sity among these amplicons exceeded 10% (see Fig. 2), representing wide genetic diversity. Some human NVs found in stools from gastroenteritis patients who lived near oyster sampling sites (within about 30 km) during the investigation period between December 2001 and February 2002 were genetically related to our strains from oysters. Approximately 30 to 40% diversity was noted between some animal calicivirus strains and some of our strains from oysters.

Quantitation of NV capsid gene by real-time PCR. As shown in Table 2, more than 10^2 copies of the NV genome were found in 11 of 191 oysters (6%) (3 from genogroup I and 8 from genogroup II). More than 10^3 copies of the NV genome were detected in four oysters harvested in area A. In January 2002, about 15% of oysters harvested in both areas (6 of 40 oysters from area A and 3 of 20 oysters from area B) had large amounts of the NV genome. In February 2002, 3 of 80 oysters harvested in area A (4%) and 1 of 15 oysters harvested in area B (7%) had relatively large amounts of the NV genome. Thus, more than 5% of oysters harvested in the two areas during January and February of 2002 carried large numbers of NVs. In a recovery test, about 85 to 90% of NV genomes were recovered (data not shown). In this study, a greater amount of NV genome was detected in oysters harvested from site A than in oysters from site B. However, no plausible sources of contamination with NVs, including wastewater treatment plants and combined sewer overflows, were present in the vicinity of either site. During the investigation period, no significant difference in seawater quality (defined as <70 coliform groups/100 ml of seawater by the Japanese Enforcement Regulation of the Food Sanitation Law for farming of oysters for raw consumption) was observed between sites (data not shown).

DISCUSSION

We demonstrated that about 10% of oysters harvested from two areas in January and February 2002 for raw consumption had NV genomes, while about 5% had large numbers of NVs (more than 10^2 copies). More than 10% nucleotide diversity was observed in our strains. No region-specific preponderance of any given genogroup was evident in our phylogenetic tree. The results suggested that Japanese oysters harvested in winter in both areas were highly contaminated with a genetically diverse population of NVs.

NV genomes have been detected in shellfish such as oysters and clams in the United States (9, 14, 22), United Kingdom (17), Canada (16), and Japan (23), and these viruses are associated with gastroenteritis in humans. NVs can be divided into two genogroups, each of which can be further divided into four to six clusters (4). Fankhauser et al. demonstrated that the polymerase gene of NVs detected during outbreaks of gastroenteritis in the United States can be divided into two clusters within genogroup I and four clusters within genogroup II (4). Capsid genes of NVs from stool specimens from patients during outbreaks of gastroenteritis in Japan can be divided into two clusters in genogroup I and four clusters in genogroup II (13). Our phylogenetic analysis revealed that NVs in Japanese oysters can be divided into two clusters in genogroup I and five clusters in genogroup II. This suggests wide genetic diversity, as has been noted in NVs from stool specimens (4, 5, 13). Transmission of NVs can occur via contaminated shellfish, especially oysters, and by person-to-person contact (10). Many reports have implicated NVs and enteroviruses in shellfish-associated outbreaks and demonstrated the presence of NV gene sequences in shellfish and in specimens from patients. Various types of NVs have been shown to cause oyster-associated gastroenteritis in Japan (23).

