TABLE 3. FG loop length correlations

Strain	Host	P type[a]	RRV Q135 equivalent[b]	SA dependence[c]	Residue at position 101	Genbank accession no.
SA11-4f	Simian	6[1]	Present	+	R	X57319
NCDV-Lin	Bovine	6[1]	Present	+[h]	R	M63267
RF	Bovine	6[1]	Present	+	R	U65924
BRV033	Bovine	6[1]	Present	+	R	U62155
SA11 cl3	Simian	5B[2]	Present	+[h]	R	M23188
RRV	Simian	5B[3]	Present	+[h]	R	AY033150
CU-1	Canine	5A[3]	Present	+	R	D13401
HCR3	Human	5A[3]	Present	+	R	L19712
K9	Canine	5A[3]	Present	+	R	D14725
Cat97	Feline	5A[3]	Present	+	R	D13402
DS-1	Human	1B[4]	Absent	−[h]	F	DQ141310
L26	Human	1B[4]	Absent	−	F	M58292
4S	Porcine	7[5]	Present	−	R	L10358
UK	Bovine	7[5]	Present	−[d,h]	R	M22306
WC3	Bovine	7[5]	Present	−	R	AY050271
B461	Bovine	7[5]	Present	−	R	M63267
678	Bovine	7[5]	Present	−	R	D32054
M37	Human	2A[6]	Absent	−	I	None[e]
1076	Human	2A[6]	Absent	−[h]	I	None[e]
McN13	Human	2A[6]	Absent	−	I	None[e]
ST3	Human	2A[6]	Absent	−	I	L33895
Gott	Porcine	2B[6]	Absent	−	V	M33516
CRW-8	Porcine	9[7]	Present	+	R	L07888
OSU	Porcine	9[7]	Present	+[h]	R	X13190
H1	Equine	9[7]	Present	+	R	D16341
TFR-41	Porcine	9[7]	Present	+	R	L07889
YM	Porcine	9[7]	Present	+	R	M63231
Wa	Human	1A[8]	Absent	−	F	M96825
KU	Human	1A[8]	Absent	−[h]	F	M21014
MO	Human	1A[8]	Absent	−	F	AB008278
YO	Human	1A[8]	Absent	−	F	AB008279
Ito	Human	1A[8]	Absent	−	F	AB008280
VA70	Human	1A[8]	Absent	−	F	AJ540229
Hochi	Human	1A[8]	Absent	−	F	AB039943
K8	Human	3[9]	Present	−[h]	R	D90260
O264	Human	3[9]	Present	−	R	AB008665
Cat2	Feline	3[9]	Present	−	R	D13403
69M	Human	4[10]	Present	−[h]	R	M60600
B233	Bovine	8[11]	Absent	−[h]	F	D13394
I321	Human	8[11]	Absent	−	F	L07657
H-2	Equine	4[12]	Present	−[h]	R	L04638
FI-14	Equine	4[12]	Present	−	R	D13398
FI23	Equine	4[12]	Present	−	R	D16342
A46	Porcine	[13]	Present	−[h]	M	AY070274
Ala	Lapine	11[14]	Present	−[h]	R	U62149
C-11	Lapine	11[14]	Present	−	R	U62150
BAP-2	Lapine	11[14]	Present	−	R	U62151
R-2	Lapine	11[14]	Present	−	R	U62152
PA169	Lapine	11[14]	Present	−	R	D14724
HAL1166	Human	11[14]	Present	−	R	L20885
Mc35	Human	11[14]	Present	−	R	D14032
Lp14	Ovine	[15]	Present	−[h]	R	L11599
EW	Murine	10[16]	Present	−	R	U08429
EC	Murine	10[16]	Present	+/−[f]	R	U08421
EB	Murine	10[16]	Present	−[h]	R	U08419
EDIM	Murine	10[16]	Present	−	R	AF039219
Ty-1	Avian	[17]	Present	−[h]	R	L41493
L338	Equine	12[18]	Present	+/−[f]	R	D13399
4F	Porcine	[19]	Absent	−[h]	V	L10359
EHP	Murine	[20]	Present	+/−[g]	R	U08424

[a] P serotype[P genotype]. Some strains have not been serotyped. Many of the strain classifications were obtained from reference 6.
[b] Indicates the presence or absence of a residue that is structurally equivalent to residue 135 of strain RRV.
[c] SA dependence is based on published data that define SA independence as a preserved infectious titer on cultured cells or enterocytes digested with *Arthrobacter ureafaciens* neuraminidase (5, 6, 10, 31).
[d] Although UK entry is not SA dependent, it binds glycosphingolipids with a specificity dependent upon the SA moiety in the oligosaccharide chain (10).
[e] Sequences from reference 17.
[f] Weak inhibition by neuraminidase digestion of MA104 cells (6).
[g] Cell-type-specific neuraminidase sensitivity (31).
[h] Strain included in the SA-dependent and SA-independent sets used to calculate variability for Table S1 in the supplemental material and Fig. 5A to C.

TABLE 4. Neutralization escape mutations selected by MAbs that recognize VP8* of human rotavirus strains

MAb	Escape mutation (strain)[a]	Immunization regimen	P genotypes neutralized (not neutralized)[b]	Immunized species	Initial screen	Reference
1-2H	G170D (KU)	Natural infection	P[4, 8] (P[5, 6, 9, 10])	Human	Binding	21
2-3E	E203K (KU)	Natural infection	P[6, 8] (P[4, 5, 9, 10])	Human	Binding	21
HS6	T721 (ST3)	IP[c] with ST3	P[6, +/−8][d] (P[4])	Mouse	Neutralization	35
HS11	E217K (ST3)	IP with ST3	P[6] (P[4, 8])	Mouse	Neutralization	35
RV-5:2	Q148R (RV-5)	IP and IV[e] with RV-5	P[4] (P[2, 3, 5, 6, 8, 10])	Mouse	Neutralization	8

[a] P genotypes of rotavirus strains: KU, P[8]; ST3, P[6]; RV-5, P[4].
[b] Only listed P genotypes were tested.
[c] IP, intraperitoneal hyperimmunization.
[d] Some, but not all, P[8] strains are neutralized.
[e] IV, intravenous hyperimmunization.

We have mapped the residues recognized by 1-2H and 2-3E in strain KU, using neutralization escape mutant analysis (Table 4). MAb 1-2H selects a unique G-to-D mutation at VP4 residue 170 (virus strain m-KU-1-2H), and MAb 2-3E selects a unique E-to-K mutation at VP4 residue 203 (virus strain m-KU-2-3E). Both mutations are in the VP8* fragment of VP4. Three other VP8*-specific antibodies that neutralize human strains of rotavirus have been described (26, 35, 36). Two of these MAbs, HS11 and RV5:2, neutralize homotypically, but the other MAb, HS6, neutralizes P[6] and some P[8] viruses (Table 4). Thus, three of five neutralizing monoclonal antibodies that recognize VP8* of human rotavirus strains are heterotypic in their neutralization specificities. A more limited degree of heterotypic neutralization has been observed among the 20 mapped MAbs that bind VP8* and neutralize animal rotavirus strains (see Table S2 in the supplemental material). Heterotypic neutralization by monoclonal antibodies derived from naturally infected humans may reflect selection for heterotypic antibodies by repeated rotavirus infection. This result correlates well with the increasingly broad serum neutralizing response against rotavirus elicited by reinfection with rotaviruses of the same or different rotavirus serotypes (19, 43).

