Table 3. Summary of cross-reactivities among VLPs

Each letter represents one strain. For example, GII/1 antiserum cross-reacted with two GII/6 strains (A and B), where A, strongly (i.e. identical to the homologous VLP titre), B, moderately strongly (i.e. twofold lower than the homologous VLP titre), and C, moderately (i.e. fourfold lower than the homologous VLP titre). For simplicity, we have excluded the homologous reactivities.

			VLPs												
	Genogroup		GI			GII									
		Genotype	4	8	11	1	2	3	4	5	6	7	10	12	17
Antiserum	GI	11 (strain #8)	C	C		C	C	CCCC	C	C	AA	C	C	C	C
	GII	1 (strain HV)						CC			AB		C	CC	
		6 (strain 7k)			C										
		10 (strain 026)				CC				C		C		CC	
		12 (strain CHV)				CC							C		

alignment of these five GII/3 VP1 sequences showed no unusual insertions, deletions or recombination sites; in fact, the shell domain was highly conserved among the GII/3 sequences (data not shown). However, the 1152 VP1 sequence had three unique amino acid residues (Thr-285, Ile-372 and Ser-508) when compared with the other four GII/3 VP1 sequences. The first two residues were located in the outermost region of the P2 domain, whilst the third residue was located within the P1 domain (data not shown). We used the PSIPRED secondary structure prediction software (McGuffin *et al.*, 2000) to compare the five GII/3 VP1 structures. We found that the predicted VP1 structures for 809, Sh5, 18-3 and 336 had a helix between residues 219 and 237, whereas this helix structure was absent for 1152 (Fig 5). These data suggested that the helix structure may play an important role in influencing the cross-reactivity among the GII/3 VLPs and antisera.

DISCUSSION

In this study, we analysed NoV capsid-based grouping and cross-reactivity among 26 different VLPs belonging to six GI and 12 GII genotypes. Using an antibody ELISA, we found that the antisera reacted strongly against the homologous VLPs with titres ranging from 102 400 to 1 638 400. As summarized in Table 3, we also observed strong, moderately strong and moderate cross-reactivities among different genotypes (i.e. equal to the homologous VLP titre and to twofold and fourfold dilutions, respectively). For example, GI/11 antiserum had a broad range of cross-reactivities, detecting two GI genotypes (GI/4 and GI/8) and 10 GII genotypes (GII/1–7, GII/10, GII/12 and GII/17); GII/1 antiserum (strain HV) had a broad range of cross-reactivities, detecting four GII genotypes (GII/3, GII/6, GII/10 and GII/12); GII/10 antisera also had a broad range of cross-reactivities, detecting four GII genotypes (GII/1, GII/5, GII/7 and GII/12); GII/6 antiserum detected GI/11 VLPs; and GII/12 antiserum (strain CHV) detected GII/1 and GII/10 VLPs.

Although antigen ELISAs are generally broadly reactive (Jiang *et al.*, 2000), this is the first report of a GI (strain #8) polyclonal antiserum cross-reacting strongly with other GII genotypes and the first report of a GII (strain HV) polyclonal antiserum cross-reacting strongly with other GII genotypes (Jiang *et al.*, 2002; Kamata *et al.*, 2005; Kitamoto *et al.*, 2002). These broad-range cross-reactivities may be due to unfolded VLPs on the microtitre plates at the high pH used (carbonate/bicarbonate buffer, pH 9·6) (White *et al.*, 1997). However, we have not found such broad-range cross-reactivities in any of our other studies (Kamata *et al.*, 2005). Conserved continuous residues in the shell and/or P1-1 domains may be the reason for these cross-reactivities against different genotypes (Fig. 4 and Table 2). However, we found that several antisera were genotype-specific, indicating that VLPs have unique epitopes.

Interestingly, we found that four types of GII/3 antisera (strains 809, Sh5, 18-3 and 336) cross-reacted moderately to weakly against GII/3 1152 VLPs (i.e. up to eightfold lower than the homologous VLP titre; Table 2). Amino acid alignments of these five GII/3 sequences revealed that 1152

Fig. 5. Schematic representations of the complete predicted secondary structures of VP1 of NoV (GII/3) strains 1152, 18-3, 336, 809 and Sh5. The level of confidence of prediction (Conf) is shown on the first line, where a tall box represents a high confidence of prediction and a short box represents a low confidence of prediction. The predicted secondary structure (Pred) is shown on the second line, where a helix is represented by a cylinder, a β-strand by an arrow and a coil by a line. The third line also shows the predicted secondary structure (Pred), where H represents a helix, E a β-strand and C a coil. The amino acid sequence (AA) is shown on the bottom line. The boxed regions in 18-3, 336, 809 and Sh5 VP1 indicate a helix structure that is absent in 1152 VP1. The amino acid residues that are unique to the 1152 sequence when compared with the other four GII/3 sequences are indicated by arrows.

had three unique amino acid residues compared with the other four GII/3 sequences (Thr-285, Ile-372 and Ser-508), two of which were located within the P2 domain (Thr-285 and Ile-372). Amino acid secondary structure predictions made using the PSIPRED secondary structural prediction software revealed that the VP1 secondary structures for 809, Sh5, 18-3 and 336 had a helix structure between residues 219 and 237; this helix structure was absent for 1152 (Fig. 5). This helix structure may, in part, influence the cross-reactivity among the GII/3 VLPs (i.e. without the helix structure); GII/3 1152 VLPs cross-reacted weakly with the other four GII/3 antisera. This suggestion may also explain NoV virulence in which some strains appear to infect a certain population over an extended period of time (Dingle, 2004; Noel *et al.*, 1999). In a recent report, single amino acid changes were suggested to represent a possible way for the virus to evade the host immunity (Dingle, 2004). In addition, one report suggested that a change in VP1 secondary structure (i.e. the disappearance of a helix structure) was responsible for a chronic NoV infection in an immunocompromised patient for over 2 years (Nilsson *et al.*, 2003).

Almost half of our constructs (strains SeV, 645, CV, HV, Ina, 809, Sh5, 18-3, 1152, 104, 754, CHV and Alph23) did not include the ORF3 sequence, which encodes a minor capsid protein (VP2) thought to increase the stability of NoV VLPs and may function in RNA genome packaging (Bertolotti-Ciarlet *et al.*, 2003). For rabbit haemorrhagic disease virus, VP2 is essential for the production of infectious virus (Sosnovtsev & Green, 2000). Nevertheless, we found that all constructs with or without ORF3 sequences expressed VLPs that were morphologically similar to native NoV (Fig. 2). Further studies are needed to determine whether VP2 has some influence on antigenicity.

In conclusion, this cross-reactivity study represents the most extensive undertaken for any genera in the family *Caliciviridae*. Since human NoV strains cannot be propagated in cell culture systems and human serological studies have found that VLPs and native virions share similar antigenic properties, VLPs have been used to understand antigenic relationships in more detail. Further studies, such as high-resolution structural analysis of other NoV genotypes and antigenic mapping, are needed in order to explain the complex NoV antigenicity, as previously suggested (Chen *et al.*, 2004). Finally, the results and reagents from this study can be used to design detection systems capable of detecting a broad-range of genotypes in clinical specimens; in particular, GI/11 antisera may be capable of detecting at least 32 % (12/37) of the recently described NoV genotypes (Kageyama *et al.*, 2004).