Although no conventional culture method for NVs is available at present (2), other NV detection methods, such as electron microscopy (EM), ELISA, and RT-PCR, can be used (2). EM and ELISA are practical for the detection of NVs in diarrheal stool specimens that contain a large amount of virus (10^5 to 10^6 particles/ml) (3). Determination of copy numbers of NV genome in foods is important, although these numbers do not exactly correspond to infective doses of NVs. However, our results and those of previous reports suggested that various foods, including shellfish, contain much smaller numbers of NVs than the number of NVs found in stool specimens (11). This hinders the detection of NVs in foods by EM and ELISA, since their sensitivity is limited (6). In contrast, RT-PCR is not quantitative but is highly sensitive, being able to detect a few copies of the NV genome in a sample (1). Recently, a quantitative real-time PCR method has been developed (12). We applied this method to determine the copy numbers of NV genomes in Japanese oysters. Some of our oyster-derived strains were found to be genetically related to strains infecting patients with gastroenteritis during the period of our investigation (Fig. 2). The results suggested that genetically similar types of NVs from oysters might be associated with NV-associated outbreaks during our investigation period. Some genetic similarities exist between animal calicivirus and human calicivirus (18, 24), and there are many types of animal caliciviruses (2). Interestingly, some of our strains had unique sequences that were genetically related to some animal calicivirus strains (30 to 40% diversity; Fig. 2). Thus, we could not rule out the inclusion of animal calicivirus strains in our study. In addition, the NV genome was detected in abundance in oysters harvested in January and February of 2002 but not those harvested in December of 2001. Japanese epidemiologic data for the interval between October and December of 2001 (http://www.mhlw.go.jp/topics/syokuchu) suggest that the incidence of oyster- or clam-associated gastroenteritis outbreaks was low at that time, although these data are not directly linked to our own. The population of NV-contaminated Japanese oysters from both areas in December of 2001 may indeed have been low. Yearlong month-to-month studies regarding the prevalence of NVs in oysters may be needed.

Fecally excreted NVs, enteroviruses, astroviruses, and hepatitis virus type A from patients with gastroenteritis or hepatitis enter sewage and seawater, where they may remain infective for long periods of time (9). In many countries, oyster beds are located near shore, in an estuary or a bay. It is possible that various shellfish (especially oysters and clams) contain high concentrations of viral pathogens, such as NV, enterovirus, and hepatitis A virus (19). In the course of this study, relatively large amounts of NV genes in oysters from site A were detected. However, no significant difference in bacteriologic quality of seawater was noted between the two sites, and no likely sources of NV contamination were nearby. Why relatively large numbers of the NV genome were detected in oysters harvested from site A is not known. To prevent food

poisoning by microorganisms in raw oysters, the Japanese government regulates the oyster industry under the Enforcement Regulation of Food Sanitation Law. However, this regulation focuses mainly on bacteriology. We detected NVs in 10% of government-approved oysters. Additional regulation based on viral sanitation protocols may be needed.

Further molecular epidemiologic studies of causative viral agents (NVs, enteroviruses, astroviruses, and hepatitis virus type A) carried out by using phylogenetic analysis and quantitative real-time PCR methods will contribute to an understanding of the epidemiology of these viruses and provide a more accurate assessment of the risk factors for shellfish-associated illnesses.

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Detection of norovirus (GI, GII), Sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR

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Abstract

A reverse transcription (RT) single-round multiplex polymerase chain reaction (smPCR) assay was developed to detect simultaneously Norovirus genogroup I and II, Sapovirus and astrovirus. A total of 377 diarrhea stool samples (screened for rotavirus- and adenorivus-negative) from four regions in Japan during July 2000 to June 2001 were examined by RT-smPCR. The positive rate was 16.4% (62 out of 377 stool samples). Norovirus, Sapovirus and astrovirus were detected in 42, 16, 4 of 60 positive samples, respectively. Coinfection was not found in these samples. Infections occurred mainly in November, December and January.

The key elements of the RT-smPCR are (i) the cDNA synthesis with the Superscript RTII and random primer at 42 °C for 1 h, at 99 °C for 5 min, and (ii) single-round multiplex PCR by using Taq polymerase mixed together with a mixture of four different primer pairs (G1-SKF/G1-SKR for Norovirus genogroup I, COG2F/G2-SKR for Norovirus genogroup II, SLV5317/SLV5749 for Sapovirus, PreCAP1/82b for astrovirus). All of the four primer pairs amplify the capsid region of target viral genome, produce four size-specific amplicons of 330, 387, 434, 719 bp for Norovirus genogroup I and II, Sapovirus and astrovirus, respectively. This assay provides a more rapid and efficient way to detect these viruses from fecal samples in a single test, and also offers the potential for their molecular detection in food and environmental samples.

