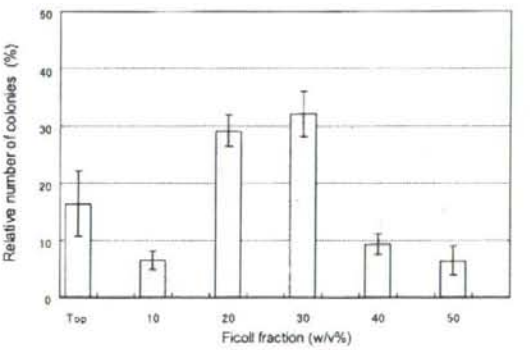

FIG. 2. Distribution of *M. morganii* collected after DGC. Sample: 200 μl of *M. morganii* added to the yellowtail extract (100 cells/100 μl). N=3

so that the cell concentration was 10^2 cells per 100 μl. After DGC, the added *M. morganii* cells were distributed in every fraction (Fig. 2). Fish meat components were collected in the top layer and 10 w/v % Ficoll layer. Therefore bacterial cells distributed in the 20-50 w/v % Ficoll layers were obtained without disturbance of the fish meat. The total cell collection rate in the 20-50 w/v % Ficoll layers was 78 %. *M. morganii* cells were principally collected from the 20 and 30 w/v % Ficoll layers.

It was revealed that the bacterial cells were attached to the fish meat components (Fig. 3). In order to separate these cells from fish meat, Tween 80 was added to PB. As depicted in Fig. 4, the addition of 0.1% Tween 80 solution decreased the cell distribution rate in the top layer to 8 %. Under this condition, the total collection rate from the 20-50 w/v % Ficoll layers reached 86 %.

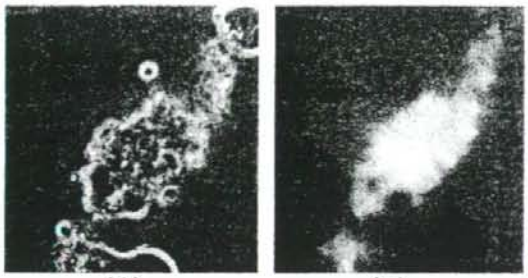

FIG. 3. Fluorescence images of *M. morganii* cells adhering to the yellowtail muscle. Sample: The top layer of Fig. 2. (A) Bright field image, (B) Fluorescence image. The test sample was stained with DAPI.

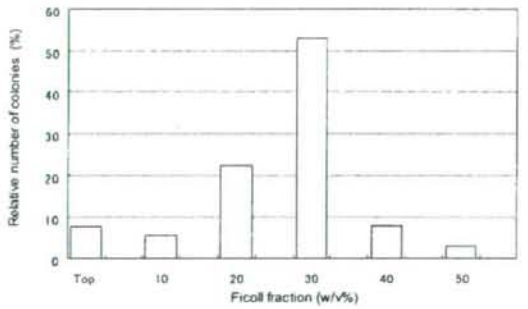

FIG. 4. Distribution of *M. morganii* collected after DGC. Sample: 200 μl of *M. morganii* added to the yellowtail extract (100 cells/100 μl) containing 0.1% Tween 80. N=2

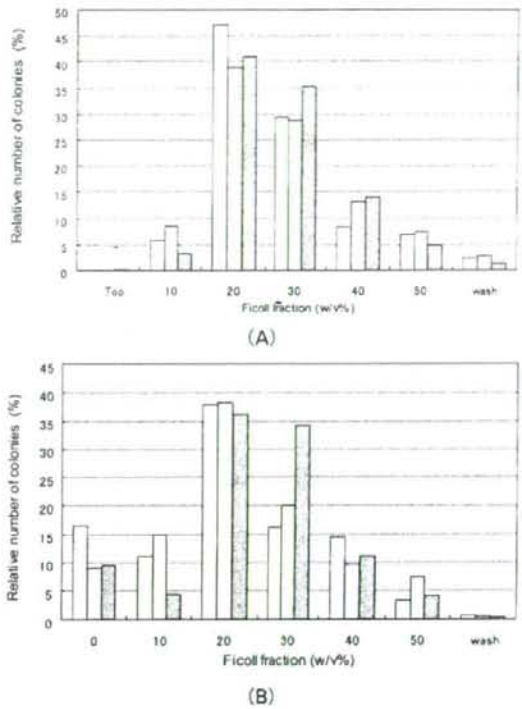

FIG. 5. Distribution of *M. morganii* collected after DGC. Sample: (A) 200 μl of *M. morganii* suspended in PB. (B) 200 μl of *M. morganii* added to the yellowtail extract containing 0.1% Tween 80. Cell density: 10^2, 10^4, 10^6cells/200 μl.

The cell distribution after DGC was thought to be influenced by the cell density in the test cell suspension. Thus test suspensions containing 10^2, 10^4, and 10^6 cells/200 μl (=cells/tube) were subjected to DGC with or without fish meat components. Without the fish meat, there was no cell density dependency (Fig. 5(A)). Total distribution rates in the 20-50 w/v % Ficoll layers were 89-96 % irrespective of the cell density from 10^2 to 10^6 cells/200 μl. Even with the fish meat, the cell density dependency was only slight (Fig. 5(B)). The total distribution rate in the 20-50 w/v % Ficoll layers was 73 % at 10^2cells/tube, while it was 86 % at 10^6cells/tube.