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Editor-Communicated Paper

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

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

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

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

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

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

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

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

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

Materials and Methods

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

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

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

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

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

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

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

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

Results

Diagnosis and Epidemiology of NV Associated Outbreaks

Fecal samples from 40 outbreaks of acute nonbacte-

Table 1. Reference strains and genetic classification of NVs

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

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

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

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

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

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

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

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

Phylogenetic Analysis and Genotyping of NVs

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

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

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

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

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

Discussion

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

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

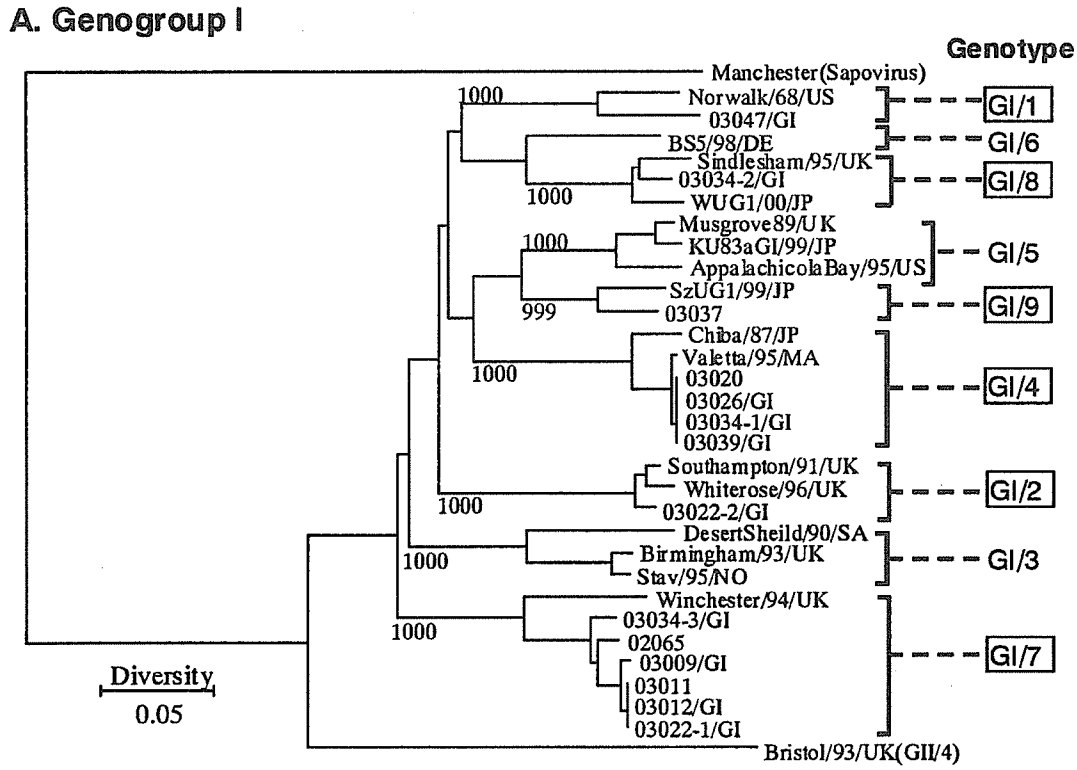

Fig. 1. Phylogenetic trees of published NV sequences and outbreak strains from this study based on the N/S domain region (GI, 290 nt; GII, 278 nt). Genogroup I NVs and Bristol/93/UK(GII/4) (A), genogroup II NVs and Norwalk/68/US(GI/1) (B) are reconstructed using Manchester *sapovirus* as the outgroup.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

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

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

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

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

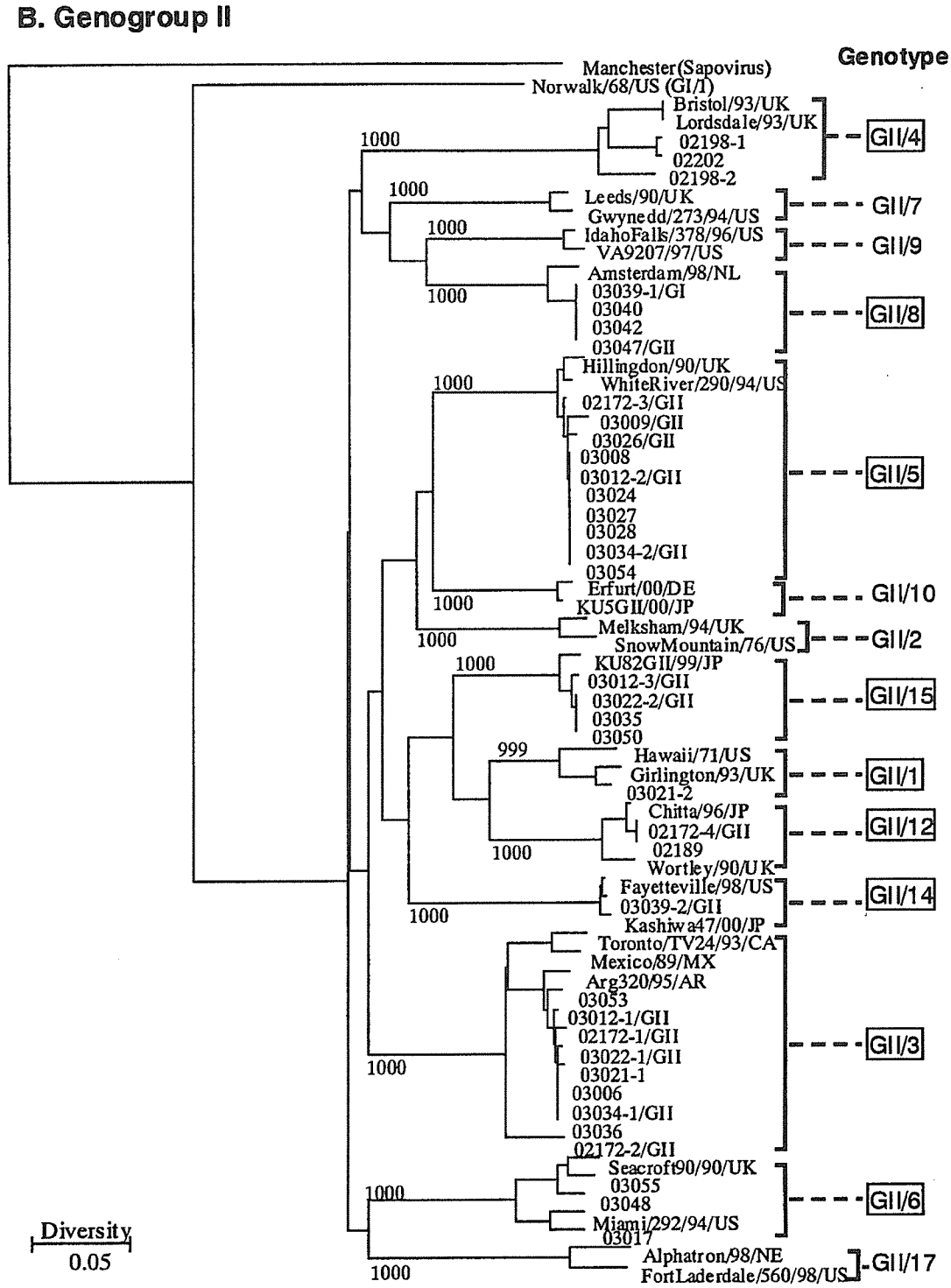

Fig. 1. B

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

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Rapid Detection of Norovirus from Fecal Specimens by Real-Time Reverse Transcription–Loop-Mediated Isothermal Amplification Assay