Although VP5* is more conserved among strains than is VP8*, the presence of heterotypic neutralization epitopes on VP8* of human strains suggests that immunization with recombinant VP8* of human strains could induce a heterotypic neutralizing antibody response. Such a response has been demonstrated against VP8* from an animal rotavirus strain, as primary immunization of laboratory animals with recombinant VP8* of the simian strain RRV does produce heterotypically neutralizing antibodies (16).

Mapping of neutralization escape mutations on the DS-1 VP8* core structure. As previously described, the 20 neutralization escape mutations mapped to VP8* of SA-dependent animal rotavirus strains cluster in four epitopes (14). The five neutralization escape mutations now mapped to VP8* of SA-independent human rotavirus strains do not cluster in these epitopes or in any easily identifiable new epitopes (Fig. 5A to C, labeled residues). Only the mutation at residue 148 of human strain RV-5 lies within one of the previously described epitopes (designated 8-1). Because the DS-1 and RRV VP8* cores have a common fold, gross structural differences do not explain the distinct distributions of escape mutations.

When the RRV VP8* core crystal structure is fitted to the spike envelope of a 12-Å-resolution electron cryomicroscopy image reconstruction of trypsin-primed, SA-dependent rotavirus particles, all of the escape mutations on VP8* of SA-dependent strains are accessible for antibody binding, forming "caps" at the tips of the spikes' heads (Fig. 5D). Although the DS-1 VP8* core fits the same molecular envelope, the escape mutations of SA-independent human strains are not in the most accessible locations, and three (at residues 72, 203, and 217) ring the base of the head (Fig. 5E and F). In fact, the escape mutation at residue 72 contributes to the point of attachment of head to body and to the surface of the gap between the paired heads, where it is not accessible for antibody binding (Fig. 5C, E, and F). The escape mutation at residue 203 also contributes to the surface of the gap between head and body and is on the boundary between accessible and inaccessible surfaces.

Thus, the distribution of escape mutations suggests that the state of the VP4 spike recognized by some VP8*-specific antibodies that neutralize SA-independent human rotavirus strains differs from the state recognized by VP8*-specific antibodies that neutralize SA-dependent animal rotavirus strains. Alternatively, the selected residues could mediate escape from neutralization by indirect steric effects, rather than by direct disruption of an epitope. No electron cryomicroscopy image reconstructions of SA-independent rotavirus virions are currently available to test the hypothesis that the conformations of trypsin-primed spikes on SA-independent and SA-dependent strains expose different molecular surfaces of the VP8* core for potential antibody binding.

VP4 spikes have multiple conformations during rotavirus entry. Prior to trypsin priming, the spikes are flexible and therefore not visible in averaged icosahedral image reconstructions (9). Trypsin priming rigidifies pairs of VP4 molecules to produce spikes. Threefold symmetry of the portion of VP4 buried under the VP7 shell (44), the trimeric appearance of altered VP4 spikes on virions that have been treated with alkali (39), and the stable trimer formed by a rearranged VP5* fragment (13) suggest that each VP4 cluster on the virion surface may contain three molecules, one of which remains flexible after trypsin priming. In addition, electron cryomicroscopy image reconstructions demonstrate subtle conformational differences in spike morphology among SA-dependent rotavirus strains (38). The 22- to 23-Å resolution limit of these reconstructions does not permit a precise determination of the boundaries of the accessible surfaces on the variants. Thus, either strain differences in spike morphology or the multiple conformational states of VP4 could explain the distribution of

neutralization escape mutations on VP8* of human rotavirus strains.

In summary, although the VP8* cores of an SA-dependent strain (RRV) and of an SA-independent strain (DS-1) are substantially similar, there are significant structural differences between the two phenotypic variants. The biochemical characteristics of both variants, including ease of expression and purification, high solubility, and chemical stability make them promising components for a potential second-generation recombinant rotavirus vaccine. In this regard, heterotypic neutralization by MAbs recognizing VP8* of human strains is a particularly promising finding. Differences between the RRV and DS-1 VP8* cores in the region that corresponds to the RRV SA binding site make it unlikely that DS-1 VP8* binds an alternative carbohydrate ligand in this location. A widened cleft between the EF β-hairpin and the six-stranded β-sheet in the DS-1 VP8* core and the binding of a peptide chain in this cleft suggest that VP8* may bind a protein ligand. The very different neutralization surfaces of SA-dependent and SA-independent viruses suggest different mechanisms of neutralization and, possibly, differences in spike morphology. Further structural studies of SA-independent rotavirus strains could reveal differences in the VP4 spike that are directly relevant to the pathogenesis of rotavirus gastroenteritis in children. .

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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

