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Genetic Analysis of Noroviruses in Chiba Prefecture, Japan, between 1999 and 2004

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Noroviruses (NVs) are common pathogens that consist of genetically divergent viruses that induce gastroenteritis in humans and animals. Between September 1999 and June 2004, 1,898 samples obtained from patients showing sporadic or outbreak gastroenteritis in Chiba Prefecture, Japan, were tested for NVs by reverse transcription-PCR. NVs were detected in 603 samples. Approximately 80% were positive for genogroup GII, 13% were positive for genogroup GI, and the remaining 7% were positive for both genogroups. Phylogenetic analysis showed that the GI and GII genogroups could be further divided into 13 and 16 genotypes (including new genotypes), respectively. The GII-4 genotype, which included five small genetic clusters (subtypes), was the most common in this study and was detected in approximately 40% of positive samples. The P2 regions of 10 strains belonging to each of the five GII-4 subtypes showed 5 to 18% amino acid diversity. The amino acid substitutions accumulated in the protruding (P) region during the 5-year study period. Our data suggest that highly variable NV strains are circulating in Chiba Prefecture, with a high rate of genetic change observed during the 5-year study period.

The genus *Norovirus* is a member of the family *Caliciviridae*. Caliciviruses contain a positive-sense single-stranded RNA genome and include a further three genera, *Vesivirus*, *Lagovirus*, and *Sapovirus* (2, 3, 8). Noroviruses (NVs) have three major open reading frames (ORFs) that encode nonstructural, capsid, and minor structural proteins, respectively (8). They are one of the most common causes of gastroenteritis and have been detected in fecal samples from both humans (12, 15, 28) and animals (20, 30, 37). Human-associated NV outbreaks resulting from ingestion of contaminated water or food, such as oysters (4, 5, 18, 23), and outbreaks in public places, particularly hospitals, schools, and cruise ships (9, 11, 22, 36), pose an important public health problem.

Reverse transcription-PCR (RT-PCR) and sequencing of the partial viral genome are the most popular and useful procedures for obtaining epidemiological and genetic information on NVs. Human NVs can be divided into two genogroups, genogroups GI and GII, by genetic analysis of the RNA polymerase and capsid regions (1, 15), with several genotype classifications having been reported independently (1, 16, 33). Recently, based on the genotype classification of Katayama et al. (16), Kageyama et al. (15) reported on a detailed scheme for the genotyping of NVs based on distribution analysis by using the pairwise distance of the capsid N-terminal/shell domain. They classified the GI and GII genogroups into 14 and 17 genotypes, respectively.

During the winter of 2002–2003, an increase in NV outbreaks was reported in Europe and the United States (6, 21). Moreover, worldwide, the GII-4 genotype (Bristol virus-like genotype) has been shown to be the predominant strain of NV associated with gastroenteritis (13, 21, 34–36). Changes in the phylogenetic and genetic characteristics of GII-4 genotype strains have also been reported (9, 21).

To clarify the genetic characteristics of NV in Chiba Prefecture, Japan, we phylogenetically analyzed nucleotide sequences at the 5′ end of ORF2, which encodes the capsid protein (8), in NVs detected in Chiba Prefecture from 1999 to 2004. Furthermore, the protruding (P) region of the capsid protein from GII-4 genotype NV strains was also analyzed.

MATERIALS AND METHODS

Collection and processing of stool samples. Between September 1999 and June 2004, 732 stool samples were collected from patients (40 adults and 692 children) with sporadic gastroenteritis from seven hospitals in Chiba Prefecture, Japan. A total of 1,166 samples were also collected through 15 public health centers from patients (1,032 adults and 134 children) representing 200 gastroenteritis outbreaks (1 to 12 samples per outbreak).

Approximately 10% (wt/vol) suspensions of stool specimens in phosphate-buffered saline were prepared by centrifugation at 1,500 × *g* for 20 min. Three milliliters of the supernatants was concentrated by ultracentrifugation at 200,000 × *g* for 2.5 h by using a 50.2Ti rotor (Beckman Coulter Inc., Fullerton, Calif.), and the concentrate was then resuspended in 200 μl of distilled water. The samples were used for RNA extraction or were stored at −80°C until use.

RT-PCR and sequencing. RNA was extracted from 25 μl of concentrated sample by using a High Pure Viral RNA kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol, and then reverse transcription was performed with ReverTra Ace reverse transcriptase (TOYOBO, Osaka, Japan), according to the manufacturer's instructions. PCR was performed with the G1F1 and G1R1 primer pair for the GI strains and the G2F1 and G2R1 primer pair for the GII strains (17). For detection of genetically divergent GII strains, primer G4R2 (CCNGCTGTGAASGCRTTNCCMGC) was used in place of primer G2R1, and for amplification of the 3′ end of the NV genome, primer dT25VN [$(T)_{25}$V(A/G/C)N(A/G/C/T)] (19) was used as the reverse primer. Primer LVPF (AGTCTCYTGTCGAGTYCTCAC) and primer LVCAPEND (CCAAGGACATCAGAYGCCA) were used to analyze the P region of the GII-4 genotype. PCR products were purified with the High Pure PCR Products Purification kit (Roche Diagnostics) and were directly sequenced using the BigDye Terminator cycle sequencing kit and Genetic Analyzer 310 (Applied Biosystems, Foster City, Calif.).

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TABLE 1. NV strains analyzed in this study

Genogroup and strain name	Type	Source[a]	Accession no.
Genogroup I			
Chiba/000600/2000	GI-1	C/S	AJ865482
Chiba/030308/2003	GI-1	A/O	AJ865496
Chiba/030542/2003	GI-1	A/O	AJ865501
Chiba/040252/2004	GI-1	A/O	AJ865507
Chiba/040501/2004	GI-1	A/O	AJ865509
Chiba/010510/2001	GI-2	A/O	AJ865487
Chiba/030547G1/2003	GI-2	A/O	AJ865503
Chiba/030658/2003	GI-2	C/O	AJ865505
Chiba/000520/2000	GI-3	A/O	AJ865481
Chiba/020251/2002	GI-3	A/O	AJ865492
Chiba/030546/2003	GI-3	A/O	AJ865502
Chiba/000016G1/1999	GI-4	A/S	AJ865476
Chiba/000516/2000	GI-4	A/O	AJ865480
Chiba/010108/2001	GI-4	A/O	AJ865485
Chiba/020097/2002	GI-4	A/O	AJ865490
Chiba/020267/2002	GI-4	A/O	AJ865493
Chiba/030403/2003	GI-4	A/O	AJ865499
Chiba/040589/2004	GI-4	A/O	AJ865510
Chiba/030360/2003	GI-5	A/O	AJ865498
Chiba/040493/2004	GI-5	A/O	AJ865508
Chiba/030654/2003	GI-6	A/O	AJ865504
Chiba/991013/1999	GI-7	C/S	AJ865475
Chiba/000782/2000	GI-7	C/S	AJ865484
Chiba/020250/2002	GI-7	A/O	AJ865491
Chiba/030541/2003	GI-7	A/O	AJ865500
Chiba/000661/2000	GI-8	A/S	AJ865483
Chiba/010433/2001	GI-8	A/O	AJ865486
Chiba/030305/2003	GI-8	A/O	AJ865495
Chiba/040001/2004	GI-8	A/O	AJ865506
Chiba/020096G1/2002	GI-9	A/O	AJ865489
Chiba/000335/2000	GI-11	A/O	AJ865477
Chiba/000337/2000	GI-12	A/O	AJ865478
Chiba/020247/2002	GI-13	C/O	AJ865511
Chiba/030335/2003	GI-13	C/O	AJ865512
Chiba/020062/2002	GI-14	A/O	AJ865488
Chiba/030358/2003	GI-14	A/O	AJ865497
Chiba/030100/2003	GI-15	A/O	AJ865494
Genogroup II			
Chiba/000485/2000	GII-1	A/O	AJ865528
Chiba/010587/2001	GII-1	C/S	AJ865539
Chiba/030510/2003	GII-1	A/O	AJ865563
Chiba/021026/2002	GII-2	C/O	AJ865553
Chiba/030322/2003	GII-2	A/O	AJ865557
Chiba/030429/2003	GII-2	A/O	AJ865560
Chiba/040554/2004	GII-2	C/O	AJ865588
Chiba/991120/1999	GII-3	C/S	AJ865517
Chiba/000022/2000	GII-3	A/O	AJ865522
Chiba/000336/2000	GII-3	A/O	AJ865525
Chiba/000344/2000	GII-3	A/O	AJ865526
Chiba/010621/2001	GII-3	C/S	AJ865540
Chiba/010998/2001	GII-3	C/S	AJ865542
Chiba/020198/2002	GII-3	A/O	AJ865547
Chiba/020301/2002	GII-3	A/O	AJ865548
Chiba/021068/2002	GII-3	A/O	AJ865555
Chiba/030486/2003	GII-3	A/O	AJ865562
Chiba/030603/2003	GII-3	C/O	AJ865568
Chiba/040230/2004	GII-3	A/O	AJ865580
Chiba/990897/1999	GII-4b	C/S	AJ865513
Chiba/990900/1999	GII-4b	C/S	AJ865474
Chiba/991173/1999	GII-4b	C/S	AJ865518
Chiba/001049/2000	GII-4b	A/O	AJ865529
Chiba/010105/2001	GII-4b	A/O	AJ865532
Chiba/021071/2002	GII-4b	A/O	AJ865556
Chiba/040092/2004	GII-4b	C/S	AJ865576
Chiba/021050/2002	GII-4c	C/S	AJ865554
Chiba/030556/2003	GII-4c	A/O	AJ865567
Chiba/020984/2002	GII-4d	C/O	AJ865551
Chiba/030517/2003	GII-4d	A/O	AJ865564
Chiba/030910/2003	GII-4d	A/O	AJ865569
Chiba/040045/2004	GII-4d	A/O	AJ865575
Chiba/040096/2004	GII-4d	C/S	AJ865577
Chiba/040140/2004	GII-4d	A/O	AJ865579
Chiba/040311/2004	GII-4d	A/S	AJ865584
Chiba/021022/2002	GII-4e	C/O	AJ865552
Chiba/031038/2003	GII-4e	A/O	AJ865572
Chiba/040110/2004	GII-4e	A/O	AJ865578
Chiba/040277/2004	GII-4e	A/O	AJ865581
Chiba/991180/1999	GII-5	A/S	AJ865519
Chiba/010751/2001	GII-5	A/O	AJ865541
Chiba/020015/2002	GII-5	A/O	AJ865543
Chiba/020040/2002	GII-5	A/O	AJ865544
Chiba/030547G2/2003	GII-5	A/O	AJ865566
Chiba/040500/2004	GII-5	A/O	AJ865585
Chiba/000459/2000	GII-6	C/O	AJ865527
Chiba/991012/1999	GII-6	C/S	AJ865515
Chiba/010522/2001	GII-6	C/S	AJ865536
Chiba/030968/2003	GII-6	A/O	AJ865570
Chiba/040002/2004	GII-6	A/O	AJ865573
Chiba/040309/2004	GII-6	A/S	AJ865583
Chiba/040545/2004	GII-6	C/O	AJ865587
Chiba/010526/2001	GII-7	C/S	AJ865537
Chiba/030981/2003	GII-7	A/O	AJ865571
Chiba/010045/2001	GII-8	A/O	AJ865523
Chiba/020475/2002	GII-8	A/O	AJ865549
Chiba/030399/2003	GII-8	A/O	AJ865558
Chiba/030412/2003	GII-8	A/O	AJ865559
Chiba/990969/1999	GII-10	A/O	AJ865514
Chiba/000325/2000	GII-10	C/S	AJ865524
Chiba/010006/2001	GII-10	A/O	AJ865531
Chiba/020555/2002	GII-10	A/O	AJ865550
Chiba/010442/2001	GII-11	A/O	AJ865534
Chiba/000016G2/2000	GII-12	A/S	AJ865521
Chiba/020096G2/2002	GII-12	A/O	AJ865546
Chiba/991118/1999	GII-13	C/S	AJ865516
Chiba/010465/2001	GII-13	C/S	AJ865535
Chiba/000010/2000	GII-14	A/O	AJ865520
Chiba/010390/2001	GII-14	A/O	AJ865533
Chiba/040300/2004	GII-14	A/O	AJ865582
Chiba/030474/2003	GII-15	A/O	AJ865561
Chiba/030522/2003	GII-15	A/O	AJ865565
Chiba/040003/2004	GII-15	A/O	AJ865574
Chiba/020042/2002	GII-16	A/O	AJ865545
Chiba/040502/2004	GII-18	A/O	AJ865586