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In this study, we developed a one-step, single-tube genogroup-specific reverse transcription–loop-mediated isothermal amplification (RT-LAMP) assay for the detection of norovirus (NoV) genomes targeting from the C terminus of the RNA-dependent RNA polymerase gene to the capsid N-terminal/shell domain region. This is the first report on the development of an RT-LAMP assay for the detection of NoV genomes. Because of the diversity of NoV genotypes, we used 9 and 13 specially designed primers containing mixed bases for genogroup I (GI) and II (GII), respectively. The RT-LAMP assay had the advantages of rapidity, simplicity, specificity, and selectively and could obtain results within 90 min, generally even within 60 min, under isothermal conditions at 62°C. The detection limits for NoV genomes were between 10^2 and 10^3 copies/tube for GI and GII with differentiation by genotype, and no cross-reactions among NoV GI and GII and other gastroenteritis viruses, such as sapovirus, human astrovirus, adenovirus type 40 and 41, and group A and C rotavirus, were found. In the evaluation tests with fecal specimens obtained from gastroenteritis outbreaks, the sensitivity and specificity of the RT-LAMP assay with regard to RT-PCR were 100 and 94% for GI and 100 and 100% for GII, respectively. These findings establish that the RT-LAMP assay is potentially useful for the rapid detection of NoV genomes from fecal specimens in outbreaks of food-borne and person-to-person-transmitted gastroenteritis.

Noroviruses (NoVs), which belong to the family *Caliciviridae* (5), commonly cause human gastroenteritis worldwide (1, 3, 7, 10, 14) and are sometimes detected in oysters (2, 7) and clams (12). In addition, they are also recognized as a cause of person-to-person transmission of gastroenteritis in nursing homes, hospitals, and schools (5, 7, 14). The detection of NoVs is usually carried out by electron microscopy and reverse transcription-PCR (RT-PCR) because they are noncultivable (5).

Recently, the detection of NoV genomes by nucleic acid sequence-based amplification (NASBA) (6, 9, 15) as an isothermal amplification method was reported, and similarly, a transcription-reverse transcription concerted (TRC) assay (8) for the detection of NoV genomes has been commercially developed in Japan. As another isothermal amplification method, the loop-mediated isothermal amplification (LAMP) assay developed by Notomi et al. (18) is a simple method for genome diagnostics and has the advantages of high specificity and selectivity. Six primers containing two loop primers in an RT-LAMP assay are required for rapid and sensitive detection of RNA. Although more primers are needed in the RT-LAMP assay than in other amplification methods, the RT-LAMP assay has recently been applied for the rapid detection of several RNA viruses in humans, such as West Nile virus (23), severe acute respiratory syndrome coronavirus (22, 25), influenza A virus (21), mumps virus (19), measles virus (4), dengue virus (20), and respiratory syncytial virus (26).

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Because the NoVs are classified into two genogroups (GI and GII) and further divided into 14 genotypes in GI and 17 genotypes in GII according to the scheme of Kageyama et al. (10), it has been thought likely that the design of consensus primers for many genotypes of NoVs might be difficult.

In this study, we developed a genogroup-specific RT-LAMP assay for detecting NoV genomes using multiple primers containing mixed bases.

MATERIALS AND METHODS

Fecal specimens. A total of 91 fecal specimens, including 75 samples obtained from 26 gastroenteritis outbreaks and 16 additional NoV-negative samples containing three sapoviruses, three human astroviruses, one adenovirus type 40, three type 41 adenoviruses, three group A rotaviruses, and three group C rotaviruses obtained from sporadic gastroenteritis in children were used for the specificity and evaluation tests of the RT-LAMP assay. Sapoviruses and human astroviruses were detected by RT-PCR with the primers of Vinjé et al. (27) and Sakon et al. (24), respectively. Adenoviruses of types 40 and 41 and group A rotaviruses were detected by enzyme immunoassay (Adenoclone 40/41 and Rotaclone; Meridian Bioscience, Cincinnati, OH), and group C rotaviruses were detected by reversed passive hemagglutination assay (DENKA SEIKEN, Tokyo, Japan).

Viral RNA extraction. The viral RNA was extracted from 140 μl of the supernatant of approximately 10% fecal specimens with a QIAamp viral RNA mini kit (QIAGEN, Valencia, CA). The extracted RNAs were kept at −80°C until use in tests.

Primer design. The NoV-specific primers were designed on the basis of the sequence of the region from the C terminus of the RNA-dependent RNA polymerase gene to the capsid N-terminal/shell domain region, and two sets of genogroup-specific primers for GI and GII were designed. For GI, the nine primers consisted of two outer primers (F3 and B3), four inner primers (FIP1, FIP2, BIP1, and BIP2), and three forward loop primers (LF1, LF2, and LF3). For GII, the 13 primers consisted of two outer primers (F3 and B3), seven inner primers (FIP1, FIP2, FIP3, BIP1, BIP2, BIP3, and BIP4), and four forward loop primers (LF1, LF2, LF3, and LF4). FIP contain F1C, a TTTT spacer, and F2, and BIP contain B1C, a TTTT spacer, and B2. The B3 reverse outer primers for GI and GII were the G1SKR and G2SKR primers, respectively, as described else-