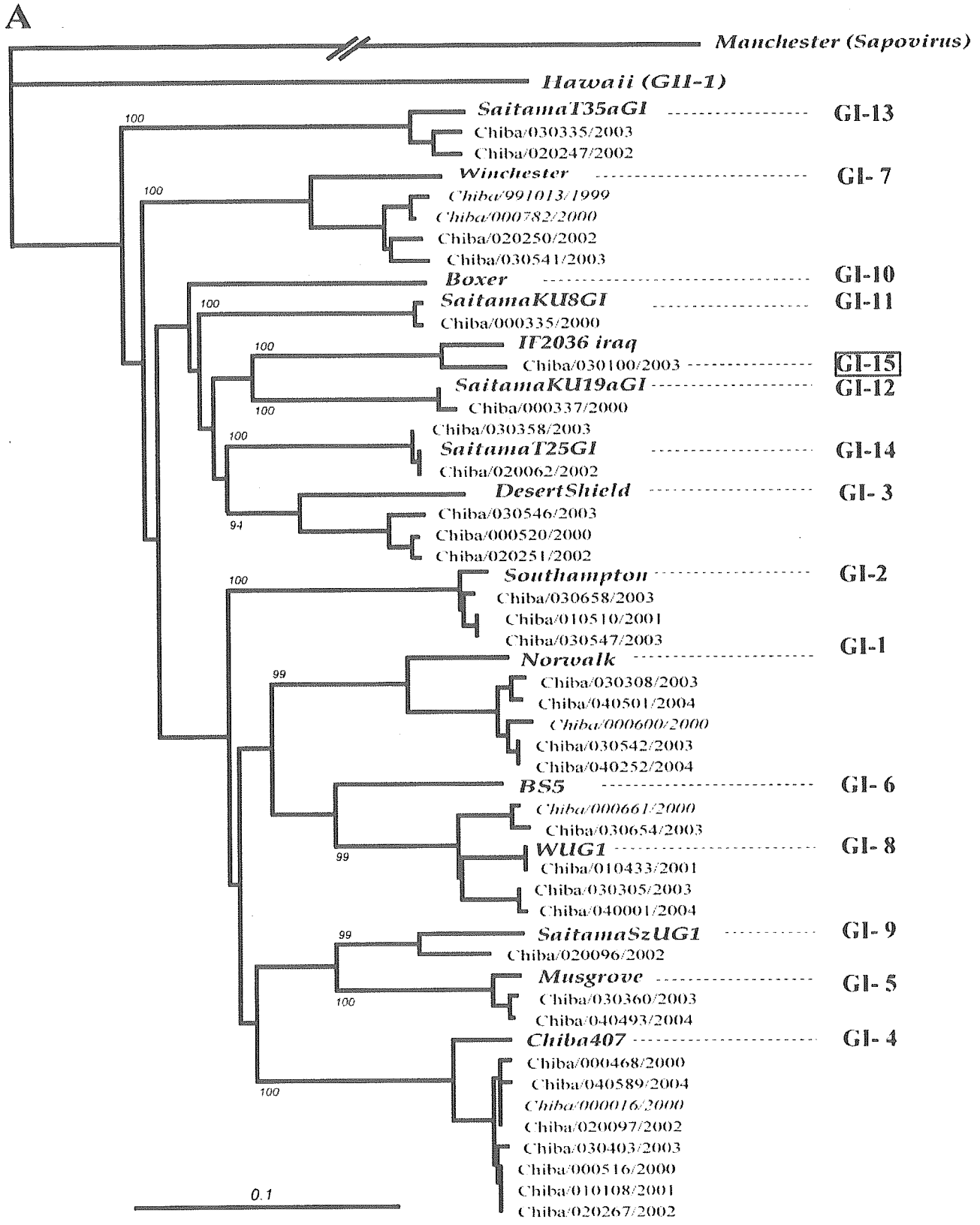

FIG. 1. Phylogenetic tree of selected norovirus strains analyzed in this study. (A) Genogroup I; (B) genogroup II. Trees were reconstructed with partial nucleotide sequences of the 5′ end of ORF2 and by using Manchester virus as the outgroup. The numbers on each branch indicate the bootstrap values of 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.