Keywords: Diarrheal viruses; Norovirus; Sapovirus; Astrovirus; Single-round multiplex PCR

1. Introduction

Norovirus, Sapovirus and human astrovirus, all are single-stranded positive sense RNA viruses, have a small-round structured morophology. These viruses are the causative agents of gastroenteritis in human worldwide (Sakamoto et al., 2000; Nakata et al., 2000; Okada et al., 2002; Rockx et al., 2002).

Norovirus and Sapovirus are the two distinct genera within the family Caliciviridae (Pringle, 1999). Both Norovirus and Sapovirus have been divided into three major genetic groups, genogroup I, II and III (GI, GII, GIII) based on the sequence analysis (Katayama et al., 2002; Schuffenecker et al., 2001). Each of these genetic groups comprises genomically and antigenically diverse strains. The Norovirus genome has three open reading frames (ORFs), which consisting of a 5′-ORF encoding for the non-structural polyproteins, including the RNA polymerase (ORF1), a second ORF encoding for the major capsid protein (ORF2), and a 3′-ORF encoding for a minor structural protein (ORF3) (Glass et al., 2000). By contrast, the genomic organization of Sapovirus was reported to be distinct from that of Norovirus. Within the Sapovirus genome, the polyprotein gene and the capsid gene are fused in a single ORF (ORF1) (Green et al., 2000). In addition, there are 3′ open reading frame (3′-ORF), ORF overlapping capsid gene (capsid overlap, only in the GI strains) and 3′ untranslated region (3′-UTR) contained in the Sapovirus genome (Schuffenecker et al., 2001). Whereas human astrovirus, one of the members of the genus Astrovirus which is the only genus in the family Astroviridae, has been classified into eight serotypes (human astrovirus 1–8, later described as astrovirus in this paper) (Taylor et al., 2001) based on their distinct antigenicity. Their genome contains three open reading frames designated ORF1a, ORF1b and ORF2, while the latter encodes the capsid gene (Jiang et al., 1993).

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Norovirus is associated with gastroenteritis in all ages, with a distinct seasonality linked to the winter months (Mounts et al., 2000). Most outbreaks of nonbacterial gastroenteritis are related to Norovirus infection. In Japan, the peak of Norovirus infection precedes that of rotavirus infection (Inouye et al., 2000). Sapovirus infection occurs less frequently. The virus appears in sporadic cases but also in outbreaks in children and adults with no seasonality reported. And yet, astrovirus causes a less severe gastroenteritis than that caused by other enteric viruses especially rotavirus. The infection occurred mainly in infants. Adults can also be affected. It was reported that astrovirus has also been implicated in outbreaks involving up to several thousand people (Oishi et al., 1994). Transmission routes of these viruses are classified into foodborne (Deneen et al., 2000; Inouye et al., 2000; Mead et al., 1999), person-to-person spread (Caceres et al., 1998; Meakins et al., 2003), and might be some other unknown modes.

It is well established for the laboratory diagnosis of viral gastroenteritis involves non-molecular assay, such as electron microscopy (EM) examination, viral isolation techniques and enzyme immunoassay (ELISA) and various molecular-based assay, including dot blot hybridization and RT-PCR. Based on the genomic sequences published, numerous RT-PCR assays have been established for sensitive and specific detection of diarrheal viruses. Most RT-PCR assays are monoplex, for which use single set of primer and can detect only one target virus. Thus conventional monoplex RT-PCR assays are potentially expensive and resource intensive, especially since diarrheal viruses may cause similar clinical syndromes, and the inability to culture some of these viruses, such as Norovirus and Sapovirus, in vitro. In contrast to monoplex assay, the multiplex RT-PCR assay incorporates different sets of specific primers for two or more targets in one reaction tube, enables simultaneous amplification of different target nucleic acids in a single test. Because of the reduction in labor and reagent costs and faster detection, multiplex RT-PCR studies have attracted much attention and been largely practised in clinical services (Casas et al., 1997; Jackson et al., 1996; Aguilar et al., 2000) as well as for the detection of viral agents in environmental samples (Tsai et al., 1994; Cho et al., 2000). Recently, the development of nested multiplex RT-PCR assay for group *F* adenovirus, rotavirus and Norovirus was reported (O'Neill et al., 2002). In this study, we have established reverse transcription single-round multiplex PCR assay (RT-smPCR) for simultaneous detection of Norovirus GI and GII, Sapovirus and astrovirus in fecal samples.