TABLE 1. Primer sets for genogroups I and II for detection of noroviruses

Genogroup	Primer name[a]	Type[b]	Target[c]	Sequence(s) (5′–3′)[d]
I	F3	OF	5270–5671	CCRGGNTGGCARGCNATGTT
	B3 (G1SKR)	OR		CCAACCCARCCATTRTACA
	FIP1	IF		F1C, CATTTACGAATTCGGGCAGG; F2, CGCTGGATGCGNTTCCATGA
	FIP2	IF		F1C, CATTTACAAAATCGGGCAGG; F2, CGCTGGATGCGNTTCCATGA
	BIP1	IR		B1C, GATGGCGTCTAAGGACGC; B2, AGCTGTRTTTGCCTCTGGWAC
	BIP2	IR		B1C, GATGGCGTCTAAGGACGC; B2, AGCWGTATTAACCTCCGGYAC
	LF1	LF		AGATYGCGATCYCCTGTCCA
	LF2	LF		AGATTGCGATCTCCTGCCCA
	LF3	LF		AGCTCGCGGTCTCCTGTCCA
II	F3	OF	4979–5389	GGNMTGGANTTTTAYGTGCCMAG
	B3 (G2SKR)	OR		CCRCCNGCATRHCCRTTRTACAT
	FIP1	IF		F1C, GGGAGCMAGATTGCGATCGC; F2, GAGBCNATGTTYAGRTGGAT
	FIP2	IF		F1C, GGGAGCMAGATTGCGATCGC; F2, GAGCCCATGTTCAGRTGGAT
	FIP3	IF		F1C, GGGAGCGAGATTGCGATCGC; F2, GAGTCAATGTTYAGGTGGAT
	BIP1	IR		B1C, TGTGAATGAAGATGGCGTCG; B2, CTCATTRTTRVTCTCTGGBACGAG
	BIP2	IR		B1C, TGTGAATGAAGATGGCGTCG; B2, CTCATTRTTGCYCTCTGGYACGAG
	BIP3	IR		B1C, TGTGAATGAAGATGGCGTCG; B2, CTCATTGTTGAYCTCTGGKACGAG
	BIP4	IR		B1C, TGTGAATGAAGATGGCGTCG; B2, CTCATTRTTACTTTCTGGCACGAG
	LF1	LF		GTGCTCARATCWGARAACCTC
	LF2	LF		GTGCTGAGGTCWGARAATCTC
	LF3	LF		GTGCTCAAATCTGAGAATCTC
	LF4	LF		GTGCTCAAGTCTGAGAAYCTC

[a] B3 primers used for genogroups I and II were G1SKR and G2SKR, respectively, as described by Kojima et al. (13). FIP and BIP primers consist of F1C plus TTTT plus F2 and B1C plus TTTT plus B2, respectively, where TTTT is a spacer.
[b] OF, outer forward; OR, outer reverse; IF, inner forward; IR, inner reverse; LF, loop forward.
[c] Nucleotide positions according to those of Norwalk/68/US (GenBank accession number M87661) for genogroup I and Lordsdale/93/UK (X86557) for genogroup II.
[d] Mixed bases in degenerate primers are as follows: K, T or G; M, A or C; R, A or G; W, A or T; Y, C or T; B, not A; H, not G; V, not T; N, any.

where (13). The FIP and BIP primers were high-performance liquid chromatography-purified primers, and the others were oligonucleotide purification cartridge-purified primers. The sequences and locations of the oligonucleotide primers are shown in Table 1 and Fig. 1.

Genogroup-specific RT-LAMP assay. The RT-LAMP assay was performed in 25 μl of the reaction mixture with an RNA amplification kit (Eiken Chemical, Tochigi, Japan). The reaction mixture contained 12.5 μl of 2× reaction mix, 1.0 μl of enzyme mix, 2.5 μl of RTmate (Nippon gene, Toyama, Japan), 5 pmol (each) of outer primers F3 and B3, 25 pmol (each) of inner primers FIP1, FIP2, BIP1, and BIP2 for GI or 40 pmol (each) of inner primers FIP1 and BIP1 and 20 pmol (each) of inner primers FIP2, FIP3, BIP2, BIP3, and BIP4 for GII, 20 pmol each of loop primers LF1, LF2, and LF3 for GI or 20 pmol each of loop primers LF1, LF2, LF3, and LF4 for GII, and 2 μl of RNA extract. The reaction mixture was incubated at 62°C for 90 min and heated at 80°C for 5 min in a Loopamp real-time turbidimeter (LA-320C; Teramecs, Kyoto, Japan). For performing the RT-LAMP assay, standard RNAs described below, as positive controls, and distilled water, as a negative control, were used in each test, and a turbidity value of ≥0.1 was considered positive. For measuring the detection limit of the RT-LAMP assay, the samples were tested in triplicate and the lowest concentration of genome copies was taken as the limit when all of the triplicate samples were positive. In addition, the fecal specimens that gave positive results at over 60 min were retested another time. Then, 2 μl of the amplified product from the RT-LAMP assay was electrophoresed on a 3% agarose gel in Tris-acetate-EDTA buffer, stained with ethidium bromide, and visualized under UV light, when necessary.

RT-PCR and real-time quantitative PCR. A conventional RT-PCR was performed to compare with the sensitivity of the RT-LAMP assay. The RT was first performed in 20 μl of a mixture containing 50 pmol of random nonamer [pd(N)$_9$; Takara Bio, Shiga, Japan], 20 U of RNase inhibitor (TOYOBO, Osaka, Japan), 100 U of reverse transcriptase (ReverTra Ace; TOYOBO), 2.5 mM concentrations of each deoxynucleoside triphosphate, and 9.5 μl of RNA extract. The RT conditions were 30°C for 10 min and then 42°C for 60 min, followed by 99°C for 5 min. After RT, PCR was performed in 50 μl of a reaction mixture containing 0.25 U of Ex *Taq* DNA polymerase (Takara), 2.5 mM concentrations of each deoxynucleoside triphosphate, 10 pmol (each) of primers G1SKF and G1SKR for GI or G2SKF and G2SKR for GII, as previously described (13), and 3 μl of cDNA.

For the measurement of the number of genome copies in the RT-LAMP-positive samples, real-time quantitative PCR was performed by using primers COG1F and COG1R and fluorogenic probe RING1-TP(a) for GI and primers COG2F and COG2R and fluorogenic probe RING2-TP for GII (11) with a LightCycler (Roche Diagnostics, Penzberg, Germany). The real-time PCR was performed in 20 μl of a reaction mixture containing 2 μl of 10× PCR master mix (LC FastStart DNA hybridization probes; Roche), 10 pmol of each of the primers, 8 pmol fluorogenic probe, 3 mM $MgCl_2$, and 5 μl of cDNA. The following real-time PCR conditions were used: 95°C for 10 min, 50 cycles of 95°C for 15 s, 56°C for 20 s, and 72°C for 4 s, and a final cooling at 40°C for 30 s.

Standard RNA. The plasmid DNAs containing the target sequences amplified with primer sets F3 and B3 (G1SKR) for GI or F3 and B3 (G2SKR) for GII were first prepared with the pDrive cloning vector (QIAGEN). The standard RNAs were transcribed from the plasmid DNAs by using SP6 polymerase (Ambion, Austin, TX) and were used to determine the sensitivity of the RT-LAMP assay. The standard RNAs for GI and GII belonged to genotypes GI/2 and GII/12, respectively, according to the scheme of Kageyama et al. (10).

Genotyping. After purification of the RT-PCR-positive products with a QIAquick PCR purification kit (QIAGEN), the sequencing of 260 bp was carried out with a SequiTherm EXCEL II DNA sequencing kit LC for 25- to 41-cm gels (EPCENTRE Technologies, Madison, WI) and a LI-COR 4200 series sequencer (LI-COR, Lincoln, NE). The sequences were compared with those of the reference strains of NoVs obtained from GenBank and classified into 31 genotypes as described by Kageyama et al. (10).