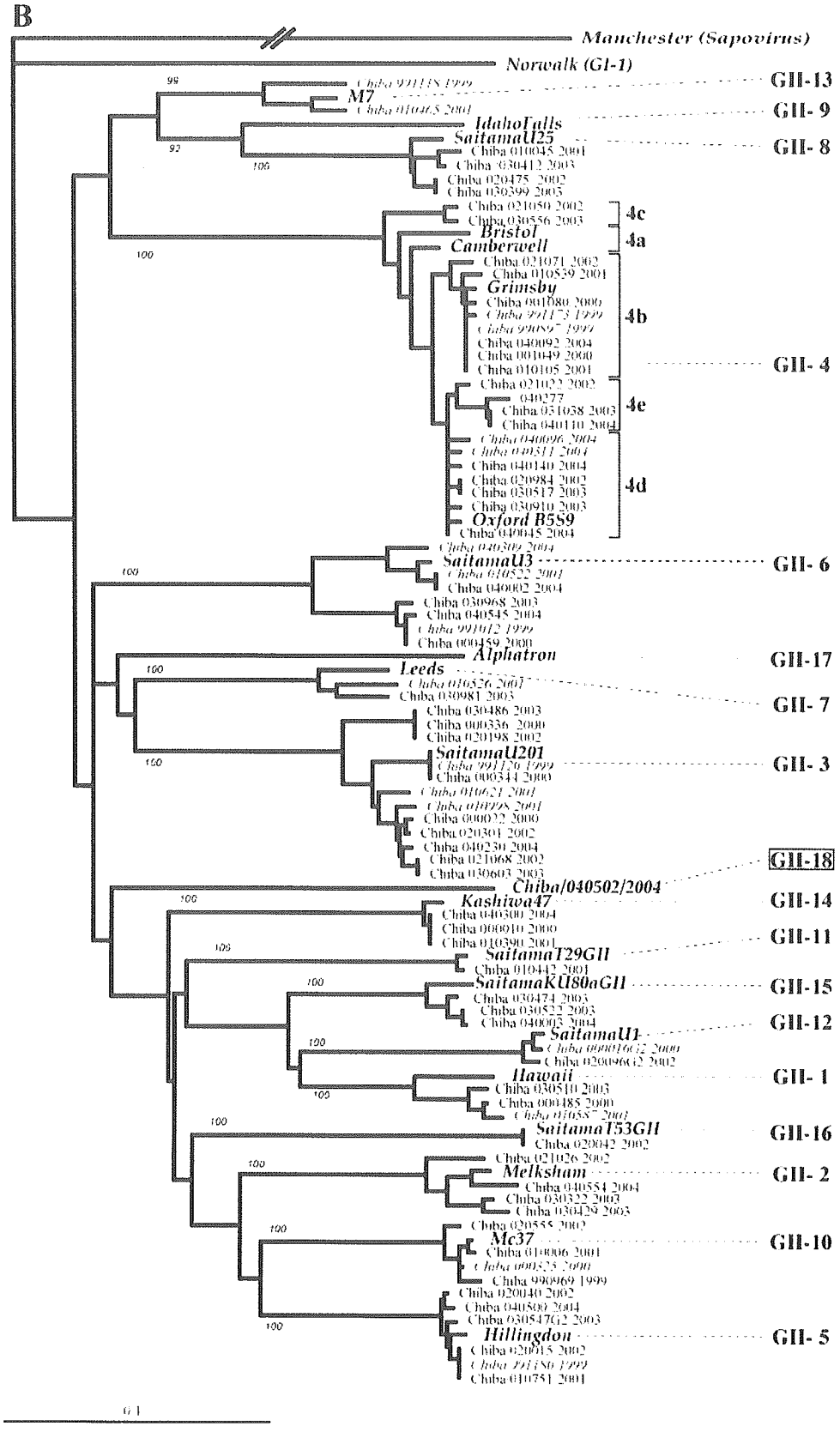

FIG. 1—*Continued.*

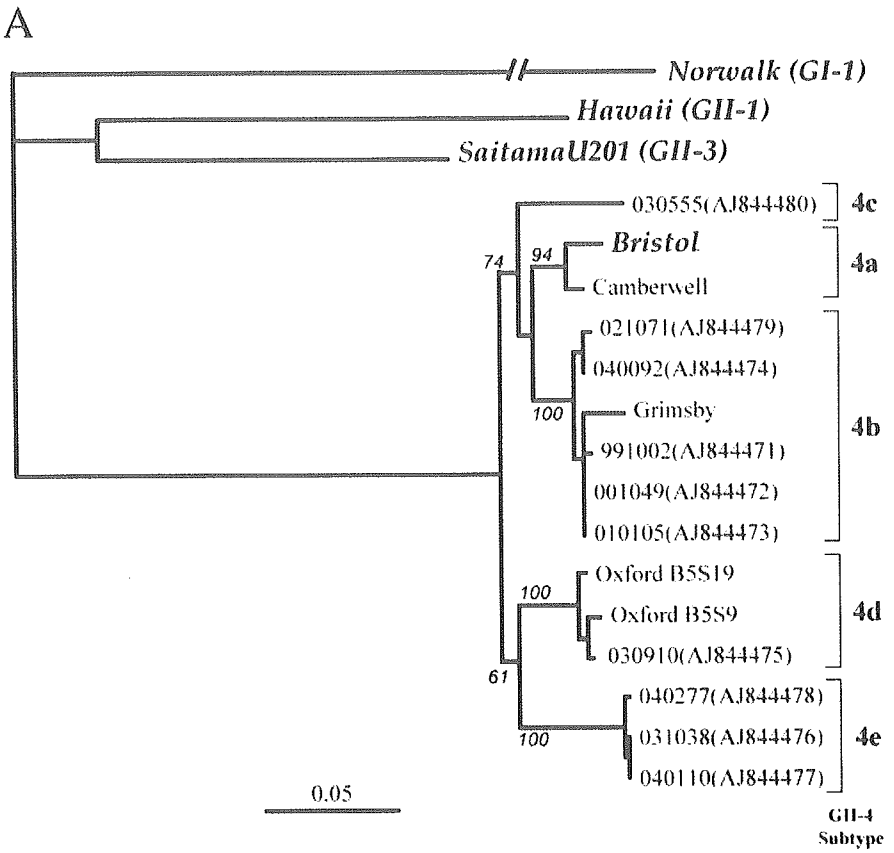

FIG. 2. Genetic analysis of the P region of the capsid protein in the GII-4 strains. (A) Phylogenetic tree of the GII-4 NV strains reconstructed by the neighbor-joining method by using the amino acid sequences of the P region of the capsid protein. The numbers on each branch indicate the bootstrap values 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

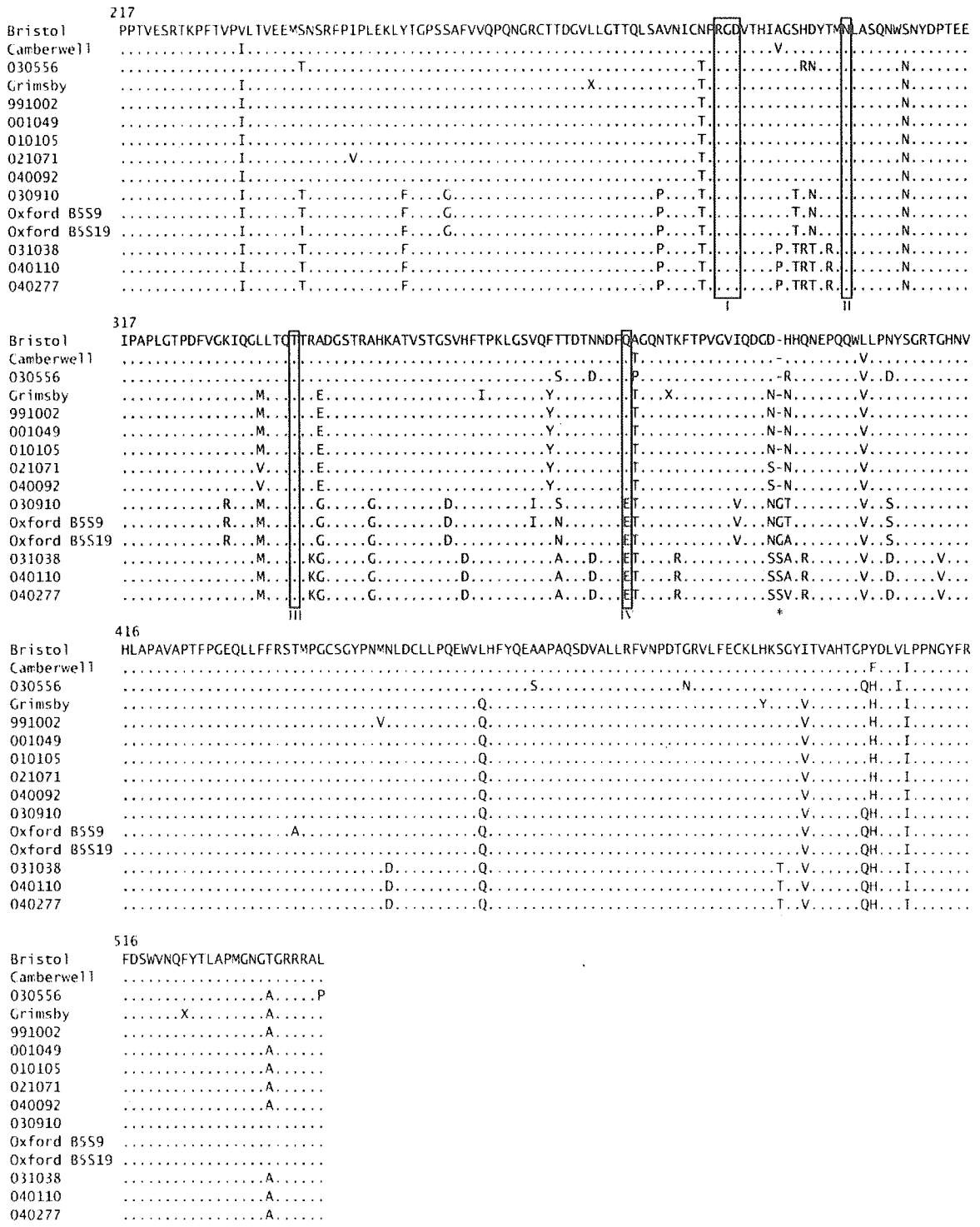

FIG. 2—*Continued.*

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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Multiprefectural Spread of Gastroenteritis Outbreaks Attributable to a Single Genogroup II Norovirus Strain from a Tourist Restaurant in Nagasaki, Japan

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A series of gastroenteritis outbreaks caused by noroviruses (NVs) among tourist groups from several prefectures was associated with eating a lunch prepared by a restaurant in Nagasaki City, Japan, on 18 and 19 November 2003. A retrospective cohort study was performed to estimate the magnitude of the outbreak and identify the source of infection. Epidemiological information was obtained through the local public health centers in the areas where the illness occurred. Stool and vomit specimens and food and environmental samples were analyzed by reverse transcription-PCR with genogroup-specific primers. Positive samples were sequenced and analyzed phylogenetically. Of 1,492 tourists who ate a lunch prepared by the restaurant during the 2-day period, 660 (44.2%) developed illness, with an average incubation time of 31.2 h. Whereas NVs were not detected in any food samples, identical sequences most closely related to the Mexico genotype of genogroup II NV were found in specimens from case patients, restaurant staff, and the kitchen table. Food handlers were concluded to be the source of the outbreak as a result of the contamination of several meals. The series of outbreaks described here exemplifies the role of tourism as a contemporary way to distribute a single infectious agent to multiple and geographically remote areas.