2. Materials and methods

2.1. Fecal specimens

Three hundred seventy-seven stool samples, which were collected from pediatric patients with gastroenteritis in four different areas (Maizuru, Saga, Sapporo, Tokyo) in Japan between July 2000 and June 2001, were used in the present study. These samples were determined previously to be rotavirus- and enteric adenovirus-negative by ELISA (Zhou et al., 1999). An approximately 10% stool suspension was made with sterilized MilliQ water by vortex mixer and clarified by centrifugation at 10,000 × *g* for 10 min at 4 °C. The supernatant was stored at −20 °C until use.

2.2. Extraction of viral RNA

Viral RNA was extracted from stool supernatant by a spin column technique using the QIAamp Viral RNA kit (QIAGEN, Germany) according to the manufacturer's protocol.

2.3. Reverse transcription (RT)

For RT, 7.5 μl of extracted RNA was mixed with a reaction mixture consisting of 2.05 μl of 5× First-Strand Buffer (Invitrogen, USA), 0.75 μl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.375 μl (1 μg/μl) of random primer (Takara, Shiga, Japan), 0.75 μl of 10 mM DTT (Invitrogen), 0.5 μl (33 U/μl) of RNase Inhibitor (Toyobo, Osaka, Japan), and 0.75 μl (200 U/μl) of SuperScript Reverse Transcriptase II (SS RTII; Invitrogen). MilliQ water was added to give a total volume of 15.0 μl. The RT step was carried out at 42 °C for 1 h, followed by heating at 99 °C for 5 min to inactivate the enzyme, and cooling at 4 °C immediately.

2.4. PCR primers

A total of four sets of primers for the capsid region of target viral genome were used in this study. Two sets of primers G1-SKF/G1-SKR (Kojima et al., 2002) and COG2F/G2-SKR (Kojima et al., 2002; Shinohara et al., 2002) were used for amplifying Norovirus GI and GII, generating a 330 bp and a 387 bp PCR product, respectively. A new primer set for Sapovirus was designed (SLV5317, 5′-CTCGCCACCTACRAWGCBTGGTT-3′ and SLV5749, 5′-CGGRCYTCAAAVSTACCBCCCCA-3) based on conserved nucleotide sequence in the 5′-ORF of reference strains of Sapovirus (GenBank database). The novel primers were used for amplifying both Sapovirus GI and GII (Fig. 1), generating a 434 bp product (Fig. 2). Positions of Sapovirus primer set were based on Manchester strain (GenBank accession no. X86560) (Fig. 1). One set of primers PreCAP1/82b (Matsui et al., 1998; Sakamoto et al., 2000) was used for amplifying all serotypes of human astrovirus, generating a 719 bp product. The sequence and relative locations of the primers are shown in Figs. 1 and 2.