[a] Sample sources: A, adult patient; C, child patient; O, outbreak case; S, sporadic case.

Sequence analysis. The nucleotide sequences were analyzed with GENETYX-MAC software. The Clustal X multiple-alignment program (version1.83) was used for multiple alignment and analysis by the neighbor-joining method (32). Molecular distance was calculated by using the DNADIST program in the PHYLIP package (10), and the phylogenetic tree was drawn by using TreeView software (27). Predictions of the secondary structures of the proteins were made by using the PSIPRED secondary structure prediction program (24).

Nucleotide sequence accession numbers. The NV strains analyzed in this study are shown in Table 1. The nucleotide sequences determined in this study were submitted to the EMBL nucleotide database and have been assigned accession numbers AJ844469 to AJ844480 and AJ865474 to AJ865588.

RESULTS

Phylogenetic analysis of NV strains. Between September 1999 and June 2004, 1,898 fecal samples were obtained from 732 sporadic cases and 1,166 outbreak-related cases of gastro-

TABLE 2. NV positivity of stool samples collected from gastroenteritis patients between 1999 and 2004

Sample type and parameter	Sept. 1999 to Aug. 2000	Sept. 2000 to Aug. 2001	Sept. 2001 to Aug. 2002	Sept. 2002 to Aug. 2003	Sept. 2003 to June 2004	Total
Sporadic samples						
No. tested	238 (75/163)[a]	329 (6/323)	72 (17/55)	41 (1/40)	52 (3/49)	732 (102/630)
No. positive	73 (8/65)	55 (4/51)	13 (2/11)	11 (4/7)	17 (3/14)	169 (21/148)
Positivity rate (%)	30.7	16.7	18.1	26.8	32.7	23.1
Outbreak samples						
No. tested	150 (137/13) [23][b]	126 (125/1) [16]	225 (217/8) [45]	352 (294/58) [60]	313 (268/45) [56]	1,166 (1,041/125) [200]
No. positive	67 (65/2) [15]	74 (73/1) [13]	60 (57/3) [22]	90 (63/27) [27]	143 (119/24) [39]	434 (377/57) [116]
Positivity rate (%)	44.7 [65.2]	58.7 [81.3]	26.7 [48.9]	25.6 [45.0]	45.7 [69.6]	37.2 [58.0]

[a] Values in parentheses show the number of samples obtained from adults/number of samples obtained from children.
[b] Values in brackets show the number of outbreak incidents.

enteritis (Table 2). Of the 732 samples obtained from sporadic cases, 169 (23.1%) were shown to be positive for NV by RT-PCR, and of the 1,166 samples obtained from 200 outbreaks, 434 (37.2%) samples from 116 outbreaks (58.0%) were positive. The rates of detection of NVs in 94 outbreaks from which two or more samples were obtained were 11 to 100% (average, 67%). During the study period, three group A rotavirus-associated outbreaks and one adenovirus-associated outbreak were observed.

NV-positive samples represented 115 GI genotypes and 513 GI genotypes. GII strains included approximately 80% of the total positive samples. All positive samples underwent direct nucleotide sequencing and were phylogenetically analyzed based on approximately 240 bp from the nucleotide sequence of the 5′ end of ORF2. The phylogenetic trees of the strains analyzed, selected by differences in the detection period and nucleotide sequences, were constructed as shown in Fig. 1. Genotype clusters were consistent with those reported by Kageyama et al. (15). GI- and GII-positive samples were classified into 13 and 16 genetic clusters, respectively, and two possible new genotypes (genotypes GI-15 and GII-18) were identified in both genogroups (Fig. 1). The results of genotype analysis in each study year are summarized in Tables 3 and 4. Of the 116 NV-positive outbreaks, GI and GII strains were identified in 34 (29.3%) and 107 (92.2%) samples, respectively. In addition, in 34 outbreaks, two or more genotypes and/or genogroups were detected in samples obtained from a single outbreak. In 53 of 55 (96.4%) outbreaks in which strains of a single genotype were detected, the nucleotide sequences of the strains were identical. Single nucleotide substitutions were observed in strains from only two outbreaks (outbreaks A and B). In outbreak A, three different nucleotide sequences [A, G or R (A+G)] were observed at the same position, and therefore, it was suggested that this outbreak was caused by at least two different strains. In outbreak B, the sequences in eight of nine samples were identical, but the sequence of the remaining sample had a nucleotide substitution of A to G, which was probably generated in this outbreak. The nucleotide changes described above were accompanied by amino acid substitutions. No significant differences were observed between the genotypes detected from sporadic cases and those detected from outbreaks (Fig. 1; Tables 3 and 4).

Regardless of whether samples were from an outbreak case or a sporadic case, the dominant genotype was GII-4; GII-4 was detected in 224 samples (47.6% of the GII-positive samples and 35.7% of the total positive samples). The GII-3, GII-4, and GII-5 genotypes were detected throughout the study period, while the other genotypes were detected intermittently. Strains representing the GI-9, GI-11, GI-12, GI-15, GII-16, and GII-18 genotypes were detected during only one period within the 5-year study period. The dominant genotypes each year were not consistent between the outbreak and the sporadic cases.

Two new strains, Chiba/030100/2003 and Chiba/040502/2004, formed genetic clusters clearly separate from previously identified genotypes (Fig. 1). The nucleotide sequences of these strains showed low levels of identity in a BLAST search of the sequences of all strains except for the identity between the sequences of Chiba/030100/2003 and NLV/IF2036/2003/Iraq (EMBL database accession number AY675555; 95% nucleotide identity). Chiba/030100/2003 and Chiba/040502/2004 were therefore tentatively classified as putative new genotypes GI-15 and GII-18, respectively.

New genetic clusters. Recent genotypic classification of NVs showed 14 and 17 genotypes within the GI and GII genogroups, respectively (15). In this study, we identified possible new genetic clusters in GI and GII (Fig. 1A and B). To characterize these NV strains, we amplified and sequenced the region between the starting codon of ORF2 and the 3′ end of the NV genome. The new GI strain, Chiba/030100/2003/JP, had 2,383 nucleotides, while the new GII strain, Chiba/040502/2004/JP, had 2,472 nucleotides. Two ORFs, corresponding to ORF2 and ORF3 of NVs, were also identified. A similarity search was performed by using the World Wide Web-based FASTA program of the DDBJ DNA database, which revealed that NLV/IF2036/2003/Iraq showed 93.5% nucleotide identity to Chiba/030100/2003/JP; however, the other NV strains showed less than 70% nucleotide identity. Detailed information on the NLV/IF2036/2003/Iraq strain is not available. No strain with more than 70% nucleotide identity to the Chiba/040502/2004/JP sequence was found in the DNA database. The results of genetic analysis therefore seem to confirm that these strains are new genotypes (genotypes GI-15 and GII-18, respectively).