The present method has been applied to the cell count in yellowtail during storage at 20°C. Yellowtail (approximately 5 kg in body weight), farmed in Miyazaki prefecture, Japan, were killed by brain piercing in order to retain freshness, and then immediately filleted. Each fillet was washed, vacuum-packed, stored in ice, and then transported. Portions of muscle (approximately 30 g from each) were taken from the dorsal region, and then packed in polyethylene zip-lock bags. Each bag was then placed in a stomacher bag and the stomacher was turned on for 60 s to smash the fish meat (control group). For the test group, the test cell suspension was added the fish meat at the time of stomaching so that there were 10^3cells per gram of fish meat. The fish samples in both the test and the control groups were stored at 20 °C in polyethylene zip-lock bags for 15, 20, 39 and 45 h. The fish samples were subjected to DGC and the number of cells was counted for each density fraction. Then total numbers of cells from the 20-50 w/v % Ficoll fractions were plotted versus the storage time. As shown in Fig. 6, the number of cells increased during the storage and reached 10^7cells/g at 45 h. In the same way, the number of cells in the control samples was measured. It was no greater than 20 cells/g at 0 h but increased markedly and reached the same level as that of the test samples at around 20-30 h.

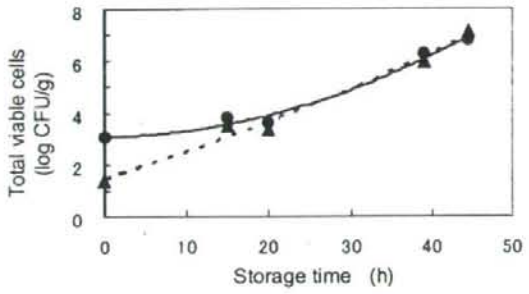

FIG. 6. Growth of *M. morganii* and other bacteria in the yellowtail during storage.
●: Number of *M. morganii* cells in 1g of yellowtail muscle. 10^3 cells/g *M. morganii* was added at t=0. Total cell count from the 20-50% Ficoll layers after DGC using Ficoll containing 0.1% Tween 80. ▲: Number of other bacterial cells in 1g of yellowtail muscle. Only the cells naturally contaminating the yellowtail were counted.

At the same time, the amount of histamine in the fish samples was also determined using high-performance liquid chromatography (HPLC) after dansylation . Histamine was found only in the test group, though the number of bacterial cells was the same in both groups. The histamine concentration in the test group was 1210 μg/g and 135 μg/g at 39 h and 45 h, respectively, while it was below the detecable limit (5 μg/g) throughout the test period in the control group. This result confirms that, even in the so-called histamine-producing fish, histamine is not produced if the fish is not contaminated with bacteria that produce histidine decarboxylase.

In the present study, it was important to separate *M. morganii* cells without degrading the cell viability. During centrifugation, bacterial cells should undergo cell-cell collisions frequently. In order to soften these collisions and protect cells from mechanical stress, we thought that the suspending solution had better be viscous. Thus we used Ficoll in the DGC. In the next step, however, it is necessary to investigate the feasibility of less viscous material such as Percoll for a more rapid and easier protocol.

In conclusion, DGC enabled the quantitative separation of the live cells of *M. morganii* from samples of yellowtail meat. The present results should provide essential data for the future design of a high throughput system for the collection of post-DGC samples.

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

Nobuhiro Iritani,[1,2]* Harry Vennema,[1] J. Joukje Siebenga,[1] Roland J. Siezen,[3] Bernadet Renckens,[3] Yoshiyuki Seto,[4] Atsushi Kaida,[2] and Marion Koopmans[1]

Laboratory for Infectious Diseases and Perinatal Screening, National Institute for Public Health and the Environment (RIVM), P.O. Box 1, 3720 BA Bilthoven, The Netherlands[1]; Department of Microbiology, Osaka City Institute of Public Health and Environmental Sciences, 8-34 Tojo-cho, Tennoji-ku, Osaka 543-0026, Japan[2]; Centre for Molecular and Biomolecular Informatics, Radboud University Medical Center, P.O. Box 9101, 6500HB Nijmegen, The Netherlands[3]; and Laboratory of Microbiology, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan[4]

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

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

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

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

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

MATERIALS AND METHODS

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

* Corresponding author. Mailing address: Department of Microbiology, Osaka City Institute of Public Health and Environmental Sciences, 8-34 Tojo-cho, Tennoji-ku, Osaka 543-0026, Japan. Phone: 81 6 6771 3147. Fax: 81 6 6772 0676. E-mail: n-iritani@city.osaka.lg.jp.