RESULTS

We developed a one-step, single-tube RT-LAMP assay for the detection of NoV genomes with genogroup-specific primers for GI and GII. The RT-LAMP assay was carried out with a set of 9 and 13 primers for GI and GII, respectively. These multiple primers used for the detection of many genotypes contained several mixed bases, which were not used in many other RT-LAMP assays (4, 19, 22, 23, 25). Taking the use of mixed bases into consideration, to achieve high sensitivity, the RT-LAMP reaction in this study was extended to 90 min at 62°C, followed by heat inactivation at 80°C for 5 min. The RT-LAMP assay amplified 402-bp (nucleotides 5270 to 5671 of Norwalk/68/US, GenBank accession number M87661) and 411-bp (nucleotides 4979 to 5389 of Lordsdale/93/UK,

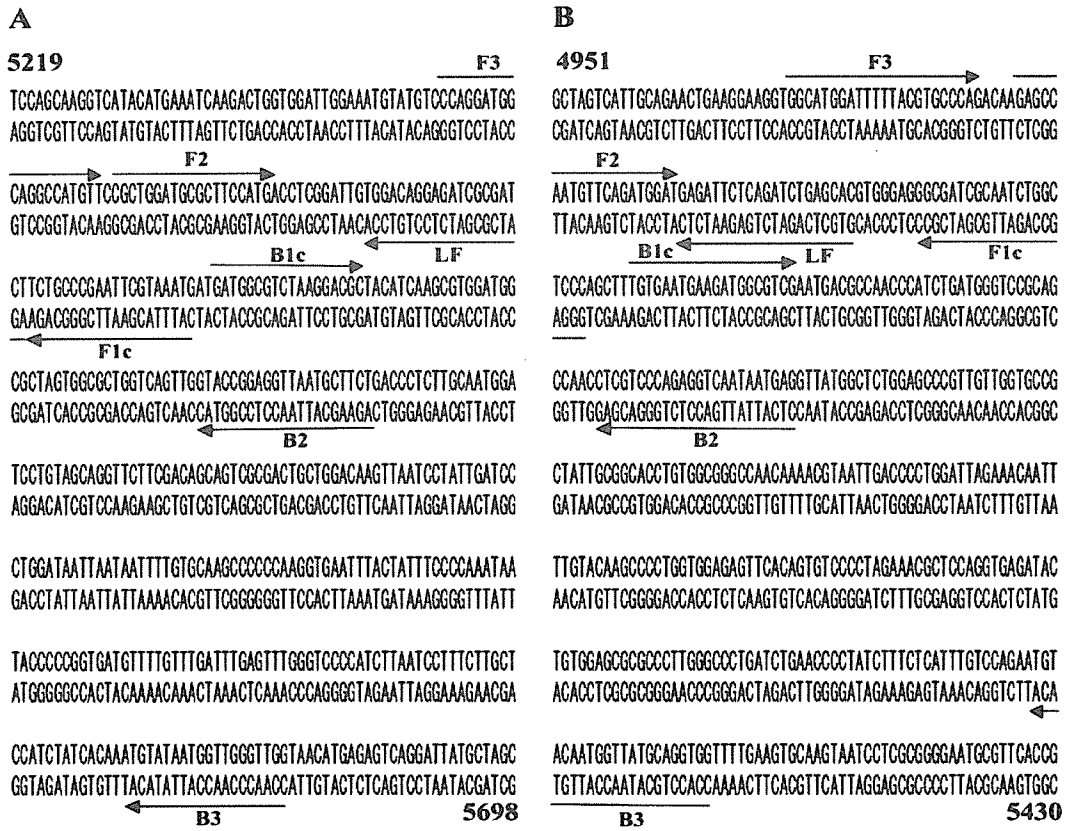

FIG. 1. Location of primers used for genogroup I (A) and II (B) for genogroup-specific RT-LAMP assay. (A) Sequence of Norwalk/68/US (GenBank accession number M87661); (B) sequence of Lordsdale/93/UK (X86557). Numbers at the beginning and end of the sequences denote nucleotide positions. Primers FIP and BIP in Table 1 consist of F1C plus TTTT plus F2 and B1C plus TTTT plus B2, respectively, where TTTT is a spacer. The B3 primers for genogroups I and II were primers G1SKR and G2SKR, respectively, as described by Kojima et al. (13).

X86557) target sequences from the C terminus of the RNA-dependent RNA polymerase gene to the capsid N-terminal/shell domain region for GI and GII, respectively.

Sensitivity and specificity of the RT-LAMP assay. The sensitivity of the RT-LAMP assay for the detection of NoV genomes was tested by using serial 10-fold dilutions of standard RNAs of GI and GII with Easy Dilution solution (Takara) and compared with that of conventional RT-PCR. The detection limits of the RT-LAMP assay for the standard RNAs of GI and GII were found to be approximately 10^2 and 10^3 copies/tube, with a time of positive change of 57 to 65 min (average, 61 min) and 57 to 60 min (average, 59 min), respectively, which were the same as for the turbidity assay, but 10-fold less reactivity than that of RT-PCR was observed (Fig. 2). A positive reaction in the RT-LAMP assay was seen as a ladder-like pattern in 3% agarose gel electrophoresis analysis. No cross-reaction was found between GI and GII with the standard RNAs of genotypes GI/2 and GII/12 at 10^8 copies/tube, respectively. Graphs of the data obtained for real-time amplification in the RT-LAMP assay are shown in Fig. 3. The times until positive change were found to be 31 min for genotype GI/2 and 20 min for GII/12. Furthermore, serial 10-fold dilutions of RNA extracts prepared from the fecal specimens were used to determine the sensitivity of the RT-LAMP assay according to genotype. For GI, the sensitivities of genotypes GI/1, GI/2, GI/4,

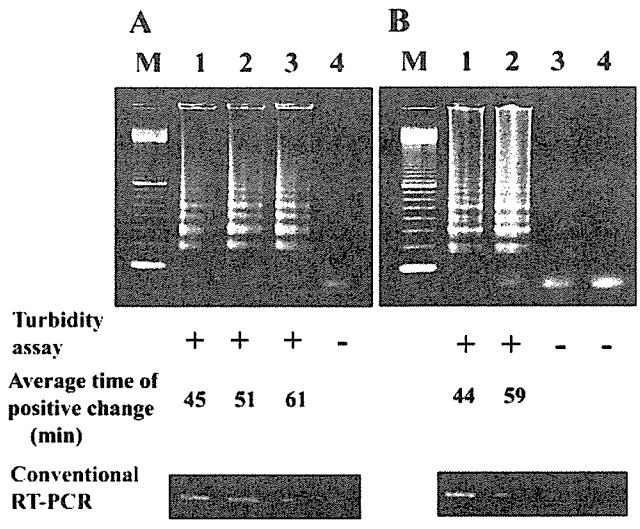

FIG. 2. Sensitivity of genogroup-specific RT-LAMP assay for standard RNAs of genotype GI/2 for genogroup I (A) and GII/12 for genogroup II (B). Lanes: M, 100-bp DNA ladder; 1, 10^4 copies/tube; 2, 10^3 copies/tube; 3, 10^2 copies/tube; 4, 10^1 copies/tube. Positive reaction shows a ladder-like pattern in 3% agarose gel electrophoresis. The turbidity was measured using an LA-320C (Teramecs), and RT-PCR was carried out with primers G1SKF and G2SKR for genogroup I and G2SKF and G2SKR for genogroup II (13). +, positive; −, negative.