Genetic transition of the GII-4 genotype. In this study, a total of 224 GII-4 genotype strains were analyzed. Of these, 94 were detected from sporadic cases and 150 were detected from 41 outbreaks. Phylogenetic analysis showed that the GII-4 cluster could be further divided into five small clusters (temporarily called subtypes), subtypes GII-4a to GII-4e (Fig. 1B and

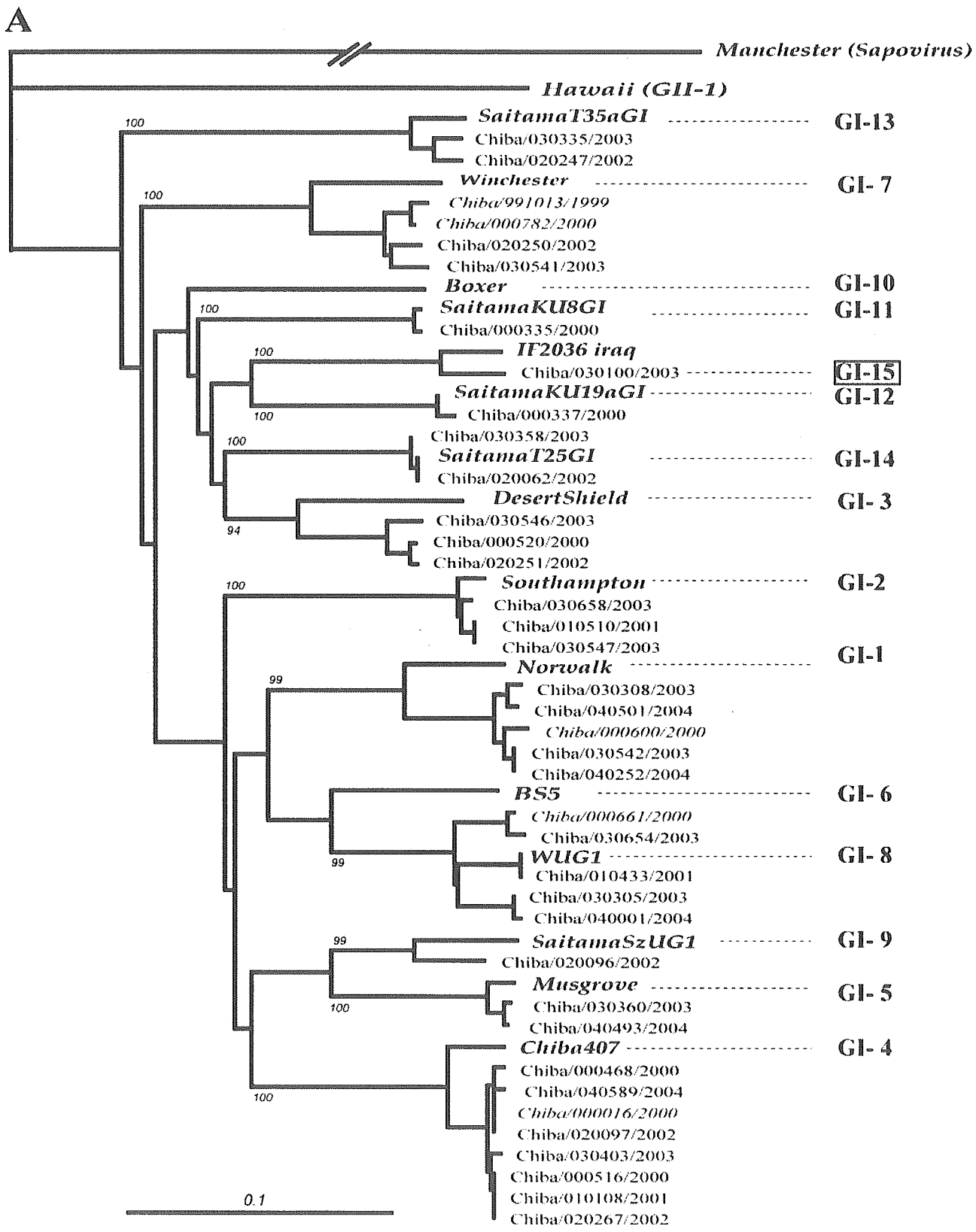

FIG. 1. Phylogenetic tree of selected norovirus strains analyzed in this study. (A) Genogroup I; (B) genogroup II. Trees were reconstructed with partial nucleotide sequences of the 5′ end of ORF2 and by using Manchester virus as the outgroup. The numbers on each branch indicate the bootstrap values of the clusters supported by that branch. Genotype classification was based on the recent scheme of Kageyama et al. (15). The accession numbers of the reference strains are included in Tables 3 and 4. The accession numbers of additional reference strains are as follows: Camberwell virus, AF145896; Grimsby virus, AJ004864; and Oxford B5S9 strain, AY588016. GI-15 and GII-18 (boxed) are the new genotypes identified in this study. The tentative subtypes observed in the GII-4 genotype are also indicated.

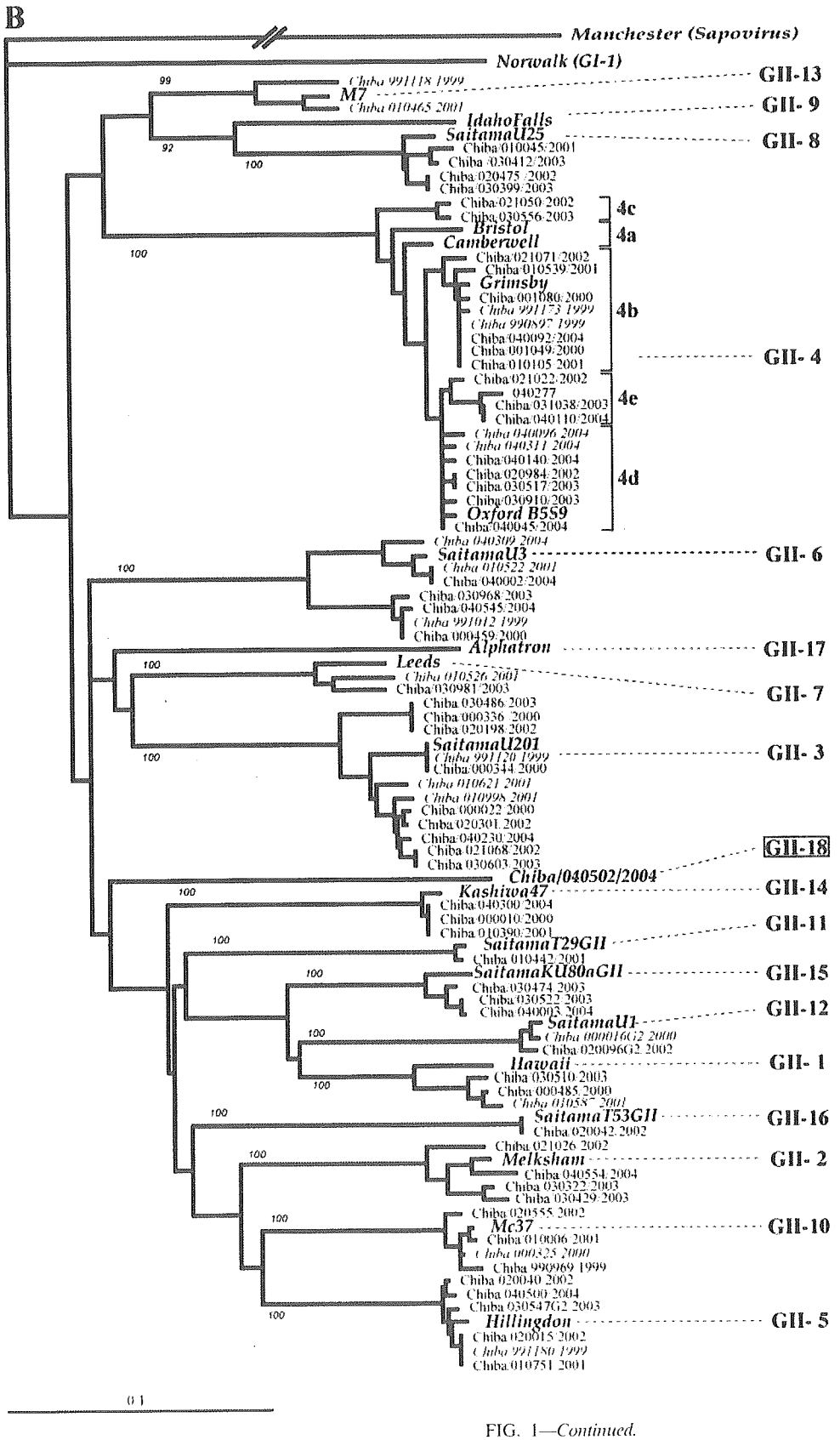

FIG. 1—*Continued.*

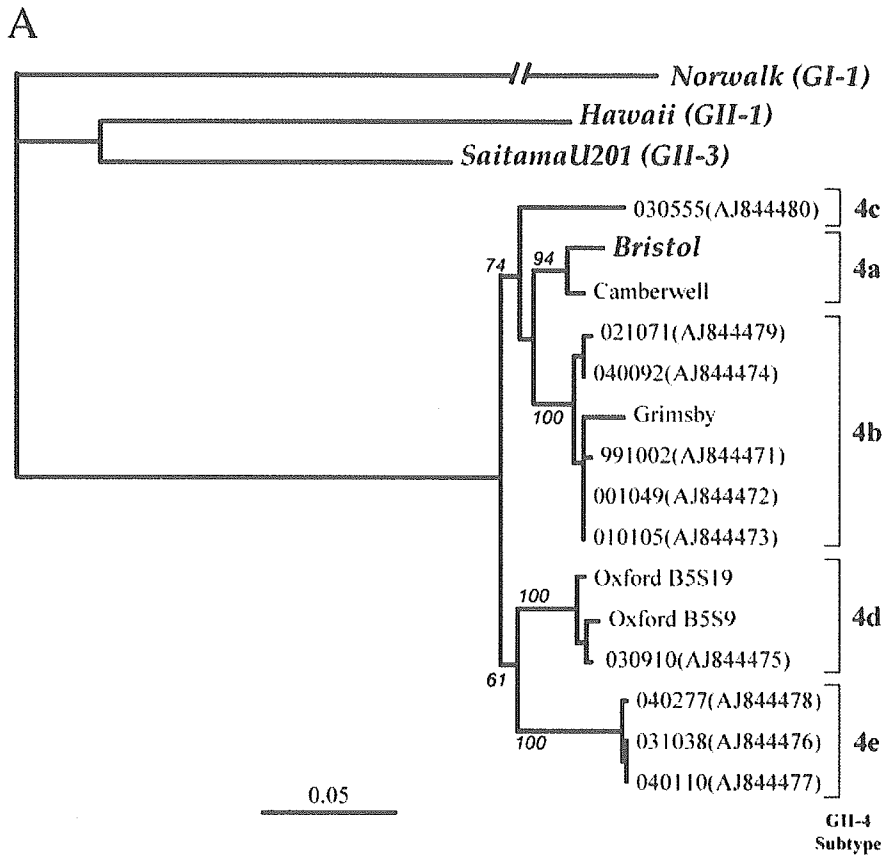

FIG. 2. Genetic analysis of the P region of the capsid protein in the GII-4 strains. (A) Phylogenetic tree of the GII-4 NV strains reconstructed by the neighbor-joining method by using the amino acid sequences of the P region of the capsid protein. The numbers on each branch indicate the bootstrap values of the clusters supported by that branch. The accession numbers of additional reference strains are as follows: Camberwell virus, AF145896; Grimsby virus, AJ004864; Oxford B5S9, AY588016; and Oxford B5S19, AY588019. (B) Amino acid alignment of the P region; the P2 region is shown in gray. The solid box (I to IV) shows the putative sites responsible for the binding pocket to histo-blood group antigens (32). The asterisk indicates the position of the single amino acid insertion in the P2 region.