2.5. Single-round multiplex PCR (smPCR) assay

In the smPCR assay, 2.5 μl of cDNA was added to a 500 μl microcentrifuge tube with 2.5 μl of 10× Taq DNA polymerase buffer (Promega, Madison, WI), 0.4 μl

```
Norovirus GI          5342    G1-SKF    5361          5671    G1-SKR    5653
                     5'-CTGCCCGAATTYGTAAATGA-3'       3'-ACATRTTACCRACCCAACC-5'
      Norwalk/68  GCGATCTCCTGCCCGAATTCGTAAATGATGATG.....ACAGATGTACAATGGATGGGTTGGCAATATGC
  Southampton/91  CGCAATCTCTGCCCGAATTTGTAAATGATGATG.....CCAAATGTATAATGGCTGGGTTGGAAATATGA
             BS5  GCGATCTCCTGCCCGAATTCGTAAATGATGATG.....ACAGATGTACAATGGATGGGTTGGCAATATGC

Norovirus GII         5003       COG2F       5028          5389       G2-SKR     5367
                     5'-CARGARBCNATGTTYAGRTGGATGAG-3'     3'-TACATRTTRCCHRTACGNCCRCC-5'
    Lordsdale/93  TGCCCAGACAAGAGCCAATGTTCAGATGGATGAGATTCT.....CCAGAATGTACAATGGTTATGCAGGTGGTTTTGAAG
      Hawaii/71  TGCCCAGGCAAGAGCCGATGTTCAGATGGATGAGATTCT.....CAAGAATGTATAATGGTTATGCCGGCGGGGTTGAAG
    Melksham/89  TGCCCAGGCAAGAACCCATGTTCAGATGGATGAGATTCT.....CAAGAATGTATAACGGGTATGCCGGTGGGATGGAGG

Sapovirus             5083      SLV5317      5105          5516      SLV5749     5494
                     5'-CTCGCCACCTACRAWGCBTGGTT-3'       3'-ACCCCBCCATSVAAACTYCRGGC-5'
   Manchester/93  ACCAGGCTCTCGCCACCTACAATGCTTGGTTCATAG.....CCGGGTGGGGCGGTAGTTTTGAGGTCCGGCTATCGA
     Sapporo/82  ACCAGGCTCTCGCCACCTACAACGCTTGGTTTATAG.....CCGGGTGGGGCGGTAGCTTTGAGGTCCGGCTGTCGA
     Houston/86  ACCAGGCTCTCGCCACCTACAATGCTTGGTTCATAG.....CCGGGTGGGGCGGTACGTTTGAGGTCCGGCTATCGA

human astrovirus      4235      PreCAP1      4255          4953       82b        4934
                     5'-GGACTGCAAAGCAGCTTCGTG-3'         3'-TCCCTACCGACCACCGAGTG-5'
       serotype 1  CCTCTCACGGACTGCAAAGCAGCTTCGTGACTCT.....GACCTAGGGATGGTTGGTGGCTCACAAACACCA
       serotype 2  CCTATCACGGACTGCAAAGCAGCTTCGTGACTCT.....GGCCAAGGGATGGCTGGTGGCTCACTAATACCA
       serotype 3  CCTGTCTCGGACTGCTAAGCAGCTTCGTGAGTCT.....GGCCCAGGGATGGGTGGTGGTTGACTAACACAA
       serotype 4  CCTCTCGCGGACTGCAAAGCAGCTTCGTGACTCT.....GACCTAGGGATGGCTGGTGGCTCACAAACACCA
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Fig. 1. Nucleotide sequence alignment of the reference strains of Norovirus GI and GII, Sapovirus and human astrovirus at consense and antisense primer regions. Conserved nucleotides among strains are given in bold letters. The position numbers of the four primer pairs indicated correspond to representative strains Norwalk/68, Lordsdale/93, Manchester/93 and Oxford for Norovirus GI and GII, Sapovirus and human astrovirus