Norovirus, a genus within the family *Caliciviridae*, has emerged as an important cause of food- and waterborne gastroenteritis outbreaks in industrialized countries (6, 8, 20). Noroviruses (NVs) are responsible for 78.5% of all nonbacterial outbreaks of gastroenteritis reported from 1995 to 2000 in Europe (21). They accounted for an estimated 6 to 14, 11 to 18, and 20% of infectious intestinal diseases in England and Wales (3, 7, 28), The Netherlands (4, 17), and Finland (27), respectively. It was reported that 96% of 90 outbreaks of nonbacterial gastroenteritis were caused by NVs (6), and it is estimated that NVs cause 23 million illnesses each year (22) in the United States. In Japan, NVs accounted for 28% of cases of food poisoning from all causes and 99% of cases from purely viral sources (24).

NVs can be classified into five genogroups, genogroups GI to GV; the three genogroups GI (prototype strain, Norwalk virus), GII (prototype strain, Snow Mountain virus), and GIV have been found in humans (1, 23, 29, 31). Reverse transcription-PCR (RT-PCR) has become a favored method for detection and classification of NVs and has extensively been used as a tool in investigations of acute gastroenteritis outbreaks (9, 13, 30, 33). Little has been reported about the genotype distribution of NVs in Japan. The GII Lordsdale genotype (GII/4) has been predominant since 1996, and the GII Mexico genotype (GII/3) suddenly appeared and spread during the 1999-2000 season in Osaka City, Japan (11). In another study, various genotypes of NVs were found in Kyushu, Japan, from 1988 to 1993, and the GII Mexico genotype was dominant in 1989 (26). In Japan, raw oysters are the primary source of transmission in small outbreaks, whereas school lunches and catered meals, banquet halls, and hospitals are most often implicated as the vehicles and settings of transmission in large outbreaks (those involving >50 patients) (10). In terms of the number of patients involved in NV gastroenteritis outbreaks in Japan, the largest one (3,236 schoolchildren) occurred in nine elementary schools in 1989 following consumption of a school lunch prepared by a lunch preparation center in which one food handler had gastroenteritis (15).

In this article we describe the investigation into a series of gastroenteritis outbreaks that occurred among tourists who had a lunch prepared by a single tourist restaurant and that were attributed to a single strain of NV.

MATERIALS AND METHODS

Outbreak description. Multiple outbreaks of acute gastroenteritis occurred among the tourists from several prefectures who visited Nagasaki City, Japan, and who had a lunch prepared by a tourist restaurant (restaurant J) in November 2003. Nagasaki City is located in the western part of the island of Kyushu, has a population of 420,000, and is visited by more than 5 million tourists a year. On 19 November, the Public Health Authority in Nagasaki City initially received two independent calls that students and teachers from schools in different prefectures who had visited Nagasaki City on a school excursion the day before had gastrointestinal symptoms, such as nausea, vomiting, diarrhea, abdominal pain, and fever. It turned out that the members of these tourist groups had lunch at restaurant J or ate box lunches prepared by that restaurant.

Thus, the Public Health Authority immediately suspended the business of restaurant J, as it was the suspected origin of the food-poisoning outbreak. Gastroenteritis cases continued to occur among tourists who had lunch prepared

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TABLE 1. Characteristics, attack rates, and incubation times for the groups that ate at restaurant J on 18 November

Group	Type of group	Time of visit	No. of tourists	No. of patients	Attack rate (%)	Incubation time (h)[a]
A	Junior high school excursion from Kagoshima	Box lunches	32	25	78.1	27.8 ± 7.1
B	Adults from inside Nagasaki Prefecture	11:30	26	2	7.6	33.5 ± 2.8
C	Elementary school excursion from Fukuoka	11:40	103	73	70.9	33.0 ± 8.6
D	Adults from Osaka	12:30	17	5	29.4	30.1 ± 10.6
E1	High school excursion from Aichi	12:30	415	322	77.6	29.8 ± 10.3
Total			593	427	72.0	30.1 ± 10.0

[a] Values are means ± standard deviations.

by restaurant J on 19 November. Restaurant J was open for tourist groups only on a subscription basis and had a kitchen staff of 10, including 2 cooks, at the time of the event. Single parties of less than 30 tourists each visited restaurant J each day between 15 and 17 November. However, a total of 11 groups ate food from restaurant J on 18 and 19 November: 593 tourists among 5 groups (groups A to E1) ate food from the restaurant on 18 November, and 931 tourists among 7 groups (groups A, E2, and F to J) ate food from the restaurant on 19 November (Tables 1 and 2).

Epidemiological investigation. A retrospective cohort study of the 11 groups that ate food from restaurant J on 18 and 19 November was conducted. Since the case patients became ill at home or during their trip after they left Nagasaki City, information was obtained through the local public health centers in the administrative regions where the case patients affected by gastroenteritis lived. The questionnaires, standardized by the Ministry of Health, Labour and Welfare, were used to obtain information about the sex and age of each of the patients, the time of onset and nature of their symptoms, and what foods they ate.

A case was defined as the development of at least two of the following symptoms in any tourist who had eaten food from restaurant J on 18 and 19 November: nausea, vomiting, diarrhea, abdominal pain, and fever.

The restaurant employees were interviewed in detail. We investigated the hotels and other restaurants in Nagasaki City that the 11 groups used during their trips. We also interviewed other tourist groups that visited Nagasaki City during the same period but that did not consume food from restaurant J. Information on the secondary cases was gathered through the local public health centers.

Environmental investigation. The facility was inspected by the Food Hygiene Section of the Nagasaki City Health Department on 20 and 21 November. The storage conditions of the meals and bulk food items were investigated, and several food samples were taken. A total of 29 smears of environmental samples were also taken from the restaurant, including the kitchen and the washroom. Stool specimens from all kitchen staff were submitted on 21 and 22 November.

Microbiological investigation. The vomit and stool specimens from the case patients were cultured for bacterial enteropathogens, including *Salmonella*; *Shigella*; enteropathogenic *Escherichia coli*, including *E. coli* O157; *Campylobacter*; *Yersinia*; *Vibrio*; *Aeromonas*; *Plesiomonas*; *Staphylococcus aureus*; *Clostridium perfringens*; and *Bacillus cereus*. Approved standard laboratory methods were used for all bacteriological investigations.

RNA extraction, RT-PCR, and sequencing. Samples and specimens were examined for NVs by RT-PCR, as described elsewhere (24, 33). Genogroup-specific primers were used to amplify the partial capsid region of NVs by RT-PCR (16, 24), as follows: primers COG1F and G1-SKR and primers COG2F and G2-SKR for amplification of the GI and GII NVs, respectively. For some samples, a nested PCR was performed with primers G1-SKF and G1-SKR (GI) and with primers G2-SKF and G2-SKR (GII). We also quantified the NV capsid genes for some PCR-positive samples by using a real-time PCR, as described previously (13, 24). The detection limits were 10^1 and 10^2 copies for the food and environmental samples and the clinical specimens, respectively (data not shown).