2). Subtype GII-4a included the prototype strain, Bristol virus; GII-4b included strains detected between 1999 and 2002 with the Grimsby virus, and one strain, 040092, detected in 2004; GII-4c included four strains obtained from one sporadic case in 2002 and three strains from a single outbreak in 2003; GII-4d included strains detected between 2002 and 2004; and GII-4e included strains detected between 2003 and 2004.

For verification of these clusters and characterization of each subtype, we analyzed the nucleotide sequences of the P regions of 10 strains belonging to each GII-4 cluster. The range of the P region was determined as described by Chen et al. (7). The resultant phylogenetic tree and amino acid alignment of the P region are shown in Fig. 2. Genetic clustering into five subtypes was supported by the high bootstrap value of each branch. The amino acid sequence diversity of the P region among these subtypes was 1 to 11% (0 to 18% diversity in the P2 region). The predicted secondary structures of the P region of each subtype are shown in Fig. 3. The predicted helix structures within the P2 region varied among the subtypes. An additional helix structure positioned at amino acid 64 was observed in subtypes GII-4a and GII-4c, and one positioned at amino acid 110 was observed only in subtype GII-4b. No additional predicted helix structures within the P2 region were observed in subtypes GII-4d and GII-4e. One of the four sites reportedly corresponding to the putative histo-blood group antigen binding pocket (31) had amino acid substitutions (Q to E in site IV; Fig. 2B) in the GII-4d and GII-4e subtypes. These subtypes also shared a single amino acid insertion at the same position within the P2 region. Amino acid substitutions converged in the P2 region and accumulated with time. These results show that the GII-4 genotype rapidly evolved and shifted genetically between 2002 and 2003.

DISCUSSION

This study genetically analyzed NV strains detected in samples from sporadic cases and outbreaks of gastroenteritis. Of 1,898 samples, 603 (31.8%) were positive for NV; GII strains represented 81.3% of these positive samples. NVs were detected in samples obtained from 58% of the outbreaks, with detection rates varying between 11 and 100% in each outbreak. NVs were detected at low rates in some outbreaks, not all of which were caused by NV; however, despite this, no other viral or bacterial pathogens were detected in almost all outbreaks.

B

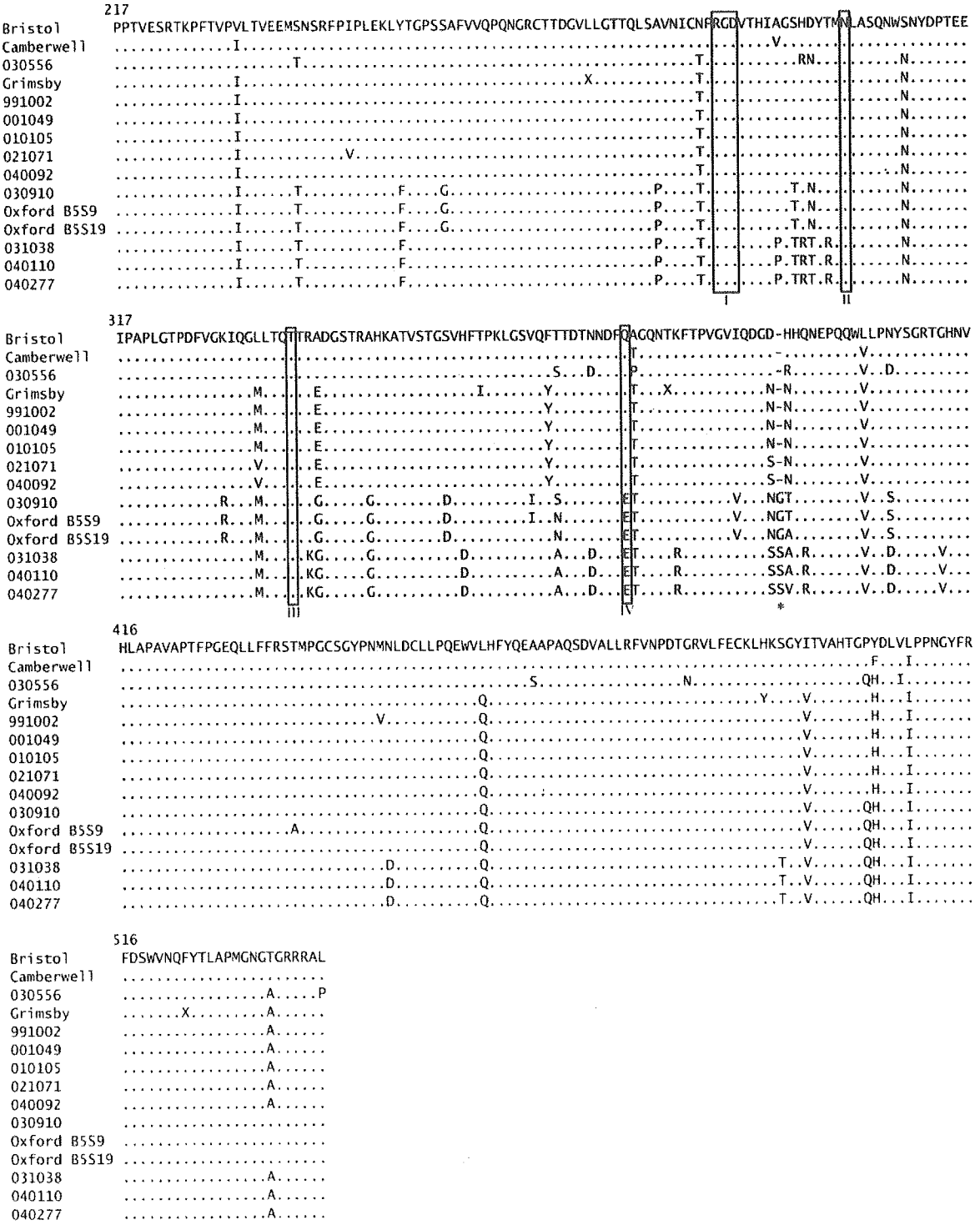

FIG. 2—*Continued.*

TABLE 3. Results of genotype analysis of genogroup I NV strains detected in Chiba Prefecture between 1999 and 2004

Type	Prototype strain	Accession no.[a]	1999–2000		2000–2001		2001–2002		2002–2003		2003–2004		Total	
			OB[b]	SP[c]	OB	SP	OB	SP	OB	SP	OB	SP	OB	SP
GI-1	Norwalk	M87661		1					2 (2)	1	13 (5)		15 (7)	2
GI-2	Southampton	L07418			9 (3)	5			7 (2)				16 (5)	5
GI-3	DesertShield	U04469	9 (2)[d]	2	2 (2)	1	2 (2)		1 (1)				14 (7)	3
GI-4	Chiba407	AB042808	4 (4)	3	9 (3)		5 (4)				3 (1)		21 (12)	3
GI-5	Musgrove	AJ277614							1 (1)		1 (1)		2 (2)	
GI-6	BS-5	AF093797												
GI-7	Winchester	AJ277609		2			3 (2)		1 (1)				4 (3)	2
GI-8	WUG1	AB081723	1 (1)		5 (2)	2			6 (3)		1 (1)		13 (7)	2
GI-9	Saitama SzUG1	AB039774					1 (1)						1 (1)	
GI-10	Boxer	AF538679												
GI-11	Saitama KU8GI	AB058547	3 (1)										3 (1)	
GI-12	Saitama KU19aGI	AB058525	1 (1)										1 (1)	
GI-13	Saitama T35aGI	AB112132					1 (1)		4 (1)				5 (2)	
GI-14	Saitama T25GI	AB112100					1 (1)		1 (1)				2 (2)	
GI-15	Chiba/030100/2003	AJ844469								1				1
Total			18	8	25	8	13	0	23	2	18	0	97	18

[a] Accession numbers of the reference strains used in this study.
[b] OB, outbreak samples.
[c] SP, sporadic samples.
[d] Values in parentheses show the number of outbreak incidents in which each genotype was detected.

A number of epidemiological reports on NV infection have shown that the GII genogroup is the predominant agent of NV-associated gastroenteritis (11, 14, 29, 36). In this study, GII strains were also predominant in both sporadic cases and outbreaks. The dominant GI genotype changed each year; therefore, no tendency with regard to the predominant GI genotype was found within the study period. However, in the GII genogroup, three predominant genotypes, GII-3, GII-4, and GII-5, were identified. These genotypes were detected each year during the study period and represented approximately 70% of the GII-positive samples. The GII-4 genotype was especially dominant throughout the 5-year study period, except in 2001 and 2002. Of the 33 known NV genotypes, 29 were identified in this study; GI-6, GI-10, GII-9, and GII-17 were not identified. These data show that most genotypes exist in Japan, inducing NV-associated outbreaks and sporadic gastroenteritis. Detection of variable strains of NV within the Japanese population and in Japanese oysters has also been reported (14, 15, 26).