serotype 1, respectively. GenBank accession number of the reference strains are: Norwalk/68 (M87661), Southampton/91 (L07418), BS5 (AF093797), Lordsdale/93 (X86557), Hawaii/71 (U07611), Melksham/89 (X81879), Manchester/93 (X86560), Sapporo/82 (U65427), Houston/86 (U95643), human astrovirus serotype 1 (L23513), human astrovirus serotype 2 (L13745), human astrovirus serotype 3 (AF141381), human astrovirus serotype 4 (NC-001943). Within nucleotide sequences of primers, B: C, G or T; H: A, C or T; N: any base; R: A or G; S: G or C; V: A, C or G; W: A or T; Y: C or T.

of the four sets of specific primers (33 μM each), 2.0 μl (2.5 mM/μl) of dNTPs (Roche, Mannheim, Germany), 0.25 μl (5 U/μl) of Taq DNA polymerase (Promega), and made up to 25.0 μl with MilliQ water. PCR was performed at 94 °C for 3 min followed by 35 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 60 s, and a final extension at 72 °C for 7 min, followed by cooling at 4 °C.

2.6. Specificity testing of the four primer pairs

Specificity testing was carried out for the four positive controls: Norovirus GI (Osaka/3626), Norovirus GII (Osaka/3581), Sapovirus (Osaka/3668) and astrovirus (human astrovirus serotype 1, H1 (Oxf), accession no. L23513). Among those, Osaka/3626, Osaka/3581 and Osaka/3668 were sequenced in both directions by using specific primer previously (data not shown), to determine the specificity of the four primer pairs. Moreover, group A rotavirus serotype G1 and G2, adenovirus F41 and poliovirus type 1 were used as negative control for RT-smPCR. For the four positive controls, various combinations were tested: the PCR mixture containing four primer pairs and each viral cDNA (name as A mixture), each primer pair and four viral cDNA which were synthesized from a mixture of Norovirus GI, Norovirus GII and Sapovirus positive controls and cultured astrovirus (name as B mixture), and the four viral cDNA and four primer pairs (name as C mixture), by using the same thermal cycler program described above. The final concentrations of each kind of premixes were the same for the procedures of smPCR mentioned above, and MilliQ water was added to give a total volume of 25.0 μl (Table 1).

2.7. Sensitivity testing of the multiplex PCR and the monoplex PCR

To compare the sensitivity level of the multiplex PCR and the monoplex PCR, 10-fold serial dilutions (10^{-1} to

Table 1
The components of PCR premix for the specificity testing

25 μl of PCR premix (μl)	A mixture	B mixture	C mixture
DDW	14.675	17.075	14.675
10× Taq buffer	2.5	2.5	2.5
dNTP (2.5 mM)	2	2	2