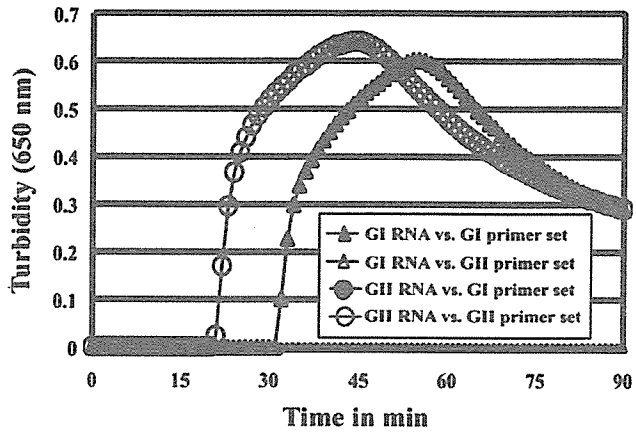

FIG. 3. Evaluation of cross-reactivity of genogroup-specific RT-LAMP assay between genogroups I and II. Standard RNAs of genotypes GI/2 and GII/12 at 10^8 copies/tube were used in genogroups I and II, respectively. The real-time turbidity was determined using an LA-320C (Teramecs).

GI/8, GI/11, and GI/14 tested were 10^2, 10^2, 10^2, 10^2, 10^2, and 10^3 copies/tube, respectively, in the turbidity assay. For GII, the sensitivities of genotypes GII/1, GII/2, GII/3, GII/4, GII/5, GII/6, GII/8, GII/12, and GII/14 tested were 10^3, 10^2, 10^3, 10^2, 10^3, 10^3, 10^3, 10^3, and 10^3 copies/tube, respectively. Thus, the sensitivity of the RT-LAMP assay was different depending on the genotype. Although the fecal specimens containing the genotypes GI/2, GI/11, and GII/5 used in this test also included other genotypes (GII/12, GII/3, and GI/14, respectively), these sensitivities were similar to the others. Furthermore, it was observed that none of the genogroup-specific primers cross-reacted between genogroups with any of the fecal specimens obtained from NoV outbreaks (Table 2).

The specificity of the RT-LAMP assay was checked using 16 fecal specimens containing the following: sapovirus (3 specimens), human astrovirus (3 specimens), adenovirus type 40 and 41 (4 specimens), and group A and C rotavirus (6 specimens). The fecal specimens containing these gastroenteritis viruses gave negative results for the RT-LAMP assay within a 90-min run (data not shown). Thus, the genogroup-specific RT-LAMP assay with the multiple primers containing mixed bases had a high degree of specificity for NoV genomes.

Evaluation of RT-LAMP assay with fecal specimens. A total of 75 fecal specimens obtained from 26 NoV outbreaks were tested by the genogroup-specific RT-LAMP assay, and the results were compared to those of conventional RT-PCR. A concordance of 95% (71 of 75) between the RT-LAMP assay and RT-PCR was observed. Of the 75 samples, 23 and 57 were positive for GI and GII, respectively, and 48 and 18 were negative for GI and GII, respectively, in both assays. The RT-LAMP assay, however, gave positive results for four RT-PCR-negative specimens of two outbreaks and detected NoV GI in these specimens. These fecal specimens were also positive by the RT-LAMP assay in retests. The sensitivity and

TABLE 2. Comparison of RT-PCR and RT-LAMP assays for detection of noroviruses in gastroenteritis outbreaks

Outbreak no.	Genogroup I			Genogroup II			Epidemiology (transmission)
	RT-PCR[a]	RT-LAMP[a]	Genotype(s)[b]	RT-PCR[a]	RT-LAMP[a]	Genotype(s)[b]	
40	●●●	●●●	GI/1, GI/1, GI/1	○○○	○○○		Person to person
38	●●○	●●○	GI/1, GI/11	●●●	●●●	GII/3, GII/3, GII/3	Food borne
35	○○●	●●●	GI/2	●●●	●●●	GII/5, GII/5, GII/5	Food borne
20	○●●	○●●	GI/2, GI/2	●●●	●●●	GII/12, GII/12, GII/14	Food borne
32	○○●	○○●	GI/2	●●●	●●●	GII/14, GII/14, GII/14	Food borne
61	●●●	●●●	GI/4, GI/4, GI/4	○○○	○○○		Person to person
43	●●	●●	GI/4, GI/4	●●	●●	GII/3, GII/3	Food borne
25	○●	○●	GI/4	●●	●●	GII/12, GII/12	Person to person
16	●●●	●●●	GI/4, GI/4, GI/8	○○○	○○○		Food borne
48	○○●	○○●	GI/8	●●○	●●○	GII/4, GII/12	Food borne
42	●○	●○	GI/14	○●	○●	GII/4	Food borne
27	●●●	●●●	GI/14, GI/14, GI/14	○●●	○●●	GII/5, GII/5	Food borne
15	○○○	○○○		●●○	●●○	GII/1, GII/1	Food borne
45	○○○	○○○		●○●	●○●	GII/1, GII/1	Food borne
60	○○○	○○○		●●●	●●●	GII/2, GII/2, GII/2	Person to person
39	○○○	○○○		●●●	●●●	GII/3, GII/3, GII/3	Person to person
49	○○○	○○○		●○●	●○●	GII/4, GII/4	Person to person
50	○○○	○○○		●●●	●●●	GII/4, GII/4, GII/4	Person to person
51	○○○	○○○		●●○	●●○	GII/4, GII/4	Person to person
55	○○○	○○○		●●●	●●●	GII/4, GII/4, GII/4	Person to person
57	○○○	○○○		○●●	○●●	GII/4, GII/4	Person to person
59	○○○	○●●		●●○	●●○	GII/4, GII/6	Food borne
28	○○○	○○○		●●●	●●●	GII/5, GII/5, GII/5	Food borne
37	○○○	○○○		●●●	●●●	GII/5, GII/5, GII/5	Food borne
54	○○○	○○○		●●●	●●●	GII/6, GII/6, GII/6	Person to person
33	○○○	○○○		●●●	●●●	GII/8, GII/8, GII/8	Person to person

[a] Same samples in order in RT-PCR and RT-LAMP assay. ●, positive; ○, negative. Each circle refers to the results from a single fecal specimen in the outbreak. Underlined results denote that positivity was detected at over 60 min in the RT-LAMP assay.

[b] The genotyping of amplicons by RT-PCR was carried out according to the scheme of Kageyama et al. (10). The column represents the genotypes identified in the outbreak and the genotypes of the positives by RT-PCR are shown in order. Eleven outbreaks were due to multiple genotypes, and one or two genotypes in each fecal specimen were identified.