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

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

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

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

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

Amplification and sequencing for the complete capsid gene of GII.2 strains. Viral RNA was extracted from stool suspensions by using a QIAamp viral RNA Mini kit (Qiagen, Hilden, Germany). RT-PCR was carried out with the reaction mixtures and enzymes as previously described (8). RT was performed at 42°C for 2 to 3 h with reverse primer, N235Rex (17), and enzyme was inactivated at 95°C for 5 min. PCR was performed using several pairs of PCR primers (Table 2) with a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) under the following conditions: denaturation at 95°C for 1 min; 40 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 1 min; and a final cycle of incubation at 72°C for 5 min. When a PCR failed to produce strong products, we performed nested PCR. The amplified fragments were sequenced directly with a Big Dye terminator cycle sequencing kit and ABI 3700 sequencer (Applied Biosystems, Foster City, CA). The nucleotide sequences were determined in both orientations using the PCR primers. DNA sequences were edited using SeqManII (DNAStar Inc., Konstanz, Germany).

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

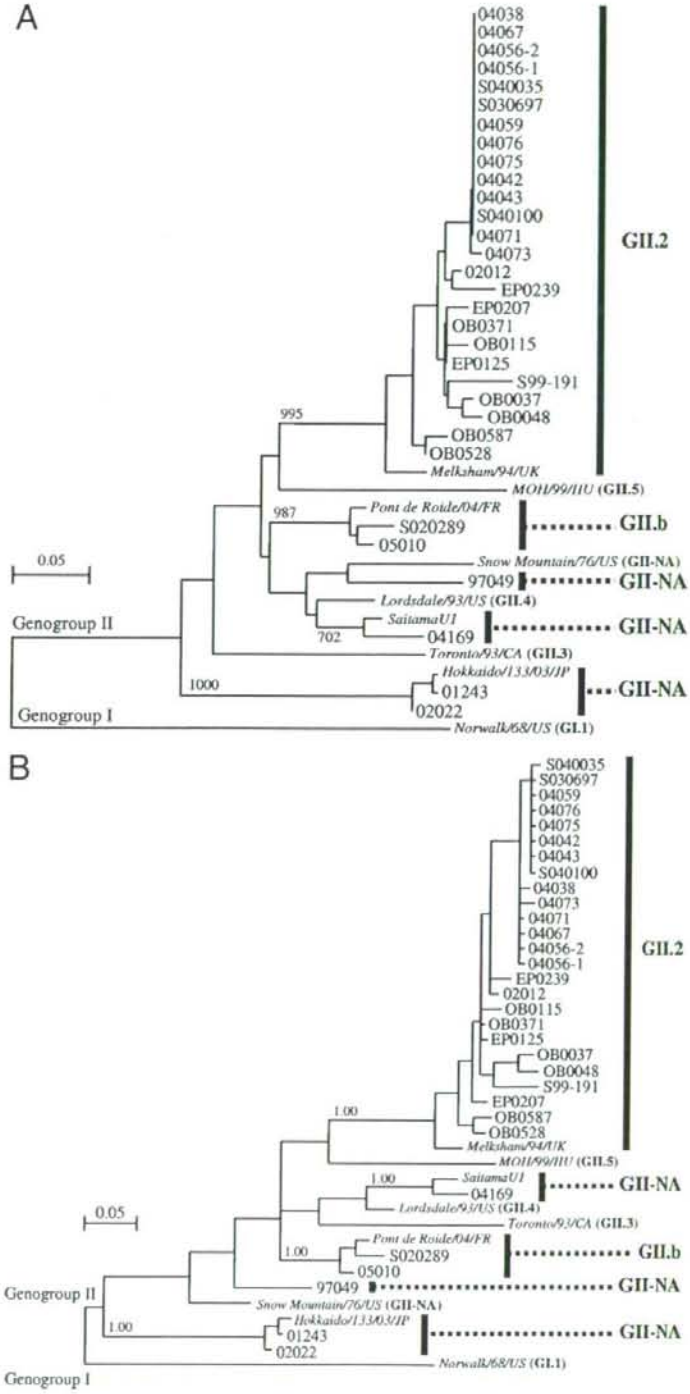

TABLE 2. Primers used to amplify the capsid gene of GII.2 NoV strains

Primer	Sequence (5′ to 3′)[a]	Polarity	Location (nt)	Reference or source
COG2F	CARGARBCNATGTTYAGRTGGATGAG	+	5003[b]	19
G2SKF	CNTGGGAGGGCGATCGCAA	+	5058[b]	22
G2SKR	CCRCCNGCATRHCCRTTRTACAT	−	5401[b]	22
N235Rex	GCWANRAAAGCTCCWGCCAT	−	6273[b]	17
MKcap508-524F	CAGAAAGATGATCCCAA	+	508[c]	This study
MKcap524-508R	TTGGGATCATCTTTCTG	−	524[c]	This study
MKcap662-649R	TCCACTGTTGGTGG	−	662[c]	This study
MKcap1109-1125F	TGGGTCAGATTCAAATT	+	1109[c]	This study
MKcap1125-1109R	AATTTGAATCTGACCCA	−	1125[c]	This study
MKcap1304-1290R	AAGAGCAGGCGCTCC	−	1304[c]	This study
MK35R	CAAAAGCTCCAGCCAT	−	1644[c]	This study

[a] Abbreviations for residues in boldface are as follows: B, not A; H, not G. In addition, N is A, C, G, or T; R is A or G; W is A or T; Y is C or T.
[b] Location of the 5′ end of the primer corresponding to the nucleotide position of Lordsdale/93/UK (X86557).
[c] Location of the 5′ end of the primer corresponding to the nucleotide position of ORF2 and ORF3 of Melksham/94/UK (X81879).

and EsyPred3D (25) servers. WHAT IF could not model residues 342, 344, and 345 based on the Norwalk/68/US capsid protein template (PDB ID 1IHM); as they are present in an inserted loop of the P2 subdomain relative to the template structure, but they are predicted to be in the same position as in the 3D model based on the GII.4 genotype capsid protein template (PDB ID 2OBR). A dimer of the GII.2 NoV capsid protein was modeled by superimposing two predicted monomers onto the trimeric template of the Norwalk/68/US capsid protein. The 3D models were visualized by the YASARA view program (version 6.813, http://www.yasara.org/) (24).