In this study, possible new genotypes in the GI and GII genogroups were identified. Kageyama et al. (15) described

TABLE 4. Results of genotype analysis of genogroup II NV strains detected in Chiba Prefecture between 1999 and 2004

Type	Prototype strain	Accession no.[a]	1999–2000		2000–2001		2001–2002		2002–2003		2003–2004		Total	
			OB[b]	SP[c]	OB	SP	OB	SP	OB	SP	OB	SP	OB	SP
GII-1	Hawaii	U07611	8 (2)[d]						5 (1)				13 (3)	
GII-2	Merksham	X81879					1 (1)		15 (6)		10 (1)		26 (8)	
GII-3	Saitama U201	AB067542	22 (6)	9	2 (2)	6	13 (5)	4	20 (8)		6 (4)	4	63 (25)	23
GII-4	Bristol	X76716	13 (3)	39	27 (9)	32	6 (2)	3	17 (4)	8	87 (23)	12	150 (41)	94
GII-5	Hillington	AJ277607	1 (1)	2	7 (3)	2	13 (6)	6	7 (2)		2 (2)		30 (14)	10
GII-6	Saitama U3	AB039776		1		2	9 (1)				16 (4)	1	25 (5)	4
GII-7	Leeds	AJ277608		1		3					4 (2)		4 (2)	4
GII-8	Saitama U25	AB067543	1 (1)		3 (1)		5 (2)		6 (3)				15 (7)	
GII-9	Idaho Fall	AY054299												
GII-10	Mc37	AY237415	7 (2)	11	3 (1)		3 (2)						13 (5)	11
GII-11	Saitama T29GII	AB112221			2 (1)						1 (1)		3 (2)	
GII-12	Saitama U1	AB039775		1	5 (1)		1 (1)						6 (2)	1
GII-13	M7	AY130761		1		2								3
GII-14	Kashiwa	AB078334	1 (1)	1	2 (1)						1 (1)		4 (3)	1
GII-15	Saitama KU80a	AB058582							4 (3)	1	1 (1)		5 (4)	1
GII-16	Saitama T53GII	AB112260					2 (2)						2 (2)	
GII-17	Alphatron	AF195847												
GII-18	Chiba/040502/2004	AJ844470									2 (1)		2 (1)	
Total			53	66	51	47	53	13	74	9	130	17	361	152

[a] Accession numbers of the reference strains used in this study.
[b] OB, outbreak samples.
[c] SP, sporadic samples.
[d] Values in parentheses show the number of outbreak incidents in which each genotype was detected.

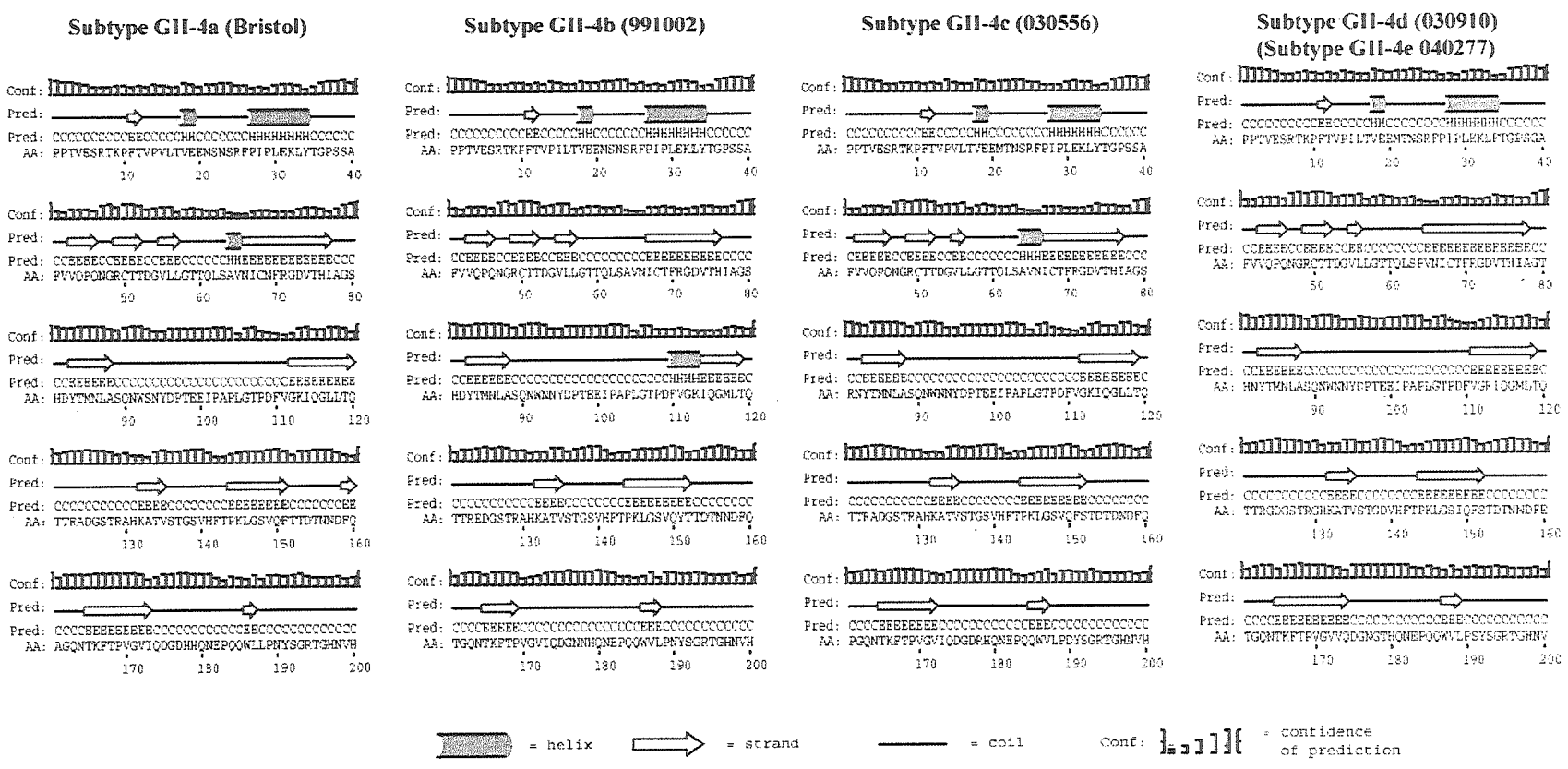

FIG. 3. Schematic representation of the secondary structure predictions of deduced proteins from isolates belonging to each GII-4 subtype, showing the helix structure pattern in the P2 region. Each panel shows a partial P region (amino acids 217 to 416 of Bristol virus) of the capsid protein.

strains as different genotypes if they showed pairwise distances in the N-terminal/shell domain of the capsid protein of more than 0.121 for the GI genogroup strains and 0.117 for the GII genogroup strains. The pairwise nucleotide distance of the N-terminal/shell domain of the capsid protein between these new strains and strains of the nearest genotype was 0.176 between Chiba/030100/2003/JP and NV/SaitamaKU19aGI/00/JP (EMBL accession number AB058525), which was used as a reference strain of GI-12, and 0.322 between Chiba/040502/2004/JP and Hu/NLV/Alphatron/98-2/1998/NET (EMBL accession number AF195847), which was used as a reference strain of GII-17. Accordingly, the two strains described in this paper, Chiba/030100/2003/JP (GI) and Chiba/040502/2004/JP (GII), were classified as new genotypes, GI-15 and GII-18, respectively.

The dominant genotype throughout the study period was GII-4, which, according to phylogenetic and genetic analyses, could also be further divided into five subtypes (subtypes GII-4a to GII-4e); they were also shown to have shifted genetically each year. The emergence of genetic variants of the GII-4 genotype was previously reported based on analysis of the RNA polymerase-coding region (21). Recently, Dingle et al. (9) analyzed 49 GII-4 strains and classified them into three subtypes with regard to the year of detection (1987 to 1994, 1995 to 2001, and 2002 to 2003, respectively) and based on the nucleotide sequences of the capsid region; these genetic clusters correspond to GII-4a, GII-4b, and GII-4d, respectively. The two additional subtypes observed here were identified in samples obtained in 2003 and 2004. As shown by Dingle et al. (9), we also identified a single amino acid insertion in subtypes GII-4d and GII-4e and an accumulation of amino acid substitutions in the P2 region. The predicted secondary structure of the P region differed among the subtypes. Four sites in the P2 region comprising the putative binding pocket of the histo-blood group antigen were reported by Tan et al. (31). In this study, we identified an amino acid substitution (Q to E) in subtypes GII-4d and GII-4e at site IV, which is involved with binding specificity to the histo-blood group antigen. This mutation and the accumulation of amino acid substitutions within the P2 region might induce changes in binding specificity to the histo-blood group antigen and in viral antigenicity.

Recently, the in vivo evolution of NV in an immunosuppressed patient was reported by Nilsson et al. (25). They reported an accumulation of amino acid substitutions in the P2 region within 1 year and also discussed the predicted structural changes that occurred in the P region. Unfortunately, human NVs cannot be propagated in vitro, and no animal infection model is available at present; consequently, evolutionary studies of NV genes are very difficult. Our results obtained from analyses of genetic changes in strains detected locally during a sequential period are therefore considered useful.

In conclusion, this study showed that NV strains with various genotypes are cocirculating in Chiba Prefecture, Japan, and revealed a pattern of viral evolution in the P2 region of the GII-4 strains. Three predominant genotypes, GII-3, GII-4, and GII-5, were detected each year and included approximately 70% of the strains identified; the remaining genotypes were detected only intermittently. The reasons for these results are unknown; however, environmental factors, host immunity to the viral genotype, and the mode of transmission of each virus might influence the epidemic spread of NV. In addition, we identified genetic changes among GII-4 genotype strains in the P region of the capsid protein. These changes are induced by repeated infections among human populations and are considered to have accumulated to escape the pressure of immunity. These data suggest a high rate of evolution in the NV capsid gene, highlighting the need for further studies on the genetic epidemiology and evolution of NVs for effective control.