The capsid sequences were aligned, and the nucleotide sequence identities were analyzed with GENETYX-MAC software (version 11.0). The nucleotide sequences were compared with those of reference strains of NVs obtained from GenBank for the phylogenetic analysis, as described previously (14).

Statistical analysis. Data are presented as means (standard deviations and ranges) or as counts or proportions. Student's *t* test was used to compare the means between the two groups. The chi-square test was used to assess the statistical significance of the associations among variables. We calculated odds ratios (ORs) using Woolf's procedure and multivariate ORs using multiple logistic regression analysis (SAS, version 8.2) for each group and Mantel-Haenszel ORs for all subjects together, with 95% confidence intervals (CIs), to assess whether there was any association between illness and an individual meal, food, or food item. A P value less than 0.05 was considered significant.

Nucleotide sequence accession number. The NV capsid sequence data have been submitted to GenBank and assigned accession number AY590117.

RESULTS

Epidemiological investigation. All 10 tourist groups in which gastroenteritis cases occurred had eaten lunch at restaurant J or ate box lunches prepared by this restaurant. By contrast, there were no reports of illness among 44 tourist groups (2,371 persons) who visited Nagasaki City during the same period but who did not dine at restaurant J ($P < 0.001$). No hotels or restaurants, other than restaurant J, where the 10 groups stayed or visited reported the occurrence of gastroenteritis. Consequently, restaurant J was concluded to be the causative facility of the outbreak.

Tables 1 and 2 show the times and the dates when the

TABLE 2. Characteristics, attack rates, and incubation times for the groups that ate at restaurant J on 19 November

Group	Type of group	Time of visit	No. of tourists	No. of patients	Attack rate (%)	Incubation time (h)[b]
F	High school excursion from Hokkaido	Box lunches	163	97	59.5	34.2 ± 10.4
G	Elementary school excursion from Kumamoto	11:00	145	37	25.5	28.3 ± 12.7
E2	High school excursion from Aichi	11:45	294	63	21.4	39.3 ± 16.4
H	Elementary school excursion from Kumamoto	12:10	169	35	20.7	24.5 ± 15.0
I	Adults from Gunma	12:10	15	0	0.0	
A	Junior high school excursion from Kagoshima	12:40	32	25[a]	78.1[a]	27.8 ± 7.1[a]
J	Junior high school excursion from Kagoshima	12:50	113	1	0.9	25.8
Total			931	233	25.9	33.1 ± 14.2

[a] The case patients were thought to be infected on the first day because of the incubation period.
[b] Values are means ± standard deviations.

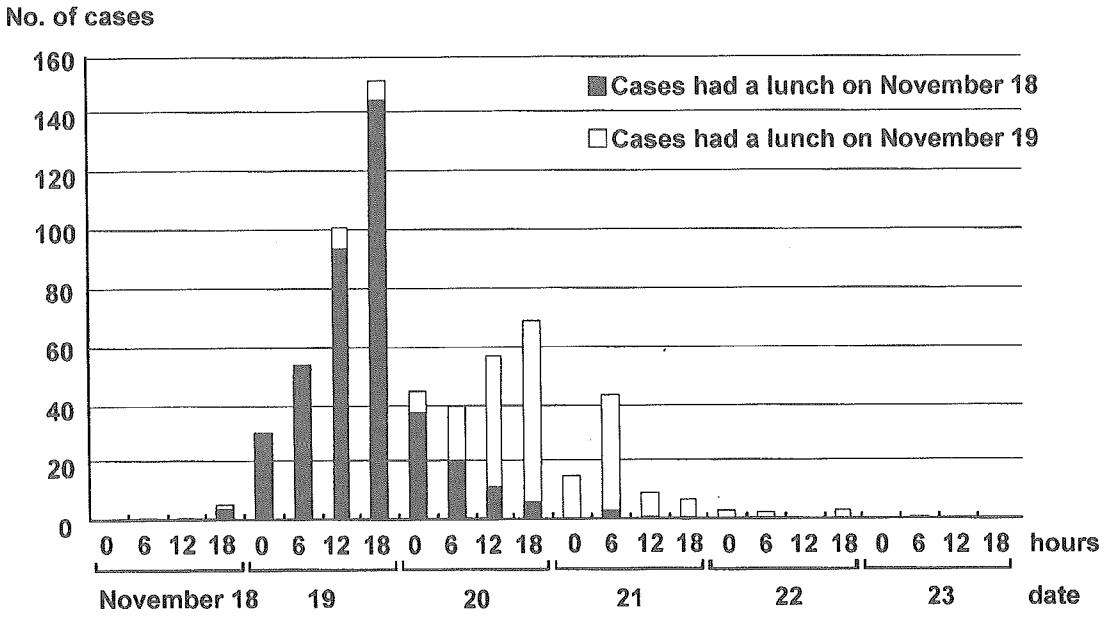

FIG. 1. Epidemic curve of cases, by hours and dates of onset of symptoms. The *x* axis presents the times (in hours) and the days when the onset of symptoms occurred.

tourists visited Restaurant J, the type of tour, the numbers of tourists and cases, the attack rates, and the incubation times for each group. Group A consumed meals from restaurant J on both 18 and 19 November. Groups E1 and E2 belonged to the same school and visited restaurant J on 18 and 19 November, respectively. The questionnaires were received from 97.3% of the tourists (35.3 to 100% for each group). Most groups responded very well (96.1 to 100%), whereas group D, which consisted of adult individuals only, responded poorly (35.3%).

Of the 1,492 tourists who used restaurant J, 660 developed illnesses that met the case definition. Thus, the overall attack rate was 44.2%. The mean age was 17.0 ± 8.4 years (age range, 11 to 74 years); and 90.6% of the cases occurred among students in elementary, junior high, and high schools (age range, 11 to 18 years). There was no sex-related difference in the attack rates, which were 46.8% for males and 44.7% for females ($P = 0.64$). The attack rates were invariably greater than 70% for the students who had lunch at restaurant J on 18 November, while they gradually decreased for those who had lunch there on the next day. There was a significant difference ($P < 0.001$) in the attack rates between the groups that ate lunch on the first day (72.0%) and the next days (25.9%) of the outbreak. The attack rate was low in groups B and D, and there were no illness in group I; although the amounts and types of foods consumed did not differ, all these groups were commonly adult tourist parties. The symptoms most commonly reported by case patients were nausea (87.0%), vomiting (71.8%; 4.0 times a day, on average), abdominal pain (69.5%), fever (68.6%), and diarrhea (54.4%; 3.1 times a day).