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the DDBJ/EMBL/GenBank with the accession numbers AB281081 to AB281090 (Table 1).

RESULTS

Capsid gene sequence analysis of GII.2 strains collected over 30 years. Sequence data from a total of 36 GII.2 strains showed that the capsid gene was 1,629 nt long and coded for a protein of 542 amino acids. There were no deletions or insertions in the capsid gene among these strains collected over a 30-year period. Sequence comparison showed ≥83.6% nucleotide and ≥93.5% amino acid identities among these GII.2 strains. The phylogenetic tree based on the 1,629 nucleotide sequences of the complete capsid gene confirmed that all strains were characterized as GII.2 genotype (Fig. 2).

Genetic analysis of GII.2 strains from a regional outbreak in Japan in the spring of 2004. Of the 21 GII.2 strains detected in Osaka City, Japan, 14 strains were detected in samples from cases in outbreaks or sporadic cases in the spring epidemic between March and May 2004 (Table 1). These strains were closely related to each other (≥99.2% nucleotide and ≥ 99.1% amino acid identities). To find out if these viruses changed genetically during circulation in the community for a short period of time (about 3 months), the complete nucleotide and amino acid sequences of the capsid gene from these 14 GII.2 strains were compared. A total of 45 nucleotide substitutions were observed (Table 3). The majority were third-base position changes (77.8%) and synonymous substitutions (82.2%). These nucleotide changes resulted in amino acid changes in eight codons, five of which were located in the P2 subdomain. In the alignment of the spring epidemic strains over the 3-month period, of the 45 nucleotide substitutions, only one nucleotide position was fixed (nt 594 in the S domain) at the end of this epidemic. This did not result in an amino acid change. All amino acid changes were sporadic. Eight of the 14 strains had an identical amino acid sequences (Table 1). Of these, strain 04038 was used for further genetic analysis.

Genetic analysis of all GII.2 strains collected between 1976 and 2005. The nucleotide and amino acid sequences of the complete capsid genes were compared for 29 GII.2 strains collected between 1975 and 2005 (30 years), excluding 7 GII.2 strains detected in Osaka City with identical amino acid sequences (10 strains from The Netherlands, 14 from Osaka City, and 5 from GenBank). A total of 488 nucleotide changes were observed (Table 4). Again, the majority of these were third-base changes (83.8%) and synonymous (85.9%). In total, 59 nucleotide changes resulted in amino acid changes (34 in the P2 subdomain) (Table 4). Twenty-five of these were informative changes (19 in the P2 subdomain), but none appeared to be fixed in the genome over time. Of the 25 informative sites, the amino acid position 345 was the most variable (Fig. 3). Nevertheless, statistical analysis showed a significantly higher rate of mutation in the P2 subdomain than in P1 and S, suggesting selective pressure ($P = 0.0018$, chi-square 5.63; and $P < 0.0001$, chi-square 12.9).

Genetic analysis of Melksham-like strains detected between 1994 and 2005. In order to understand the apparent discrepancy between selective changes in the P2 subdomain and the absence of fixation of these mutations, we repeated our anal-

FIG. 1. Phylogenetic analysis of partial RdRp gene sequences of GII.2 NoV strains by the neighbor-joining method (A) and the Bayesian method (B). The genotypes at the RdRp regions that are not assigned numbers are represented as GII-NA. The scale indicates the number of substitutions per site. Reference strains of NoV used in this analysis are given in italics. (A) The bootstrap values are indicated on each branch. (B) Tree topology was evaluated on the basis of 1,500,000 generations. The posterior probabilities are indicated on each branch (≥0.95 of the posterior probability means that the branch has high credibility). The GenBank accession numbers of the additional reference strains used in this analysis are as follows: Hokkaido/133/03/JP, AB212306; Lordsdale/93/UK, X86557; MOH/99/HU, AF397256; Norwalk/68/US, M87661; Pont de Roide 673/04/FR, AY682549; Saitama U1, AB039775; Toronto/93/CA, U02030. For strain abbreviations, see Table 1.