TABLE 3. Numbers of genome copies in RT-LAMP-positive fecal specimens, as determined by RT-PCR

Genogroup	Genotype[a]	No. of copies/tube[b]	
		Minimum value	Maximum value
I	GI/1	4.2×10^5	6.8×10^6
	GI/2	7.2×10^2	1.4×10^5
	GI/4	1.7×10^2	3.7×10^8
	GI/8	2.0×10^4	4.5×10^5
	GI/11[c]	8.0×10^5	
	GI/14	1.6×10^3	3.0×10^6
II	GII/1	2.0×10^4	1.6×10^6
	GII/2	1.5×10^5	1.1×10^6
	GII/3	1.4×10^4	3.2×10^6
	GII/4	1.3×10^3	3.7×10^5
	GII/5	3.2×10^3	1.1×10^6
	GII/6	1.9×10^4	3.7×10^5
	GII/8	7.2×10^5	8.2×10^5
	GII/12	2.8×10^4	7.0×10^5
	GII/14	5.0×10^5	7.0×10^5

[a] Genotypes were determined according to the scheme of Kageyama et al. (10).
[b] Genome copy numbers were determined by real-time PCR with primers COG1F and COG1R and fluorogenic probe RING1-TP(a) for genogroup I and primers COG2F and COG2R and probe RING2-TP for genogroup II, as described by Kageyama et al. (11).
[c] One fecal specimen was tested in genotype GI/11.

specificity of the RT-LAMP assay with regard to RT-PCR were 100 and 94% for GI and 100 and 100% for GII, respectively. Of 27 NoV-positive specimens in GI, 22 (81%) reacted within 60 min in the RT-LAMP assay and 50 (88%) of 57 positive specimens in GII reacted within 60 min (Table 2). The genotypes detected from the 26 NoV outbreaks were divided into six types (GI/1, GI/2, GI/4, GI/8, GI/11, and GI/14) for GI and nine types (GII/1, GII/2, GII/3, GII/4, GII/5, GII/6, GII/8, GII/12, and GII/14) for GII. The RT-LAMP assay was able to detect all of these genotypes of NoV with a high degree of specificity. The number of genome copies in the RT-LAMP-positive specimens was estimated to range from 1.7×10^2 to 3.7×10^8 copies/tube in GI and from 1.3×10^3 to 3.2×10^6 copies/tube in GII (Table 3). The numbers of copies of NoV genomes in the fecal specimens obtained from gastroenteritis outbreaks in this study were higher than the levels of sensitivity of the RT-LAMP assay.

DISCUSSION

NoVs are major causative agents of nonbacterial gastroenteritis in many countries in the cold season (16). Therefore, the rapid detection of NoV genomes is needed when acute gastroenteritis outbreaks occur. The detection of NoV genomes is usually performed by RT-PCR (5), which requires 3 to 4 h. Isothermal amplification methods such as LAMP, NASBA, and TRC are available for the detection of NoV genomes within a shorter time (6, 8, 9, 15, 18), but the NASBA assay requires a further test for the confirmation of NoV genomes and the TRC assay requires a precision instrument such as a real-time fluorometer. On the other hand, the RT-LAMP assay has extremely high specificity and selectivity because of its use (generally) of six primers, with two loop primers recognizing eight distinct regions on the target sequence. The specificity and selectively of the RT-LAMP assay have been documented in previous studies (4, 19–23, 25, 26). Furthermore, the RT-LAMP reaction yields a white precipitate of magnesium pyrophosphate in the reaction mixture, and the positive reaction can be identified at the end point of the assay by the appearance of this white precipitate without a turbidimeter.

We could achieve rapid detection of NoV genomes by using a one-step, single-tube genogroup-specific RT-LAMP assay. This is the first report of the development of an RT-LAMP assay for the detection of NoV genomes. The specificity of the RT-LAMP assay for NoV genomes was established by examining the cross-reaction with standard RNAs of GI and GII, RNA templates of 15 genotypes of NoVs, and RNAs of other viruses causing gastroenteritis. The RT-LAMP assay for the detection of NoV genomes was genogroup and genus specific and can be used for the detection of NoVs from outbreaks of food-borne and person-to-person-transmitted gastroenteritis and sporadic gastroenteritis cases.

At least 14 and 17 genotypes have been found for GI and GII, respectively (10). It is difficult to detect many genotypes using an RT-LAMP assay system with a high degree of sensitivity because of the diversity of NoV genotypes. Our RT-LAMP assay was able to detect NoV genomes with sensitivity levels of approximately 10^2 to 10^3 copies/tube in serial 10-fold dilution tests of RNA templates and had good performance with regard to concordance, specificity, and sensitivity in comparison to RT-PCR. Although the sensitivity of the RT-LAMP assay differed by genotype and was not influenced by the presence of multiple genotypes in a fecal specimen, our RT-LAMP assay was sufficient to detect NoV genomes from fecal specimens. However, it may be necessary to further lower the limit of detection because the numbers of copies of the NoV genome in foods such as oysters are low (17).

A total of 15 genotypes (GI/1, GI/2, GI/4, GI/8, GI/11, and GI/14 of GI and GII/1, GII/2, GII/3, GII/4, GII/5, GII/6, GII/8, GII/12, and GII/14 of GII) tested could be detected by our RT-LAMP assay. The sensitivity of the detection of the remaining 16 genotypes (GI/3, GI/5, GI/6, GI/7, GI/9, GI/10, GI/12, and GI/13 of GI and GII/7, GII/9, GII/10, GII/11, GII/13, GII/15, GII/16, and GII/17 of GII) by our RT-LAMP assay must be tested in further studies, although the primers used in the RT-LAMP assay were probably designed as consensus primers for the NoV sequences obtained from GenBank, and it is expected that the RT-LAMP assay will also be able to detect the remaining genotypes not tested in this study.

We expect that the genogroup-specific RT-LAMP assay will be routinely used in most laboratories because of its simplicity, specificity, and selectively. The greatest advantage of the RT-LAMP assay is the substantial reduction in required time compared to that required for RT-PCR. Our simple-to-use genogroup-specific RT-LAMP assay shortens the amplification time to within 90 min or even 60 min in most cases.

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Shifting Seroepidemiology of Hepatitis A in Japan, 1973–2003

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***Abstract*: *Background.* Hepatitis A infection is caused by hepatitis A virus (HAV) contracted through fecal-oral transmission. Life-long immunity is conferred after infection. Improved sanitary conditions have generally resulted in a significant decline in the incidence of hepatitis A. However, a low incidence of infection results in increased HAV susceptibility. The present study investigates the prevalence of anti-HAV antibody and clarifies the current HAV status and HAV susceptibility in Japan at 2003. *Methods.* A total of 2,430 serum specimens collected during 2003 from Japanese individuals ranging in age from 0–92 years, were tested for anti-HAV antibody using an inhibition enzyme linked immunosorbent assay. All specimens were obtained from the WHO and the National Serum Reference Bank/National Institute of Infectious Diseases, Tokyo, Japan. *Results.* The overall seroprevalence was 12.2%. Anti-HAV antibodies were rarely detected in individuals between 0–44 years of age. Starting from the age of 45–49 years, seropositivity gradually increased through age 65 years and above. Seroprevalence was not affected by gender, and geographic distribution did not affect age-specific seroprevalence until the age of 60 years. *Conclusions.* HAV susceptibility in Japan is increasing annually. Particularly, the prevalence of anti-HAV antibody in individuals older than 50 years in 2003 was 50.3%, which is significantly lower than that of corresponding studies in 1994 (74.3%), 1984 (96.9%) and 1973 (96.9%). The growing susceptible population of advanced age results in more frequent HAV infection among them. The surveillance of anti-HAV antibody prevalence is useful for implementing preventive measures and for controlling the spread of HAV.**

***Key words*: Hepatitis A, Seroepidemiology, Anti-HAV antibody**

Hepatitis A virus (HAV) is a pathogen of human acute hepatitis and is considered to be one of epidemiologically important viruses. It is a positive-strand RNA virus belonging to the Family *Picornaviridae*, genus *hepatovirus* (17).