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Editor-Communicated Paper

Genotyping of *Norovirus* Strains Detected in Outbreaks between April 2002 and March 2003 in Osaka City, Japan

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***Abstract*: *Noroviruses* (NVs) are the major cause of food- and waterborne nonbacterial gastroenteritis in Japan. Between April 2002 and March 2003, a total of 111 fecal specimens from 40 outbreaks of acute nonbacterial gastroenteritis in Osaka City, Japan were subject to NV detection. Seventy-two samples (64.9%) from 31 outbreaks (77.5%) were NV positive by a real time reverse transcription (RT)-PCR assay. To further determine the genotype of individual NV strains, we sequenced the capsid N-terminal/shell (N/S) domain of some representative strains from each outbreak. The 51 NV strains detected in this study were segregated into 15 genotypes (6 in genogroup I and 9 in genogroup II), and GII/5 genotype NV was a dominant outbreak genotype.**

***Key words*: *Norovirus*, N/S domain, Genotype, Epidemiology**

Norovirus (NV) is a genus within the family *Caliciviridae* (www.ictvdb.iacr.ac.uk/Ictv), which has been previously termed Norwalk-like virus or small round structured virus. The NV prototype strain, Norwalk/68/US, has been entirely sequenced from cDNA clones derived from stool specimens, and its genome is a single-stranded, positive-sense RNA molecule of 7.5 kb that comprises three open reading frames (ORFs) (17, 19). NVs are the major cause of acute nonbacterial gastroenteritis worldwide, and illness occurs in people of all ages. NVs are transmitted not only by a fecal-oral route but also by direct person-to-person contact (11). There have been numerous outbreaks due to NV-contaminated foods, such as shellfish, salads, and deli sandwiches (6, 8, 12, 13), and due to NV-contaminated water (5, 26).

Since NVs have not yet been cultivated *in vitro*, electron microscopy (EM) or immuno-EM had been routinely used to detect NV particles in stool specimens in the laboratory. After the cloning and sequencing of Norwalk/68/US (17) and Southampton/91/UK (27), a reverse transcription-PCR (RT-PCR) assay was developed to target the RNA-dependent RNA polymerase gene in ORF1 of the NV genome (18, 28). Using sequence information of additional NV strains, different primer sets targeting the polymerase region have been used for the diagnosis of NV in fecal specimens from both outbreaks and sporadic cases (2, 9, 34, 35). Based on the sequence information obtained from the polymerase region, the NV strains can be divided into two genogroups, genogroup I (GI) and genogroup II (GII), each comprising a large number of genetically diverse strains (1, 10, 30).

A classification system has been proposed for NVs, in which the sequence of ORF2 (the gene for the major capsid protein) of the strains is compared with that of reference strains (3, 11, 22, 33). A recent study indicated that NV GI and GII strains consist of at least 14 and

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Abbreviations: EM, electron microscopy; G, genogroup; N/S, N-terminal/shell; NV, *Norovirus*; ORF, open reading frame; RT-PCR, reverse-transcription polymerase chain reaction.

17 genotypes, respectively (21). ORF2 encodes the major structural capsid protein, including a shell domain (S) and a protruding (P) domain (31). Several reports suggested a good correlation between the clustering NV strains by the sequence of the 5′ end of ORF2 and antigenic grouping confirmed by patient immune response against NVs (4, 23, 24, 30). Therefore, it may be possible to predict antigenic types by phylogenetic analysis of the capsid region.

Previously, we described classification of NV strains detected in Osaka City, Japan by probe types, based on hybridization of the amplified viral genomes with six NV-specific DNA probes (13–15). However, we often came across NV strains to which none the probes would hybridize, making their classification difficult. In this report, we describe the characterization of the NVs in fecal specimens from 31 nonbacterial gastroenteritis outbreaks occurring in Osaka City, Japan, between April 2002 and March 2003.

Materials and Methods

Outbreaks and specimens. Fecal specimens were collected from 40 outbreaks of acute nonbacterial gastroenteritis, including 22 outbreaks associated with oysters, in Osaka City, Japan, between April 2002 and March 2003. A total of 111 fecal specimens were examined by real time RT-PCR.

RNA extraction. A 10% stool suspension was prepared as described previously (13). Viral RNA was extracted from 140 μl of the suspension with a QIAamp viral RNA Mini kit (Qiagen, Valencia, Calif., U.S.A.) according to the manufacturer's instructions. RNA was eluted with 60 μl of diethyl pyrocarbonate-treated water and kept at −80 C until use in RT-PCR.

Real time RT-PCR. Real time RT-PCR was carried out as described by Kageyama et al. (20). Viral RNA (15 μl) was added to 15 μl of the mixture containing 100 mM Tris-HCl (pH 8.3), 150 mM KCl, 6 mM $MgCl_2$, 1 mM of each deoxynucleoside triphosphate, 10 mM dithiothreitol, 75 pmol of random hexamer (pdN6; Amersham Pharmacia Biotech, Piscataway, N.J., U.S.A.), 30 U of RNase inhibitor (TaKaRa Shuzo, Kyoto, Japan), and 7.5 U of avian myeloblastosis virus reverse transcriptase XL (Life Science Inc., St. Petersburg, Fla., U.S.A.). RT was performed at 42 C for 60 min, and the enzyme was inactivated at 70 C for 15 min. cDNA was stored at −20 C.

The real time quantitative PCR was carried out in 50 μl reactions containing 4 μl of cDNA, 25 μl of *Taq*Man Universal PCR Master Mix (Applied Biosystems, Branchburg, N.J., U.S.A.), 5 pmol each of primers COG1F and COG1R and 15 pmol of RING1 (a)-*Taq*Man probe (TP) and 5 pmol RING1 (b)-TP fluorogenic probe for GI NV detection, or 5 pmol each of primers COG2F and COG2R and 5 pmol RING2-TP for GII NV detection. PCR amplification was performed with an ABI7700 sequence detector (Applied Biosystems) under the following conditions: 2 min at 50 C and 10 min at 95 C, then 40 cycles of 95 C for 15 sec and 56 C for 1 min. Data were corrected by using internal standards as described by Kageyama et al. (20).

Sequencing of the N/S domain. When more than 2 samples from an outbreak appeared to be positive by real time RT-PCR, several samples, including these 2, were selected for sequencing analysis. To amplify the N-terminal/shell (N/S) domain of the capsid region, PCR was carried out with primers G1SKF and G1SKR for GI NV strains or G2SKF and G2SKR for GII NV strains as described by Kojima et al. (25). After purification of the amplicon with a QIAquick PCR purification kit (Qiagen), the nucleotide sequences were determined with the BigDye Terminator Cycle sequence kit and ABI 310 sequencer (Applied Biosystems).

Phylogenetic analysis. Capsid sequences of the reference strains of NV were obtained from GenBank. These strains and accession numbers are shown in Table 1. Phylogenetic analysis was performed as described by Katayama et al. (22). Briefly, the sequences of N/S domain (GI, 290 nt; GII, 278 nt) were aligned by using Clustal X (version 1.81) with parameters provided in Clustal W1.6. A phylogenetic tree was constructed by the neighbor-joining method, and the genetic distances were calculated according to the Kimura two-parameter method. Reliability of the tree was estimated by 1,000 bootstrap replications, and bootstrap values of 950 or higher were considered statistically significant for the grouping (7).

Probe typing. The polymerase region was amplified by RT-PCR, using SR primers (G1 sets: SR33, SR48, SR50, and SR52; G2 sets: SR33, SR46, OC0281B) as described in Ando et al. (1). PCR products were analyzed by Southern hybridization with probes P1-A, P1-B, P2-A, P2-B, SOV, and 96065 (13), and strains were classified according to the probe to which they hybridized.

Nucleotide sequences and accession numbers. The nucleotide sequences of the N/S shell domains determined in this study were submitted to DNA Databank of Japan with the accession numbers AB186057 to AB186107.

Results

Diagnosis and Epidemiology of NV Associated Outbreaks

Fecal samples from 40 outbreaks of acute nonbacte-

Table 1. Reference strains and genetic classification of NVs

Genotype[a]	Reference strains (GenBank accession no.)	Genetic classification			
		Katayama[b]	Vinje[c]	Ando[d]	Green[e]
GI/1	Norwalk/68/US (M87661)	GI/1	GI/1	GI/1	GI/1
GI/2	Southampton/91/UK (L07418) Whiterose/96/UK (AJ277610)	GI/4	GI/2	GI/2	GI/2
GI/3	DesertShield/90/SA (U04469) Birmingham/93/UK (AJ277612) LittleRocks/94/US (AF414405) Stav/95/NO (AF145709)	GI/9	GI/3	GI/3A GI/3B	GI/3
GI/4	Chiba/87/JP (AB022679) Valetta/95/MA (AJ277616)	GI/7	GI/4	GI/4	GI/4
GI/5	Musgrove/89/UK (AJ277614) AppalachicolaBay/95/US (AF414406) KU83aGI/99/JP (AB058545)	GI/6	GI/6	GI/5	GI/5
GI/6	BS5/97/GE (AF093797)	GI/2	NA[f]	NA	GI/6
GI/7	Winchester/94/UK (AJ277809)	GI/8	GI/5	NA	GI/7
GI/8	Sindlesham/95/UK (AJ277615) WUG1/00/JP (AB081723)	GI/3	GI/7	NA	GI/6
GI/9	SzUG1/99/JP (AB039774)	GI/5	NA	NA	NA
GII/1	Hawaii/71/US (U07611) Girlington/93/UK (AJ277606)	GII/7	GII/3	GII/1A	GII/1
GII/2	Melksham/94/UK (X81879) SnowMountain/76/US (U70059, U75682)	GII/4	GII/4	GII/2	GII/2
GII/3	Toronto/TV24/91/CA (U02030) Mexico/89/MX (U22498) Arg320/95/AR (AF190817)	GII/2	GII/1	GII/3	GII/3
GII/4	Bristol/93/UK (X76716) Lordsdale/93/UK (X86557)	GII/1	GII/2	GII/4	GII/4
GII/5	Hillingdon/90/UK (AJ277607) WhiteRiver/290/94/US (AF414423)	GII/5	GII/5	GII/5	GII/5
GII/6	Seacroft/90/UK (AJ277620) Miami/292/94/US (AF414410)	GII/8	GII/7	GII/6	GII/6
GII/7	Leeds/90/UK (AJ277608) Gwynedd/273/94/US (AF414409)	GII/3	GII/6	GII/7	GII/7
GII/8	Amsterdam/98/NL (AF195848)	GII/10	NA	GII/8	NA
GII/9	Idaho Falls/378/1996/US (AY054299) VA9207/97/US (AY038599)	NA	NA	GII/9	NA
GII/10	Erfurt/00/DE (AF427118) KU5GII/00/JP (AB058575)	NA	NA	NA	NA
GII/12	Chitta/96/JP (AB032758) Wortley/90/UK (AJ277618)	GII/6	GII/8	GII/1B	GII/1
GII/14	Fayetteville/1998/US (AY113106) Kashiwa47/00/JP (AB078334)	NA	NA	NA	NA
GII/15	Saitama KU82GII/99/JP (AB058588)	NA	NA	NA	NA
GII/17	Alphatron/98/NL (AF195847) Fort Lauderdale/560/98/US (AF414426)	GII/9	NA	NA	NA