The epidemic curve shows two peaks (Fig. 1), but each peak represents a cluster of cases among those who ate food from the restaurant on either 18 or 19 November and has a pattern characteristic of a single-exposure, common-vehicle outbreak. The mean incubation time was 31.2 ± 11.7 h, and there was no difference in the incubation times between the tourists who consumed food from restaurant J on the first day (30.1 ± 10.1 h) and those who consumed food from the restaurant on the next day (33.1 ± 14.2 h) ($P = 0.32$).

Groups A and F had box lunches prepared by restaurant J and commercially available tea in a plastic bottle and consumed the box lunches on a ferry and a train, respectively. The same food items were assorted in the box lunches for these two groups. All other groups had lunch at restaurant J and had cold tea prepared by the restaurant. Although the combination of foods was not always identical, most foods were common in the lunches served to each group. When analysis was performed for each group separately, illness was statistically significantly associated with a specific food in three groups: Sara-Udon (thin fried rice noodles with mixed vegetables and seafood) in group C (OR, 3.1; 95% CI, 1.1 to 8.7; $P = 0.03$), deep-fried spring roll in group E1 (OR, 2.3; 95% CI, 1.1 to 4.7; $P = 0.02$), and boiled broccoli in group F (OR, 2.4; 95% CI, 1.2 to 4.6; $P = 0.01$). When analysis was performed for all subjects stratified together by group and day, deep-fried spring roll (Mantel-Haenszel OR, 2.06; 95% CI, 1.39 to 3.05; $P = 0.0004$), boiled broccoli (Mantel-Haenszel OR, 2.41; 95% CI, 1.29 to 4.51; $P = 0.009$), and raw lettuce (Mantel-Haenszel OR, 2.12; 95% CI, 1.13 to 3.95; $P = 0.03$) were significantly associated with illness. It may deserve to be mentioned that the four food items described above, the Sara-Udon, deep-fried spring roll, boiled broccoli, and raw lettuce, were handled with bare hands after cooking or washing. However, none of the groups were served all four of these items together. When deep-fried spring roll, boiled broccoli, and raw lettuce were included in the same model simultaneously, only boiled broccoli was significantly associated with illness (multivariate OR, 2.0; 95% CI, 1.0 to 3.9; $P = 0.05$) in groups A and F, to which all three of these

food items were served. However, none of these items that was significantly associated with illness was common to all groups.

There were two reports on the occurrence of secondary cases, besides the tourists: (i) NVs were detected in 2 sick employees of the hotel where group E stayed on the trip after visiting Nagasaki City, and (ii) 21 family members of 16 case patients in group C became sick.

Environmental investigation. On 14 November, the chief cook who was in charge of food hygiene at the kitchen had quit his job. This loss of staff, together with an extraordinary number of guests, made the business in the kitchen of the restaurant hectic during the 2-day period. One of the cooks felt general fatigue from 16 November and took an over-the-counter cold medicine on 19 November, although he allegedly had no gastrointestinal symptoms. No other restaurant staff allegedly had any illness during or immediately before the event. None of the employees reported that they had eaten raw shellfish, such as oysters, during the several days prior to the outbreak, and no family members of the employees were sick. All kitchen staff had eaten at least one meal at restaurant J on 18 and/or 19 November.

Restaurant J had only one washroom, which was located adjacent to the kitchen and which was used by both employees and tourists. Since there was no sink for hand-washing in the kitchen, the cooks washed their hands in the sink used to wash vegetables and kitchenware and wiped their hands on their aprons. The cooks and the other food handlers mostly handled the food items with their bare hands. Containers were commonly used for the food items before and after cooking. The same chopping board was used for different food items. The lettuce for the box lunches was washed with bare hands and soaked in water overnight, as was the boiled broccoli. The cold tea was prepared in a big bucket with hot water and then cooled with cubes of ice made in the ice machine in the kitchen.

In addition to the 29 environmental samples, a total of 58 meals served between 15 and 19 November were stored for the investigation and 9 bulk food items, such as frozen seafood, including bivalves similar to clams (*Paphia vernicosa*), had been kept during the inspection and were available for the investigation.

Microbiological investigation. Stool specimens (from 77 case patients) and vomit specimens (from 54 case patients) were obtained from a total of 124 case patients. Although *S. aureus* enterotoxins were detected in two vomit specimens from students in group E, the toxins from the two case patients were different: enterotoxin A and enterotoxin B, respectively. *Aeromonas hydrophila* was detected in a stool specimen from a case patient in group F. No enteropathogenic bacteria were detected in the other case patients, stool specimens from the kitchen staff, or the environmental samples from restaurant J.

RT-PCR and sequencing. Amplification by RT-PCR with genogroup-specific primers demonstrated the presence of 387-bp bands corresponding to GII NV (Fig. 2). GII NVs were detected in 87 of 124 case patients (70.2%; 44 of 54 vomit specimens [81.5%] and 48 of 77 stool specimens [62.3%]). No food samples were positive for NV, even after the nested PCR. Of the 29 environmental samples tested, only 1 was positive for GII NVs by the nested PCR (product size, 344 bp) (data not shown), and this sample was taken from the table where Sara-

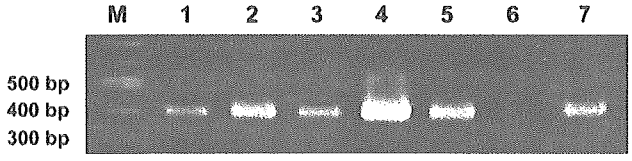

FIG. 2. Detection of NV capsid genes from specimens and samples by RT-PCR with genogroup-specific primers. The PCR products were electrophoresed on a 1.5% agarose gel. Lane M, marker (100-bp ladder; New England BioLabs Inc., Beverly, Mass.); lane 1, fecal specimen from the cook with general fatigue; lane 2, fecal specimen from another member of the kitchen staff (server); lanes 3 to 5, fecal specimens from representative case patients; lane 6, negative control (free of viral DNA); lane 7, positive control for genogroup II (strain Arg320; GenBank accession number AF190817). The GII NVs capsid gene (387 bp) was amplified and detected in the fecal specimens (lanes 1 to 5).

Udon was dished up. GII NVs were also detected in the stool specimens from 5 of 10 kitchen staff, including 2 cooks and 3 servers.

Real-time PCR quantification of the NVs revealed 61.5 copies/cm^2 in the table sample and 3.7×10^8 to 9.4×10^9 copies/g in the stool specimens from the kitchen staff. The capsid sequence analysis revealed that the NVs in all samples from the case patients, the kitchen staff, and the environmental sample had identical sequences (GenBank accession number AY590117). The genotype is most closely related to the well-characterized genotype Mexico/89/MX (GenBank accession number U22498), with 94.9% identity at the nucleotide sequence level (Fig. 3). The sequence in GenBank most closely related to the sequence that we obtained was Oberhausen455/01/DE (GenBank accession number AF425768), with which our sequence had 98.9% identity at the nucleotide level and which was originally from an outbreak in Germany.