Primer mixture	3.2[a]	0.8[b]	3.2[a]
Taq (5 U/μl)	0.125	0.125	0.125
cDNA (template)	2.5	2.5[c]	2.5[c]

[a] The mixture of four sets of specific-primers (0.4 μl of 33 μM each).
[b] The mixture of single specific-primer pair (0.4 μl of 33 μM each).
[c] Synthesized from the mixture of Norovirus GI, Norovirus GII, and Sapovirus positive controls and cultured astrovirus, such that contains the four kinds of cDNA.

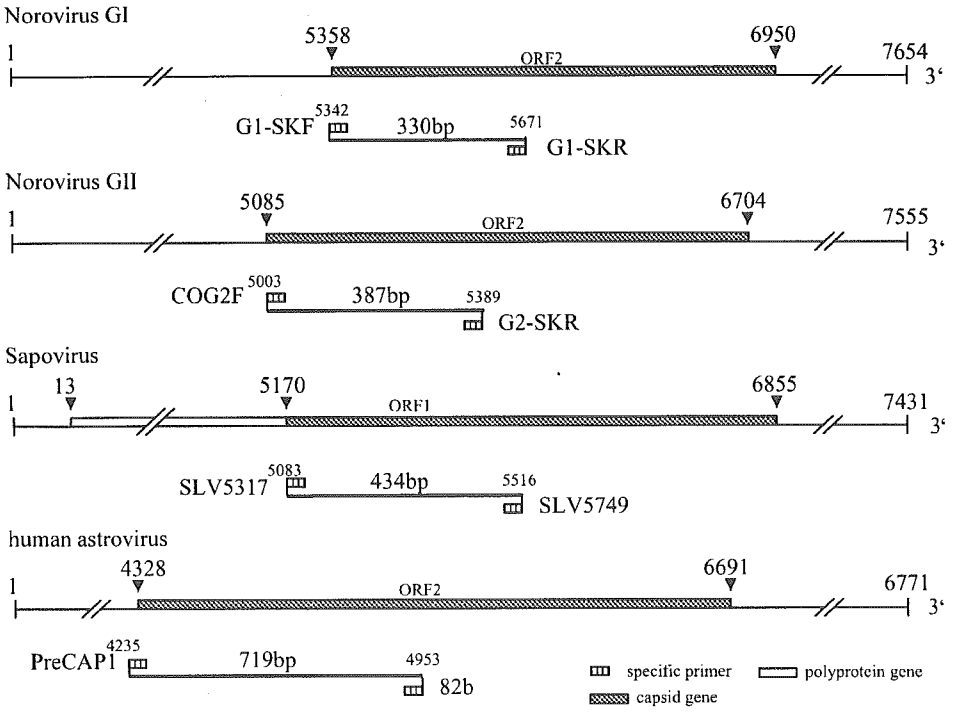

Fig. 2. Diagrams of RNA genome are shown for representative strains Norwalk/68 (GenBank accession no. M87661), Lordsdale/93 (GenBank accession no. X86557), Manchester/93 (GenBank accession no. X86560), and human astrovirus serotype 1 Oxford (GenBank accession no. L23513) in Norovirus GI and GII, Sapovirus and astrovirus, respectively. Position of the primer pairs and sizes of the resulting PCR products are indicated (in base pairs). Arrows denote location of first AUG in predicted open reading frame (ORF) encoding viral capsid protein. Capsid gene sites in ORF2 in Norovirus GI and GII and astrovirus genome. In Sapovirus genome, capsid gene is fused with polyprotein gene in a single ORF (ORF1) as described in text.

10^{-4}) in MilliQ water of the four different viral cDNA, one positive control of Norovirus GI (as which was not found in stool samples), three selected from positive samples of Norovirus GII, Sapovirus and astrovirus, respectively, were tested by the multiplex PCR and the monoplex PCR. For the monoplex PCR, the final concentrations of premix were same to that of smPCR, except single specific primer pair (0.4 μl of 33 μM each) was contained: Norovirus GI primer G1-SKF and G1-SKR, Norovirus GII primer COG2F and G2-SKR, Sapovirus primer SLV5317 and SLV5749, astrovirus primer PreCAP1 and 82b, respectively, was added in separate reaction tube for each target virus. The multiplex PCR and the monoplex PCR were performed simultaneously for the same dilution series, by using the same PCR machine and in the same thermal cycler program described above.

2.8. Electrophoresis

PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (EtBr, 0.5 μg/ml) for 20 min then visualized under ultraviolet (UV) light, and the results were recorded by photography (Ushijima et al., 1992).

3. Results

3.1. Specificity testing of the four primer pairs