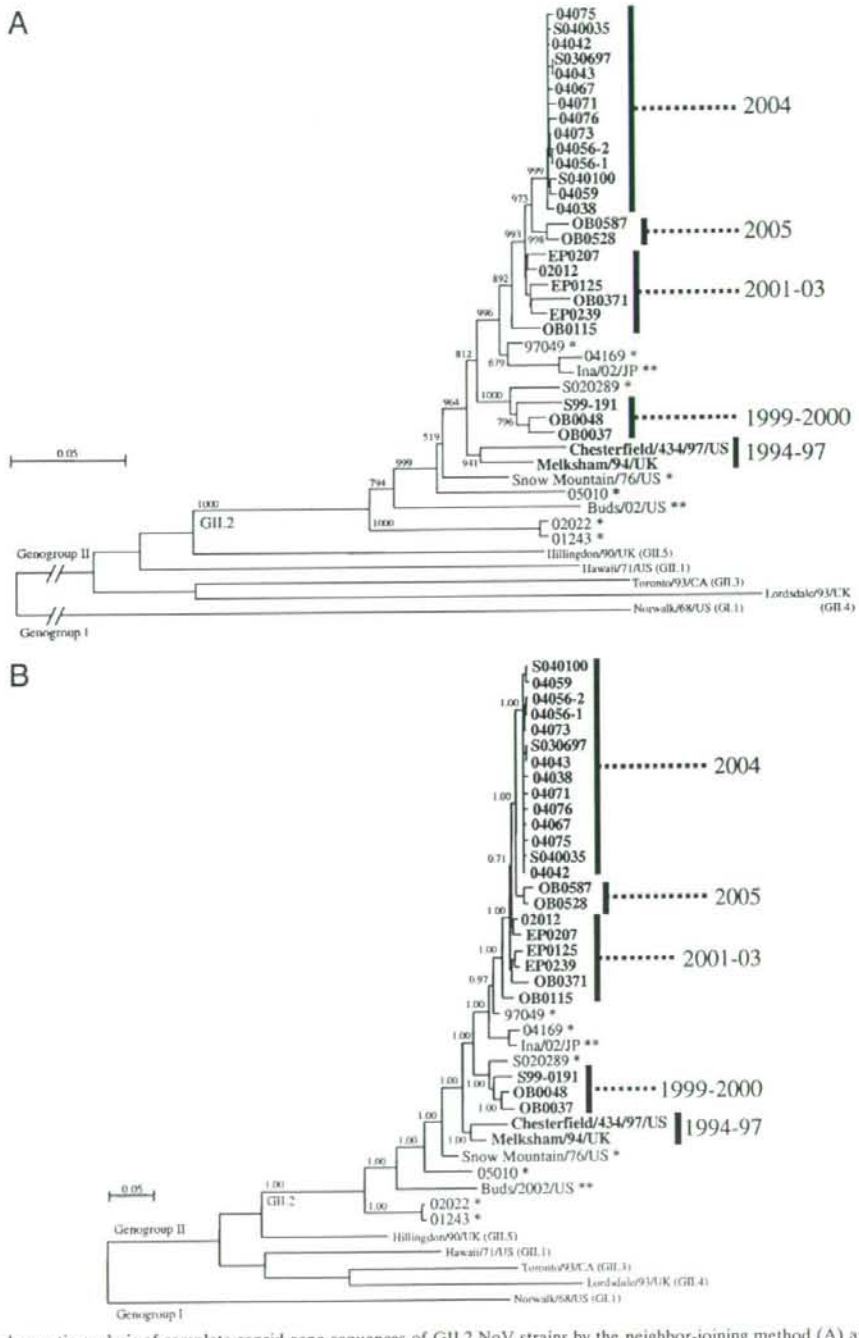

FIG. 2. Phylogenetic analysis of complete capsid gene sequences of GII.2 NoV strains by the neighbor-joining method (A) and the Bayesian method (B). Melksham-like strains, which have a matching (GII.2) RdRp sequence, are shown in boldface. Asterisks indicate the GII.2 strains that have different (*) or unknown (**) genotypes in the RdRp region. The scale indicates the number of substitutions per site. (A) The bootstrap values are indicated on each branch. (B) Tree topology was evaluated on the basis of 500,000 generations. The posterior probabilities are indicated on each branch. The GenBank accession numbers of the additional reference strains used in this analysis are as follows: Hawaii/71/US, U07611; Hillingdon/90/UK, AJ277607; Lordsdale/93/UK, X86557; Norwalk/68/US, M87661; Toronto/93/CA, U02030. For strain abbreviations, see Table 1.

TABLE 3. The numbers of nucleotide and amino acid substitutions in the complete capsid gene among 14 GII.2 strains detected in the spring epidemic of 2004 in Osaka City, Japan

Domain or subdomain[a]	Nucleotide substitutions						Amino acid substitutions	
	No. of substitutions	No. at the indicated position of the changed codon			Synonymous changes (no.)	Nonsynonymous changes (no.)	No. of substitutions	Informative changes (no.)
		1st	2nd	3rd				
N	1	0	0	1	1	0	0	0
S	18	3	2	13	16	2	2	0
P1	7	0	0	7	6	1	1	0
P1-1	4	0	0	4	4	0	0	0
P1-2	3	0	0	3	2	1	1	0
P2	19	3	2	14	14	5	5	0
Total	45	6	4	35	37	8	8	0

[a] Each domain of GII.2 NoV capsid gene was determined according to Chen et al. (7). The region and abbreviation of each domain are as follows: N, 5′ end of ORF2 and N terminal domain (residues 1 to 45); S, shell domain (residues 46 to 216); P1, P1 subdomain (P1-1, residues 217 to 274; P1-2, residues 421 to 542); P2, P2 subdomain (residues 275 to 420).

