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 $1,500 \times g$ for 20 min. Three milliliters of the supernatants was concentrated by ultracentrifugation at $200,000 \times g$ for 2.5 h by using a 50.2Ti rotor (Beckman Coulter Inc., Fullerton, Calif.), and the concentrate was then resuspended in 200 μ l 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 μ l 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 (CCNGCTGTGAASGCRITNCCMGC) was used in place of primer G2R1, and for amplification of the 3' end of the NV genome, primer dT25VN [(T)₂₅V(A/G/C)N(A/G/C/T)] (19) was used as the reverse primer. Primer LVPF (AGTCTCTGTGTCGAGTYCTCAC) and primer LVCAPE (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 and strain name	Type	Source ^a	Accession no.
Genogroup I				Chiba/040230/2004	GII-3	A/O	AJ865580
Chiba/000600/2000	GI-1	C/S	AJ865482	Chiba/990897/1999	GII-4b	C/S	AJ865513
Chiba/030308/2003	GI-1	A/O	AJ865496	Chiba/990900/1999	GII-4b	C/S	AJ865474
Chiba/030542/2003	GI-1	A/O	AJ865501	Chiba/991173/1999	GII-4b	C/S	AJ865518
Chiba/040252/2004	GI-1	A/O	AJ865507	Chiba/001049/2000	GII-4b	A/O	AJ865529
Chiba/040501/2004	GI-1	A/O	AJ865509	Chiba/010105/2001	GII-4b	A/O	AJ865532
Chiba/010510/2001	GI-2	A/O	AJ865487	Chiba/021071/2002	GII-4b	A/O	AJ865556
Chiba/030547G1/2003	GI-2	A/O	AJ865503	Chiba/040092/2004	GII-4b	C/S	AJ865576
Chiba/030658/2003	GI-2	C/O	AJ865505	Chiba/021050/2002	GII-4c	C/S	AJ865554
Chiba/000520/2000	GI-3	A/O	AJ865481	Chiba/030556/2003	GII-4c	A/O	AJ865567
Chiba/020251/2002	GI-3	A/O	AJ865492	Chiba/020984/2002	GII-4d	C/O	AJ865551
Chiba/030546/2003	GI-3	A/O	AJ865502	Chiba/030517/2003	GII-4d	A/O	AJ865564
Chiba/000016G1/1999	GI-4	A/S	AJ865476	Chiba/021050/2002	GII-4d	A/O	AJ865569
Chiba/000516/2000	GI-4	A/O	AJ865480	Chiba/040045/2004	GII-4d	A/O	AJ865575
Chiba/010108/2001	GI-4	A/O	AJ865485	Chiba/040096/2004	GII-4d	C/S	AJ865577
Chiba/020097/2002	GI-4	A/O	AJ865490	Chiba/040140/2004	GII-4d	A/O	AJ865579
Chiba/020267/2002	GI-4	A/O	AJ865493	Chiba/040311/2004	GII-4d	A/S	AJ865584
Chiba/030403/2003	GI-4	A/O	AJ865499	Chiba/021022/2002	GII-4e	C/O	AJ865552
Chiba/040589/2004	GI-4	A/O	AJ865510	Chiba/031038/2003	GII-4e	A/O	AJ865572
Chiba/030360/2003	GI-5	A/O	AJ865498	Chiba/040110/2004	GII-4e	A/O	AJ865578
Chiba/040493/2004	GI-5	A/O	AJ865508	Chiba/040277/2004	GII-4e	A/O	AJ865581
Chiba/030654/2003	GI-6	A/O	AJ865504	Chiba/991180/1999	GII-5	A/S	AJ865519
Chiba/991013/1999	GI-7	C/S	AJ865475	Chiba/010751/2001	GII-5	A/O	AJ865541
Chiba/000782/2000	GI-7	C/S	AJ865484	Chiba/020015/2002	GII-5	A/O	AJ865543
Chiba/020250/2002	GI-7	A/O	AJ865491	Chiba/020040/2002	GII-5	A/O	AJ865544
Chiba/030541/2003	GI-7	A/O	AJ865500	Chiba/030547G2/2003	GII-5	A/O	AJ865566
Chiba/000661/2000	GI-8	A/S	AJ865483	Chiba/040500/2004	GII-5	A/O	AJ865585
Chiba/010433/2001	GI-8	A/O	AJ865486	Chiba/000459/2000	GII-6	C/O	AJ865527
Chiba/030305/2003	GI-8	A/O	AJ865495	Chiba/991012/1999	GII-6	C/S	AJ865515
Chiba/040001/2004	GI-8	A/O	AJ865506	Chiba/010522/2001	GII-6	C/S	AJ865536
Chiba/020096G1/2002	GI-9	A/O	AJ865489	Chiba/030968/2003	GII-6	A/O	AJ865570
Chiba/000335/2000	GI-11	A/O	AJ865477	Chiba/040002/2004	GII-6	A/O	AJ865573
Chiba/000337/2000	GI-12	A/O	AJ865478	Chiba/040309/2004	GII-6	A/S	AJ865583
Chiba/020247/2002	GI-13	C/O	AJ865511	Chiba/040545/2004	GII-6	C/O	AJ865587
Chiba/030335/2003	GI-13	C/O	AJ865512	Chiba/010526/2001	GII-7	C/S	AJ865537
Chiba/020062/2002	GI-14	A/O	AJ865488	Chiba/030981/2003	GII-7	A/O	AJ865571
Chiba/030358/2003	GI-14	A/O	AJ865497	Chiba/010045/2001	GII-8	A/O	AJ865523
Chiba/030100/2003	GI-15	A/O	AJ865494	Chiba/020475/2002	GII-8	A/O	AJ865549
Genogroup II				Chiba/030399/2003	GII-8	A/O	AJ865558
Chiba/000485/2000	GII-1	A/O	AJ865528	Chiba/030412/2003	GII-8	A/O	AJ865559
Chiba/010587/2001	GII-1	C/S	AJ865539	Chiba/990969/1999	GII-10	A/O	AJ865514
Chiba/030510/2003	GII-1	A/O	AJ865563	Chiba/000325/2000	GII-10	C/S	AJ865524
Chiba/021026/2002	GII-2	C/O	AJ865553	Chiba/010006/2001	GII-10	A/O	AJ865531
Chiba/030322/2003	GII-2	A/O	AJ865557	Chiba/020555/2002	GII-10	A/O	AJ865550
Chiba/030429/2003	GII-2	A/O	AJ865560	Chiba/010442/2001	GII-11	A/O	AJ865534
Chiba/040554/2004	GII-2	C/O	AJ865588	Chiba/000016G2/2000	GII-12	A/S	AJ865521
Chiba/991120/1999	GII-3	C/S	AJ865517	Chiba/020096G2/2002	GII-12	A/O	AJ865546
Chiba/000022/2000	GII-3	A/O	AJ865522	Chiba/991118/1999	GII-13	C/S	AJ865516
Chiba/000336/2000	GII-3	A/O	AJ865525	Chiba/010465/2001	GII-13	C/S	AJ865535
Chiba/000344/2000	GII-3	A/O	AJ865526	Chiba/000010/2000	GII-14	A/O	AJ865520
Chiba/010621/2001	GII-3	C/S	AJ865540	Chiba/010390/2001	GII-14	A/O	AJ865533
Chiba/010998/2001	GII-3	C/S	AJ865542	Chiba/040300/2004	GII-14	A/O	AJ865582
Chiba/020198/2002	GII-3	A/O	AJ865547	Chiba/030474/2003	GII-15	A/O	AJ865561
Chiba/020301/2002	GII-3	A/O	AJ865548	Chiba/030522/2003	GII-15	A/O	AJ865565
Chiba/021068/2002	GII-3	A/O	AJ865555	Chiba/040003/2004	GII-15	A/O	AJ865574
Chiba/030486/2003	GII-3	A/O	AJ865562	Chiba/020042/2002	GII-16	A/O	AJ865545
Chiba/030603/2003	GII-3	C/O	AJ865568	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 GII 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 sam-