[a] Kageyama et al. (21).
[b] Katayama et al. (22).
[c] Vinje et al. (33).
[d] Ando et al. (3).
[e] Green et al. (11).
[f] Not assigned.

Table 2. Description of outbreaks in which NVs were detected in Osaka City, Japan, between April 2002 and March 2003

Outbreak no.	Mo/yr	Source	Attack rate ill/risk	No. of specimens	No. of NV-positive	Probe type	Genotype
02065	Apr/02	UK[a]	2/5	2	1 (GI)	P1A	GI/7
02172	Sep/02	Oyster	5/28	5	5 (GI, GII)	P1A	ND[b]
						P2B	GII/5, GII/3, GII/12
02189	Nov/02	UK	2/3	2	2 (GII)	P2B	GII/12
02198	Dec/02	UK	UK/25	3	3 (GII)	P2B	GII/4
02202	Dec/02	UK	4/5	1	1 (GII)	P2B	GII/4
03006	Jan/03	Oyster	1/1	1	1 (GII)	P2B	GII/3
03008	Jan/03	Oyster	28/35	1	1 (GII)	—	GII/5
03009	Jan/03	Oyster	3/3	2	2 (GI, GII)	P1A	GI/7
						P1B	ND
						P2B	GII/5
03011	Jan/03	Oyster	1/1	1	1 (GI)	—	GI/7
03012	Jan/03	Oyster	77/295	12	9 (G1, GII)	—	GI/7
						P1B	ND
						P2B	GII/5, GII/3, GII/15
03017	Jan/03	UK	10/13	9	8 (GII)	P1B	GII/6
03020	Feb/03	Oyster	3/3	1	1 (GI)	P1A	GI/4
						SOV	ND
03021	Feb/03	Oyster	2/2	2	2 (GII)	P2B	GII/3, GII/1
03022	Feb/03	Oyster	5/5	4	4 (GI, GII)	—	GI/7
						SOV	GI/2
						P2B	GII/3, GII/15
03024	Feb/03	UK	6/15	5	3 (GII)	P2B	GII/5
03026	Feb/03	Oyster	26/331	2	2 (GI, GII)	P1A	GI/4
						SOV	ND
						P2B	GII/5
03027	Feb/03	Oyster	2/2	1	1 (GII)	—	GII/5
03028	Feb/03	Oyster	3/3	1	1 (GII)	P2B	GII/5
03034	Feb/03	Oyster	3/3	3	3 (GI, GII)	P1A	GI/7, GI/4
						SOV	ND
						UT[c]/GI	GI/8
						P2B	GII/5, GII/3
03035	Feb/03	Oyster	6/6	2	1 (GII)	P2B	GII/15
03036	Feb/03	Oyster	3/5	1	1 (GII)	P2B	GII/3
03037	Feb/03	UK	3/15	3	2 (GI)	P1A	GI/9
03039	Feb/03	Oyster	5/9	4	3 (GI, GII)	P1A	GI/4
						P2B	GII/14, GII/8
03040	Mar/03	UK	5/9	4	2 (GII)	P2B	GII/8
03042	Mar/03	UK	20/47	1	1 (GII)	—	GII/8
03047	Mar/03	UK	2/8	1	1 (GI, GII)	P1A	GI/1
						P2B	GII/8
03048	Mar/03	UK	UK	1	1 (GII)	—	GII/6
03050	Mar/03	UK	27/62	7	4 (GII)	P2B	GII/15
03053	Mar/03	UK	11/50	3	1 (GII)	P2B	GII/3
03054	Mar/03	Oyster	3/6	2	2 (GII)	P2B	GII/5
03055	Mar/03	UK	157/283	2	2 (GII)	P1B	GII/6

[a] Unknown.
[b] Not determined.
[c] Untype.

rial gastroenteritis were tested for NV by real time RT-PCR. Seventy-two of 111 fecal specimens (64.9%) from 31 outbreaks (77.5%) were positive for NV (Table 2). GII NV was detected from 28 outbreaks, including 8 outbreaks also positive for GI. The 31 NV-positive outbreaks occurred in different settings, including restaurant, party, hotel, and home. The most common viral transmission mode in these outbreaks was ingestion of

contaminated oysters (54.8%). The NV-positive gastroenteritis outbreaks in Osaka City occurred mostly between January 2003 and May 2003 (83.9%).

All NV-positive specimens were tested for the probe type. Twenty-six of the 31 NV-positive outbreaks could be classified as 2 P1A, 2 P1B, 13 P2B, and 9 mixed probe types (Table 2). P2B strains were detected in 8 of 9 mixed probe-type outbreaks. In total, the P2B type was detected in 21 outbreaks (67.7%) and was, therefore, a predominant probe type during the 2002–03 seasons in Osaka City. The probe type could not be determined for 5 outbreaks (03008, 03011, 03027, 03042, and 03048), nor for one specimen each in outbreaks 03012 and 03022; all of these specimens were NV-positive by real time RT-PCR, but could not be amplified by RT-PCR using G1 or G2 primer sets. In addition, a single specimen from outbreak 03034, which was positive by RT-PCR using the G1 primer set, did not react with any probes (represented as UT/GI in Table 2).

Phylogenetic Analysis and Genotyping of NVs

To analyze the genetic relationships among the NV strains from the 31 outbreaks, the nucleotide sequence encoding the N/S domain of the capsid protein was determined. A total of 58 NV-positive specimens, including 18 GI and 51 GII NV-positive, were sequenced. Any strains from a single outbreak having identical nucleotide sequence in this region were considered to be identical strains.

The 51 nucleotide sequences (14 GI, 37 GII) of the N/S domain were aligned with the reference strains described in Table 1, and the genotype of each strain was determined. Phylogenetic trees based on the N/S domain were constructed by the neighbor-joining method for GI and GII NVs (Fig. 1, A and B). The bootstrap values of each genetic cluster was greater than 999, except for the GI/6 (Hesse cluster) type in GI NVs. The 51 NV strains were classified into 15 genotypes (6 GI and 9 GII genotypes) based on reference strains, and the number of each genotype was according to Kageyama's report (21). The genotypes of the 31 outbreaks strains were classified as follows: 2 GI/7, 1 GI/9, 5 GII/5, 3 GII/3, 3 GII/6, 2 GII/4, 2 GII/8, 2 GII/15, 1 GII/12, and 10 mixed genotype outbreaks.

In the outbreaks caused by mixed genotype NVs, GI/7 NV was detected in 4 outbreaks, and GII/5 NV in 5 outbreaks. Therefore, GI/7 NV was detected in a total of 6 outbreaks (19.4%), making it a dominant outbreak genotype in GI NV, and GII/5 NV was detected in 10 outbreaks (32.3%) and a dominant outbreak genotype in GII NV. In 9 of the outbreaks involving the GII/5 NV, oyster was the causal food.

In 7 outbreaks, strains of a single probe type were classified into multiple genotypes: 03021/P2B strains were of the GII/1 and GII/3, 03022/P2B strains were GII/3 and GII/15, 03034/P1A strains were GI/4 and GI/7, 03039/P2B strains were GII/8 and GII/14, and 02172/P2B, 03012/P2B, and 03034/P2B strains were of the GII/3 and GII/5 genotypes. The strain 03034-2/GI, which did not hybridize with any probes, was classified into the GI/8 genotype. Within each outbreak, the NV strains classified into the same genotype shared identical nucleotide sequence, except for NVs from outbreaks 02172 (02172-1 and 02172-2 in the GII/3 genotype) and 02198 (02198-1 and 02198-2 in the GII/4 genotype).

The relationship between probe types and genotypes of the NV outbreak strains detected in this study is as follows: P1A probe reacted with 4 genotypes (GI/1, GI/4, GI/7, and GI/9), SOV probe reacted with the GI/2 genotype, P1B reacted with the GII/6 genotype, and P2B reacted with 8 genotypes (GII/1, GII/3, GII/4, GII/5, GII/8, GII/12, GII/4, and GII/15).

Discussion

Molecular epidemiological studies of NV infections have been based on the phylogenetic analysis of the polymerase and capsid regions. The RNA polymerase region, which is relatively conserved among NV strains, has been used for detection of a wide variety of field strains, and most epidemiological studies of NV infection have been based on the sequence in this region (1, 28, 34, 35). We also reported epidemiological studies of NV infection in Osaka City, Japan targeting the polymerase region (13–15).