ysis after removing recombinant genomes from the alignment (Table 5) (nucleotide and amino acid sequences of the complete capsid gene from 20 Melksham-like strains: 10 strains from The Netherlands, 8 from Osaka City, and 2 from GenBank). Sequence comparison showed ≥91.5% nucleotide and ≥97.4% amino acid identities among these Melksham-like strains. A total of 301 nucleotide changes were observed (Table 5), the majority of which were third-base changes (87%) and synonymous (88.7%). These nucleotide changes resulted in amino acid changes in 32 codons, half of which were located in the P2 subdomain. Twelve of 32 amino acid positions were informative (9 in the P2 subdomain) (Fig. 3). In contrast with the previous finding, several mutations were fixed: of the 12 informative sites, 2 amino acid substitutions (amino acid positions 245 and 440) in the P1 subdomain and 3 amino acid substitutions (amino acid positions 342, 344, and 345) in P2 subdomain were cumulative (Table 6), segregating the strains into five genetic groups (1994 to 1997, 1999 to 2000, 2001 to 2003, 2004, and 2005) by the neighbor-joining method (Fig. 2A) and Bayesian method (Fig. 2B).The strains detected in the spring epidemic in Osaka City had a unique sequence, with S or P residues at amino acid position 364 (Fig. 3 and Table 6). The other six informative sites were not fixed.

The 3D structure of the P domain of a monomer of the NoV capsid protein was predicted by WHAT IF, 3D-Jigsaw, and EsyPred3D, based on the known 3D structure of the VA387/98/US GII.4 genotype capsid protein, which has 55% amino acid sequence identity in the P domain to the Melksham capsid protein. A comparison of the positions of the six fixed mutations to the predicted 3D structure indicated that all six residues were predicted to be located at the surface of the capsid protein, with three residues (342, 344, and 345) close to each

TABLE 4. The numbers of nucleotide and amino acid substitutions in the complete capsid gene among 29 GII.2 strains collected in the GenBank, The Netherlands, and Japan over a 30-year period

Domain or subdomain[a]	Nucleotide substitutions						Amino acid substitutions	
	No. of substitutions	No. at the indicated position of the changed codon			Synonymous changes (no.)	Nonsynonymous changes (no.)	No. of substitutions	Informative changes (no.)
		1st	2nd	3rd				
N	20	0	2	18	17	3	3	1
S	137	9	3	125	131	6	6	1
P1	162	17	7	138	143	19	16	4
P1-1	53	4	2	47	49	4	3	2
P1-2	109	13	5	91	94	15	13	2
P2	169	28	13	128	128	41	34	19
Total	488	54	25	409	419	69	59	25

[a] See note to Table 3.

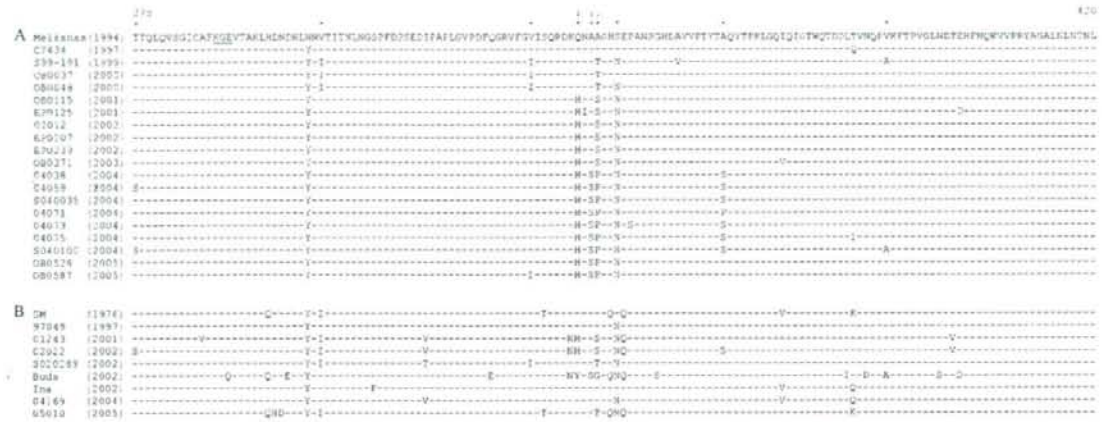

FIG. 3. Amino acid alignment of the P2 subdomains of GII.2 NoV strains showing mutations along the aligned sequences. The upper sequence alignment group (A) includes the Melksham-like strains and the lower group (B)includes the other GII.2 strains, which were recombinant genomes. In each group, sequences are arranged chronologically from top (oldest) to bottom (most recent). The detection years of the strains are indicated in parentheses. The asterisk indicates informative sites among Melksham-like strains. The arrow denotes cumulatively changing amino acid positions (342, 344, and 345) among Melksham-like strains. The numbers above the sequences indicate the sequence position relative to the position in the capsid protein of strain Melksham. Underlined sequences indicate the KGE motif that corresponds to the RGD-like motif of other NoVs and was determined with the amino acid sequence alignment of other NoVs according to Tan et al. (34). For strain abbreviations, see Table 1.

other in the P2 subdomain (Fig. 4). Furthermore, residues 342, 344, 345 of the P2 subdomain and residues 245 and 440 of the P1 subdomain were grouped closely together on the predicted 3D structure of a dimer (3D-modeling by WHAT IF) (Fig. 5).