ples 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 ad-

ditional 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

	217	
Bristo1	PPTVESRTKPFTVPVLTVVEEMSNRFPIPLEKLYTGPSSAFVVPQNGRCTTDGVLLGTTQLSAVNICNFRG	DTVTHIAGSHDYTMNLASQNWSNYDPTEE
Camberwell	I	V
030556	T	RN
Grimsby	I	T
991002	I	T
001049	I	T
010105	I	T
021071	I	V
040092	I	T
030910	I	T
Oxford B5S9	I	T
Oxford B5S19	I	T
031038	I	T
040110	I	T
040277	I	T
		I II
	317	
Bristo1	IPAPLGTPDFVGKIQGLLT	ITRADGSTRAHKATVSTGSHFTPKLGSVQFTDTNNDFOACQNTKFTPVGVIQDGD-HHQN
Camberwell		P
030556		S D
Grimsby	M	E I Y
991002	M	E Y
001049	M	E Y
010105	M	E Y
021071	V	E Y
040092	V	E Y
030910	R M	G G D I S
Oxford B5S9	R M	G G D I N
Oxford B5S19	R M	G G D N
031038	M	KG G D A D
040110	M	KG G D A D
040277	M	KG G D A D
		III IV *
	416	
Bristo1	HLAPAVAPT	FPGEQLLFFRSTMPGCSGYPNMNLDCLLPQEWLHFYQEAAPQSDVALLRFVNPDTGRVLFECKLHKSGYITVAHTGPYDLVLPNGYFR
Camberwell		F I
030556		S N
Grimsby		Q
991002		V Q
001049		Q
010105		Q
021071		Q
040092		Q
030910		Q
Oxford B5S9	A	Q
Oxford B5S19		Q
031038		D Q
040110		D Q
040277		D Q
	516	
Bristo1	FDSWVNQFYTLAPMGNGTGRRRAL	
Camberwell		
030556		A P
Grimsby	X	A
991002		A
001049		A
010105		A
021071		A
040092		A
030910		
Oxford B5S9		
Oxford B5S19		
031038		A
040110		A
040277		A

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.

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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