Most HAV infections occur through fecal-oral transmission, either by direct contact with an infected person or by ingestion of food or water contaminated with HAV (5). Improved sanitary conditions and hygiene practices have reduced the incidence of hepatitis A infection. Children who become infected are usually asymptomatic or develop mild symptoms, whereas adults infected with hepatitis A develop fever, fatigue, malaise and jaundice that lasts between 4 and 10 weeks. Regardless of symptoms, seroconversion occurs after infection and convalescent individuals develop permanent immunity (13).

Hepatitis A virus has only one known serotype and anti-HAV antibody (anti-HAV) induced by infection or vaccination protects individuals against infection with any HAV strains (14). Both immunoglobulin M (IgM) and immunoglobulin G (IgG) types of anti-HAV antibodies are detectable during the early stages of illness. Levels of IgM antibody usually diminish within 2 or 3 months after infection, but IgG antibody confers life-long immunity. Individuals without anti-HAV antibodies are susceptible to HAV infection.

Improved socioeconomic status, urbanization, ethnic origin, and access to clean water and sanitation have

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Abbreviations: anti-HAV, anti-hepatitis A virus antibody; ELISA, enzyme linked immunosorbent assay; GMT, geometric mean titer; HAV, hepatitis A virus; IgG, immunoglobulin G; IgM, immunoglobulin M; OD, optical density.

changed hepatitis A epidemiology. The distribution of anti-HAV seroprevalence by age group might reflect current hepatitis A status in various countries and regions (7, 11).

In highly endemic areas, people become infected with HAV during early childhood and acquire anti-HAV antibodies before 10 years of age. As infections in early childhood are either asymptomatic or mild, hepatitis A is not a clinical problem and the virus is recognizable only by serological studies or the occurrence of infection among visitors.

In intermediate or transition areas, HAV circulation has been reduced by improving hygiene conditions and socioeconomic status. Those who become infected are older in such areas than in highly endemic areas. The incidence of symptomatic hepatitis A is higher in adolescents and young adults. Frequent outbreaks of hepatitis A are recognized as serious public health problems (21).

In low endemic areas, rare incidence results in the accumulation of non-immune populations. Susceptible adults who are exposed to HAV infection and symptomatic hepatitis A are mostly high risk groups such as child care providers, hospital workers or family members with direct patient contact, travelers to endemic areas, drug abusers and men who have sex with men.

The incidence of hepatitis A has decreased in Japan mainly due to improved sanitary conditions and hygiene practices (Fig. 1) (15) and thus an HAV-susceptible population has developed.

This study examines the current seroprevalence of hepatitis A in Japan, and describes the seroepidemiological shift over the past three decades. The results should provide useful epidemiological information upon which to base decisions that will prevent and control the spread of hepatitis A.

Materials and Methods

Serum specimens. A total of 2,430 serum specimens (1,242 and 1,188 from males and females, respectively, aged between 0 and 92 years) were collected from 12 Japanese prefectures during 2003 and tested for anti-HAV. All specimens were obtained through the WHO and National Serum Reference Bank/National Institute of Infectious Diseases, Tokyo, Japan and stored at −20 C. The specimens were separated into groups based on age, gender and geographic location for further analysis.

Anti-HAV screening. Total anti-HAV antibody was determined for all specimens using an inhibition enzyme linked immunosorbent assay (ELISA) as described (12). Briefly, purified inactivated HAV (KRM003 strain, genotype IIIB) was bound to microtiter plates (Nunc, F96 CERT.MAXISOAP, Denmark) coated with anti-HAV rabbit serum (HAV-plates). Non HAV-binding wells prepared on each HAV-plate comprised positive controls (100% inhibition). Negative controls comprised several HAV-binding wells. Diluted test specimens (1:32) were added to appropriate wells. Equal volumes of diluents (phosphate-buffered saline (pH 7.2) containing 0.5% skim milk and 0.05% Tween 20) were added to both positive and negative control wells. Plates were incubated overnight at 4 C and then the wells were emptied. Diluted horseradish peroxidase (HRPO)-conjugated anti-HAV rabbit IgG was added to the wells and incubated at 37 C for 2 hr in

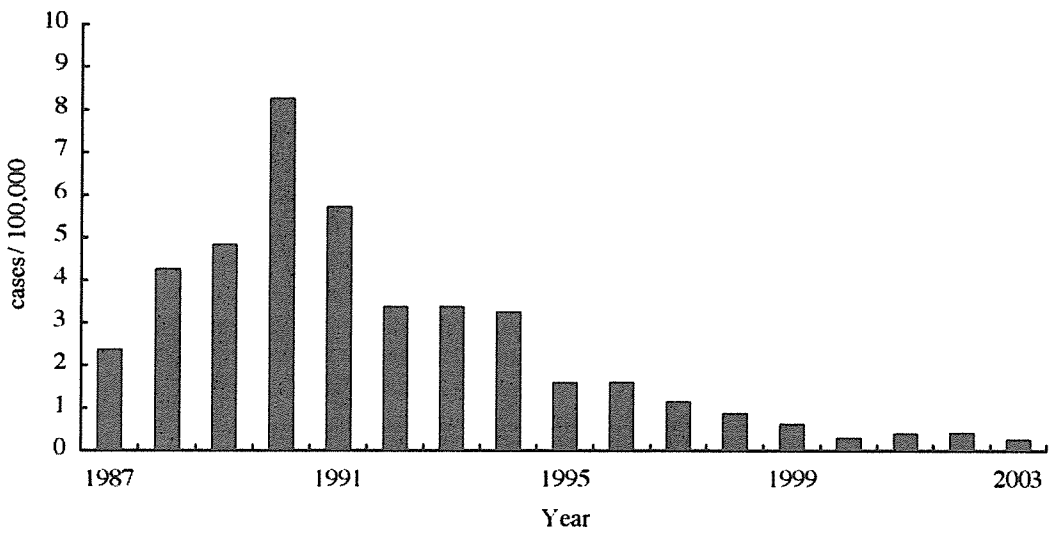

Fig. 1. Reported incidence of hepatitis A, Japan, 1987–2003. Data are cited from Ref. 15.