DISCUSSION

In this study, we analyzed the complete capsid gene of GII.2 NoVs collected over a 30-year period. The collection included a relatively large sample from an epidemic that was observed only in Japan in the spring period of 2004. In this 3-month period, no evolutionary changes were observed, but compared with other GII.2 Melksham-like strains these variants had unique amino acid sequences (S or P) at position 364. One of the recombinant genomes, strain 02022, had the same amino acid residues, suggesting that the presence of this mutation was not causally related to the epidemic pattern. The sequence analysis confirmed that the spring epidemic was an outbreak.

Molecular characterization of the GII.2 capsids over the full study period showed an interesting difference between recombinant genomes and nonrecombinant (Melksham-like) genomes. The Melksham-like strains clearly evolved over time, with accumulation of mutations particularly in the P2 subdomain. In contrast, GII.2 capsids from recombinant genomes did not fit this pattern and had a seemingly erratic pattern of mutations. Melksham-like strains are occasionally observed in molecular surveillance data from The Netherlands and Osaka

TABLE 5. The numbers of nucleotide and amino acid substitutions in the complete capsid gene among 20 Melksham-like strains over a 12-year period

Domain or subdomain[a]	Nucleotide substitutions						Amino acid substitutions	
	No. of substitutions	No. at the indicated position of the changed codon			Synonymous changes (no.)	Nonsynonymous changes (no.)	No. of substitutions	Informative changes (no.)
		1st	2nd	3rd				
N	10	0	2	8	8	2	2	0
S	81	4	3	74	76	5	6	1
P1	98	8	3	87	89	9	8	2
P1-1	34	2	1	31	32	2	2	1
P1-2	64	6	2	56	57	7	6	1
P2	112	13	6	93	94	18	16	9
Total	301	25	14	262	267	34	32	12

[a] See note to Table 3.

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-

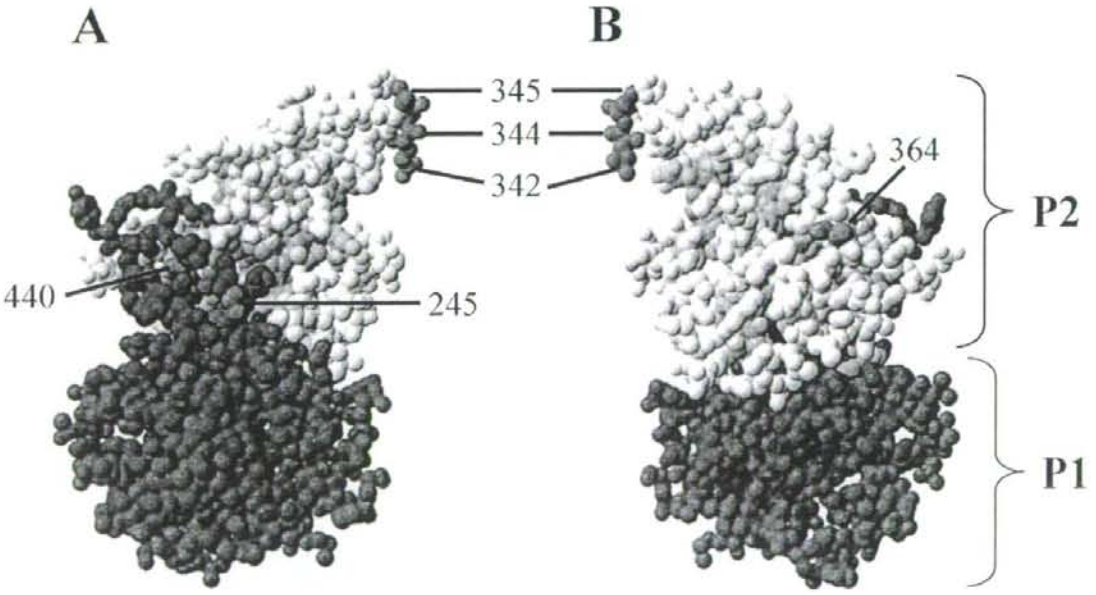

FIG. 4. Location of six fixed amino acid residues (positions 245, 342, 344, 345, 364, and 440, shown in red) on the monomer of the capsid protein. This 3D structure for the monomer P domain of the GII.2 NoV capsid protein was made by WHAT IF. The P1 and P2 subdomains are shown in blue and gray, respectively. 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.