DISCUSSION

To our knowledge, this is the largest food-borne gastroenteritis outbreak in terms of the distribution from a single causative facility into diverse geographic locations across the country, and the existence of an outbreak was unambiguously shown by linking classical and molecular epidemiological measures to a single GII NV strain of the Mexico genotype. Although recent papers have shown that new GII/4 NVs emerged in Europe (18) and on cruise ships in the Unites States (32), the causative NV in our study was classified as a different subtype, subtype GII/3. The outbreak described here is thought to be unique in that several tourist groups from across Japan were affected with gastroenteritis by exposure to NVs from a specific restaurant during a defined period of time and became ill at home or on the continuation of their trips; consequently, the specific virus has since spread into multiple prefectures. Such spread of a single infectious agent by travelers who play the role of disease transmission vehicle should be cautionary, as the outbreak is further proof of one of the contemporary modes of transmission of infectious diseases. Actually, Beller et al. (2) reported on a waterborne outbreak of illness caused by NVs in tourists traveling by bus between the United States and Canada. Furthermore, Noel et al. (25) reported that NV outbreaks due to a single virus occurred in seven countries on five continents during the 1995-1996 sea-

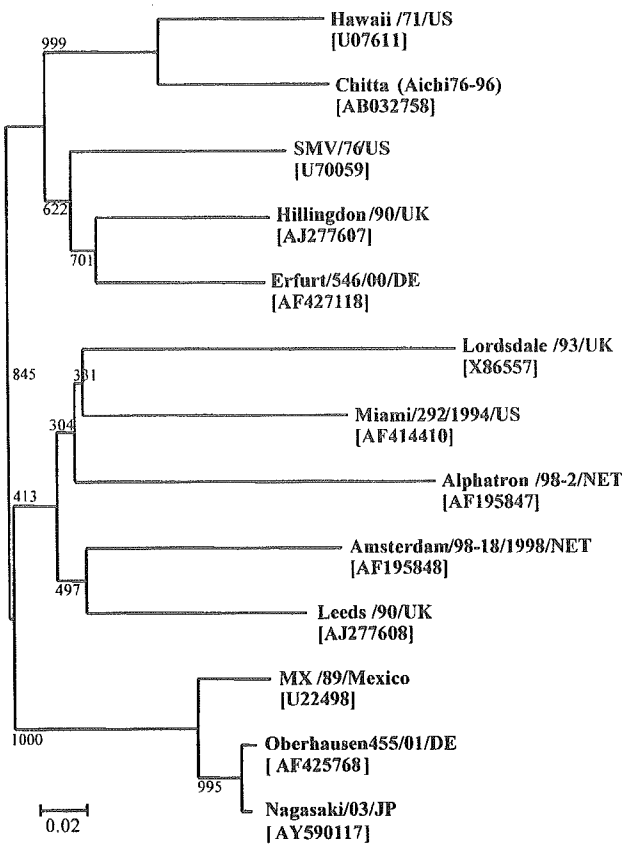

FIG. 3. Phylogenetic tree constructed on the basis of the sequences of a part of the capsid gene of GII NVs from the present outbreak and known strains from the GenBank database. GenBank accession numbers for the strains are indicated in the parenthesis. The causative viral strain of the present outbreak is shown as Nagasaki/03/JP. The numbers at each branch indicate bootstrap values for the clusters supported by that branch.

son, suggesting that the circulation of the strains might involve patterns of transmission not previously considered.

Food-borne vehicles of NVs are typically contaminated bivalve shellfish, such as oysters, items contaminated by infected food handlers, or vegetables or fruit contaminated by irrigation or washing (20). In restaurant J, frozen imported bivalve shellfish was initially suspected as the cause of infection, but no NV was detected in either the shellfish or other food samples. In outbreaks originating from infected food handlers, specific food is not always identified as the main source of the infection (5, 12, 15). Lopman et al. (19) have recently reported that specific vehicles were implicated in 39.1% of NV food-borne outbreaks and that multiple food vehicles contributed to some outbreaks. In the present outbreak, it is still unknown whether a sick cook was first infected with NV and subsequently other kitchen staff and tourists were infected or whether the kitchen staff was infected simultaneously with tourists by unknown transmission routes. However, we believe that several foods were contaminated by employees working at restaurant J. This is supported by the facts that (i) identical NVs were detected from the kitchen staff, the kitchen environment, and case patients; (ii) no NVs were found in meal or food samples; (iii) no common foods were suspected as the main source of infection; (iv) there were no differences in the attack rates between groups of tourists who ate box lunches prepared by the restaurant and those who ate at the restaurant, even though there was a great difference in the combinations of foods consumed; (v) the kitchen staff mostly handled food items with bare hands; and (vi) the kitchen staff used poor food-handling hygiene.

Although we failed to obtain a sample of ice tea prepared by the restaurant, the attack rate among the students who consumed commercially available bottled tea did not differ at all from that among those who consumed ice tea prepared by the restaurant, suggesting that waterborne transmission was much less likely. Indirect contamination in the washroom was also less likely because NV was not detected in the washroom and the illness occurred in the tourists who did not visit the restaurant. Unfortunately, we failed to obtain a sample of water in which the lettuce and broccoli were soaked overnight. The attack rates were significantly lower in the tourists who ate food from the restaurant on 19 November than those who ate food from the restaurant on 18 November ($P < 0.001$), and the rates dropped steeply on 19 November, suggesting that the foods were substantially more contaminated on 18 November (Tables 1 and 2). The fact that the attack rates for groups E1 and E2 (77.6 and 21.4%, respectively), which had the same background, showed a significant difference ($P < 0.001$) supports this hypothesis (Tables 1 and 2).

Although the highest incidence of NV infections is in children under 5 years, NV infections can occur at any age (20). In the outbreak reported here, all tourists ate a similar combination of foods at the restaurant, while the attack rates for adult tourist groups were much lower than those for student tourist groups. This suggests that NV gastroenteritis may tend to cause more severe illness in children and adolescents than in adults. This is consistent with the findings of a proportion analysis study conducted in The Netherlands (4), which showed that individuals in the age group of 18 to 64 years demonstrated a lower infection rate than individuals in younger and older age groups. Although the average incubation time in the present outbreak was thought to be typical for primary NV gastroenteritis, it is possible that some cases with apparently longer incubation periods were probably due to secondary person-to-person transmission, since most tourist groups continued their tours after they left Nagasaki City.

The sudden emergence and spread of a single strain raise important public health implications about the mode of transmission that permitted the rapid radiation of a single virus (6). It is generally believed that the movement of people from one place to another, whether it is through tourism or other means, may have profound effects on the dissemination of NVs into different populations, but there is not much evidence that directly supports such a hypothesis. In this regard, this study provides a unique opportunity to gain insight into the question of how various genotypes of NVs emerge, cocirculate, and disappear in different geographic locations. It is important and interesting to use modern molecular biology-based techniques to keep track of where this NV outbreak strain will spread and if it will cause outbreaks in Japan or elsewhere in the world. For this purpose, enhanced vigilance that includes the pursuit and characterization of secondary cases that follow outbreak