In general, good correlation has been reported between phylogenetic analyses of the polymerase region and capsid region (30, 33). However, recent studies indicated that phylogenetic analysis of the polymerase region sequence did not facilitate the classification of strains into genotypes (22), and a system has been proposed for the identification of NVs in which the capsid sequences are compared to those of reference strains. Ando et al. (3) used sequences encoding the capsid N-terminal 94 amino acids to divide G1 NVs into 5 "genetic clusters" and GII NVs into 10 clusters. Vinje et al. (33) demonstrated that the NVs could be divided into 7 "phylogenetic groups" within GI and 5 within GII using the capsid N-terminal region sequence (GI; 278 nt, GII; 249 nt). Katayama et al. (22) demonstrated that the NVs could be divided 9 "genotypes" within GI and 10 within GII using the capsid N/S domain. Furthermore, Green et al. (11) demonstrated that the NVs could be divided 7 "genetic clusters" within GI and 7 within GII using the complete capsid

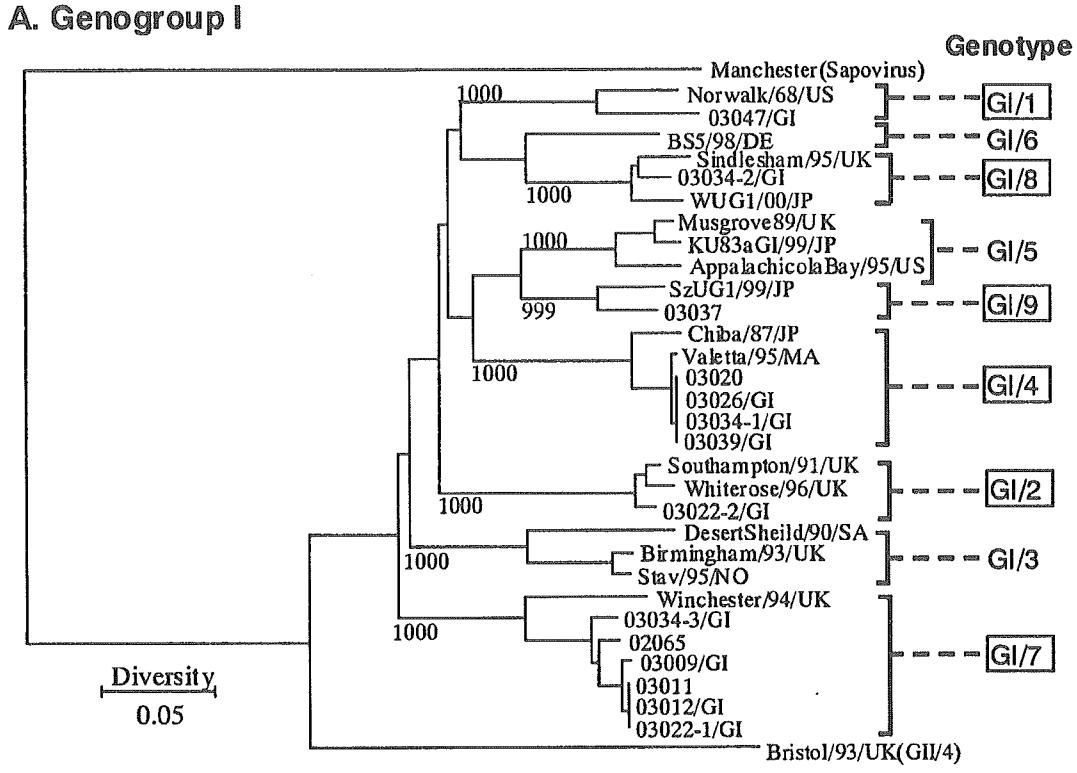

Fig. 1. Phylogenetic trees of published NV sequences and outbreak strains from this study based on the N/S domain region (GI, 290 nt; GII, 278 nt). Genogroup I NVs and Bristol/93/UK(GII/4) (A), genogroup II NVs and Norwalk/68/US(GI/1) (B) are reconstructed using Manchester *sapovirus* as the outgroup. The numbers on each branch indicate the bootstrap value for the clusters supported by that branch. The genotypes that include an outbreak strain are boxed.

region. The genetic classification described in these four reports is summarized in Table 1. The comparison indicates that their constellations of genotypes or genetic clusters are almost identical except for the names. The phylogeny based on the ORF2 region is related to antigenic types, as indicated by solid phase immuno-EM for 8 genetic types (33). The phylogenetic analysis of the N/S domain (22) was well correlated with antigenic analysis using recombinant virus-like particles and their antisera (23, 24). Therefore, the 5′ end of ORF 2 region (N/S domain) may be suitable for the molecular typing of NV strains.

In this study, a phylogenetic analysis of the N/S domain incorporating new outbreak strains, NV reference strains from previous reports, and additional reference strains, GI NVs were segregated into 9 genotypes, as reported by Katayama et al. (22), but GII NVs were segregated into 14 genotypes (Fig. 1, A and B). Kageyama et al. reported that the NV could be divided into 14 genotypes within GI and 17 genotypes within GII based on the capsid N/S domain (21).

The P2B outbreak strains, the predominant probe type in this season, were characterized into 8 genotypes (Table 2). However, there was no predominant genotype of NV outbreak strains; multiple genotypes of NV were prevalent in Osaka City, Japan. The outbreaks in which mixed NV genotypes were detected mainly occurred by consumption of oysters. It may be that concurrent infections with more than one strain occurred by ingesting the contaminated oysters. Similar results of coinfection have been reported previously (21, 32), and various types of NVs have been detected from oysters in Japan (29). Ninety percent of GII/5 NV outbreaks were associated with consumption of oysters, indicating that the GII/5 NV was closely related to oysters in this season. The 9 GII/3 NV strains, classified as P2B, detected in this study, in Fig. 1B, were closely related to Arg-320/95/AR which might be occurred a genetic recombination between ORF1 and ORF2 (15, 16, 33).

In summary, we applied a recently developed quantitative real time PCR a method (20) to detect NV genomes from stool specimens in Osaka City, Japan. This method is useful for routine diagnosis, because of

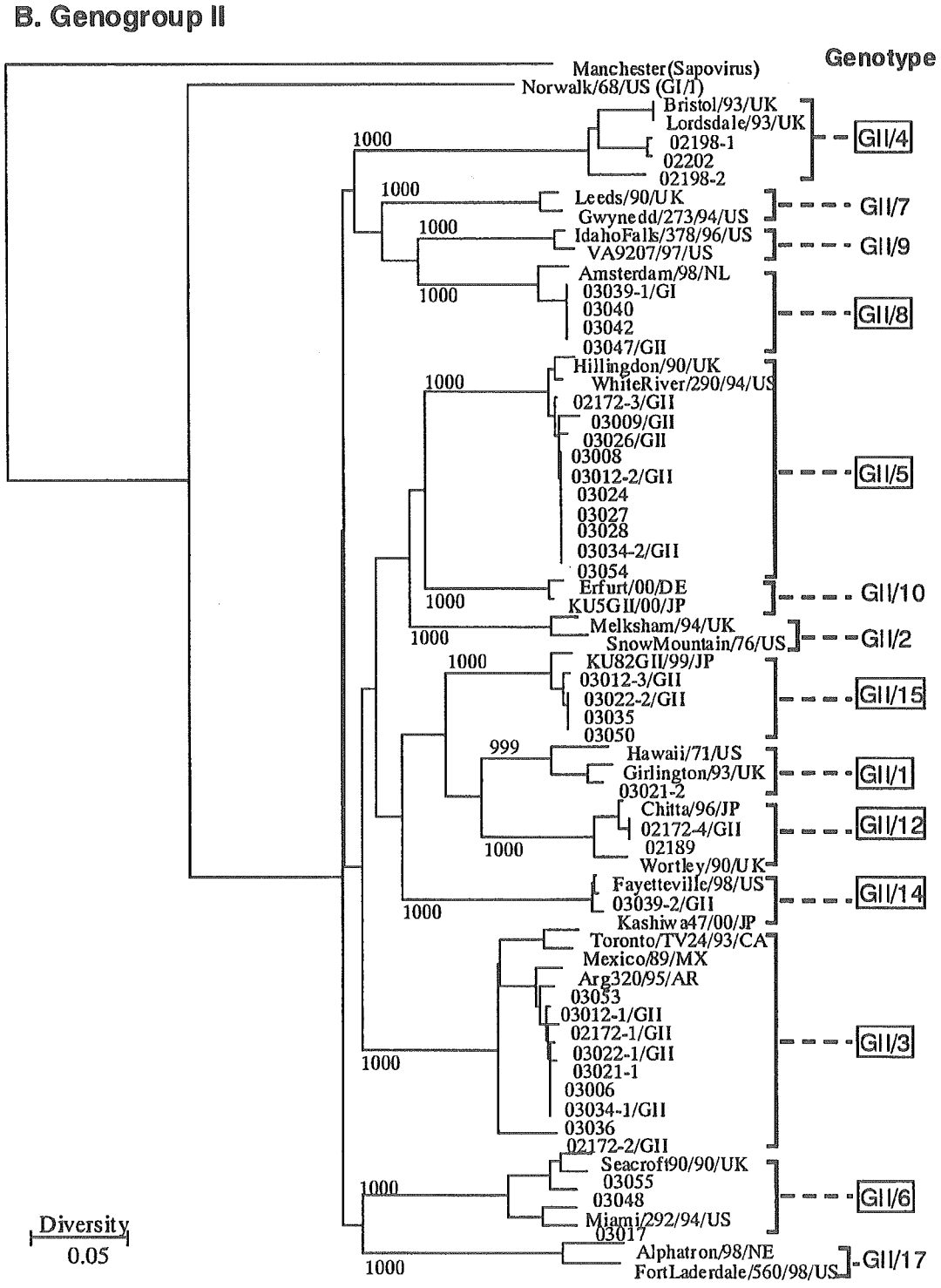

Fig. 1. B

its broad reactivity and high sensitivity compared to our previous diagnostic method using RT-PCR and hybridization. Application of genotyping methods has provided information on disease transmission for epidemiological investigations of public health significance. Further molecular phylogenetic studies of NVs will contribute to an understanding of the epidemiology of NV infection.

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