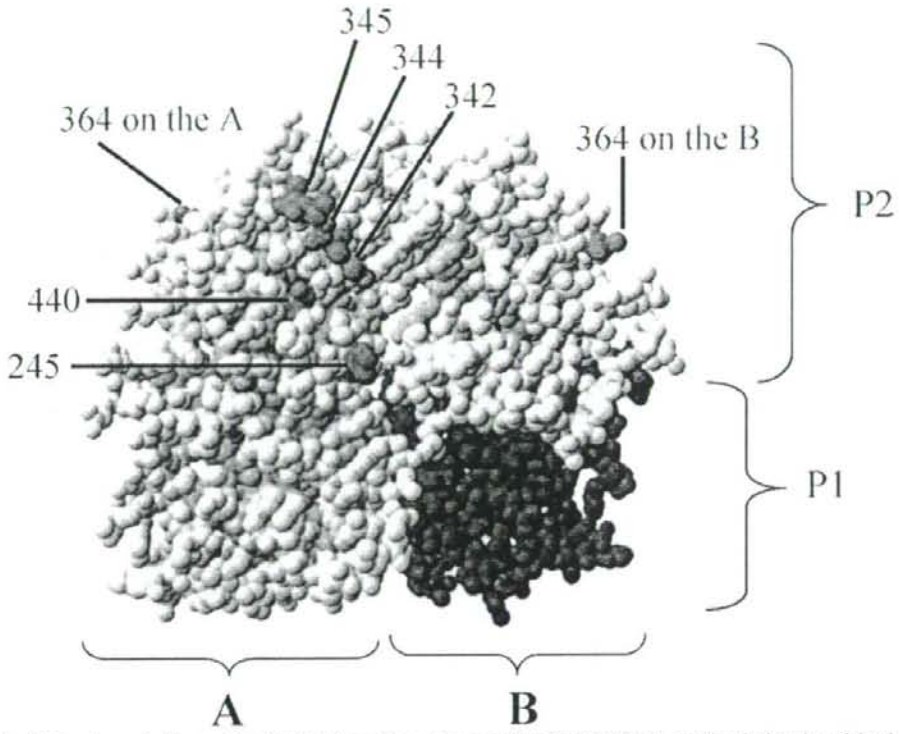

FIG. 5. The 3D structure of a dimer of the GII.2 NoV capsid protein, as predicted by WHAT IF, showing the location of the six informative amino acid residues (red). Monomer A is shown in yellow, while the monomer B is shown in blue (P1) and gray (P2). The S domains are not shown. The KGE motif (green)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

Nobuhiro Iritani,[1,2] Atsushi Kaida,[1] Hideyuki Kubo,[1] Niichiro Abe,[1] Tsukasa Murakami,[1] Harry Vennema,[2] Marion Koopmans,[2] Naokazu Takeda,[3] Hisashi Ogura,[4] and Yoshiyuki Seto[4,5]*

Department of Microbiology, Osaka City Institute of Public Health and Environmental Sciences, 8-34 Tojo-cho, Tennoji-ku, Osaka 543-0026, Japan[1]; Laboratory for Infectious Diseases and Perinatal Screening, National Institute for Public Health and the Environment (RIVM), P.O. Box 1, 3720 BA Bilthoven, The Netherlands[2]; Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-Murayama, Tokyo 208-0011, Japan[3]; Department of Virology, Osaka City University Medical School, Asahimachi, Abeno-ku, Osaka 545-8585, Japan[4]; and Laboratory of Microbiology, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan[5]

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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.

* Corresponding author. Mailing address: Laboratory of Microbiology, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan. Phone: 81-72-254-9484. Fax: 81-72-254-9918. E-mail: seto@vet.osakafu-u.ac.jp.

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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

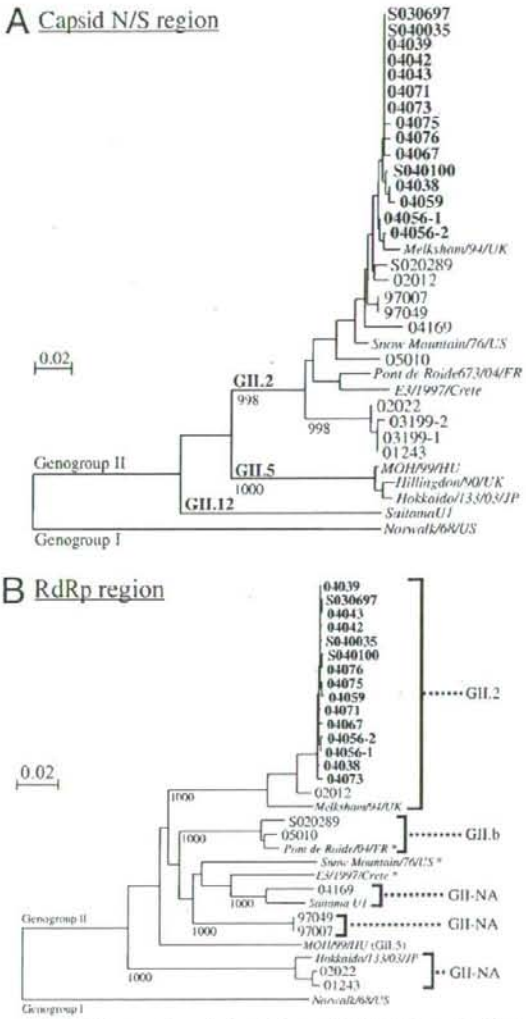

FIG. 1. Phylogenetic analysis of the capsid N/S (278 nucleotides) (A) and the partial RdRp (B) regions of the GII.2-cap strains detected in Osaka City. The GII.2-cap strains detected between March and May 2004 (04spring strains) are represented in boldface. Reference strains of NoV used in this study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