The specificity of each set of primers used in this study was tested. For the four positive controls, various combinations, exactly, the PCR reaction mixture containing four primer pairs and each cDNA (Fig. 3, lanes 1–4), the four cDNA and each primer pair (data not shown), and the four cDNA and four primer pairs (Fig. 3, lane 5), amplified each expected product independently and specifically. No cross-reaction was found between the individual primers and non-target virus in the monoplex PCR (data not shown). Each PCR product was obtained as a clear band at 330, 387, 434 and 719 bp generated by G1-SKF/G1-SKR, CO-G2F/G2-SKR, SLV5317/SLV5749, and PreCAP1/82b, respectively (Fig. 3). For the negative controls adenovirus F41, poliovirus type 1, rotavirus G1 and G2, none amplicon was generated.

3.2. Sensitivity testing of the multiplex PCR and the monoplex PCR

For the 10-fold dilution series, the highest dilution at which the multiplex PCR exhibited a positive result was 10^{-2}, 10^{-1}, 10^{-4} and 10^{-4} for Norovirus GI,

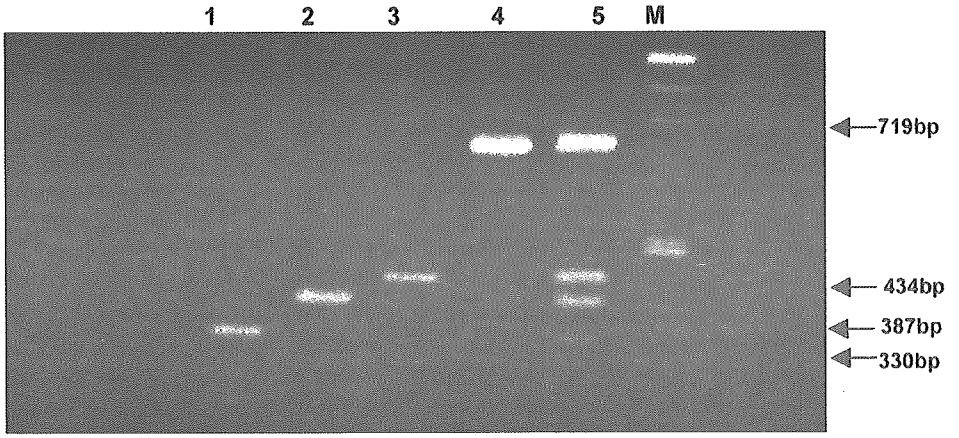

Fig. 3. Specificity testing of the multiplex PCR assay with a mixture of the four primer pairs for the four positive controls. Lanes: (1–3) Norovirus GI, Norovirus GII, Sapovirus positive control, respectively; (4) cultured astrovirus; (5) the mixture of Norovirus GI and GII, Sapovirus positive controls and cultured astrovirus; (M) DNA molecular size markers.

Norovirus GII, Sapovirus and astrovirus, respectively. Whereas, the positive results of the highest dilution in the monoplex PCR were 10^{-3} for Norovirus GI, 10^{-2} for Norovirus GII, 10^{-4} for Sapovirus and astrovirus. However, product of 10^{-4} dilution of Sapovirus and astrovirus in the multiplex PCR is poorer by visualization on agarose gels than that in the monoplex PCR (Fig. 4). Overall, the sensitivity level of the multiplex PCR dropped about 10-fold compared with the monoplex PCR.

3.3. Detection of target viruses in stool samples by RT-smPCR

In total, 62 out of 377 (16.4%) stool samples were positive for the target viruses, of these, 42 were Norovirus GII, 16 were Sapovirus, 4 were astrovirus, and none of Norovirus GI was detected. Fig. 5 shows an example of Norovirus GII-, Sapovirus-, astrovirus-positive samples, which out of the stool samples collected from Japan during 2000 July and 2001 June, by using RT-smPCR. Infections occurred mainly

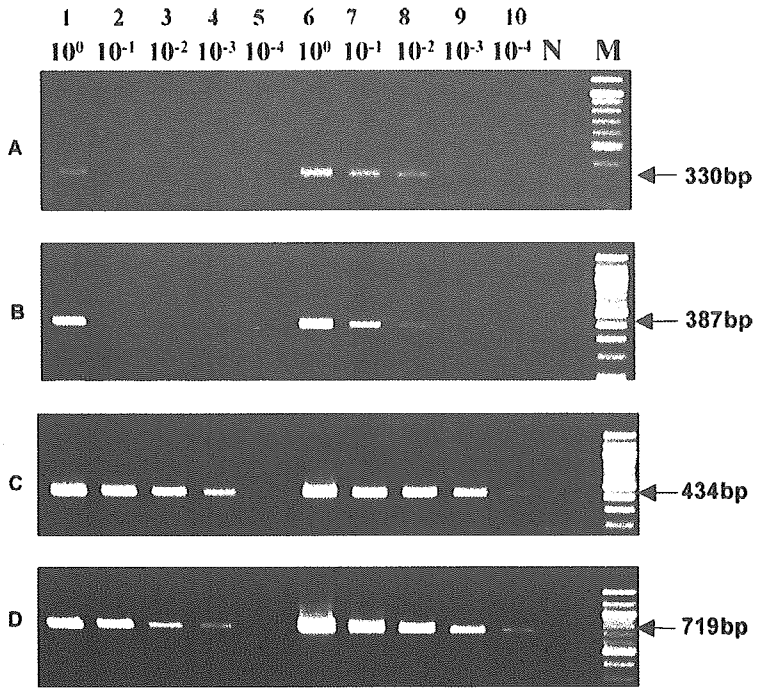

Fig. 4. Comparison of the sensitivity between the multiplex PCR and the monoplex PCR for the 10-fold serial dilutions of Norovirus GI cDNA (A), Norovirus GII cDNA (B), Sapovirus cDNA (C), and astrovirus cDNA (D). Lanes: (1–5) sensitivity testing of the multiplex PCR; (6–10) sensitivity testing of the monoplex PCR; (N) negative control; (M) DNA molecular size markers.

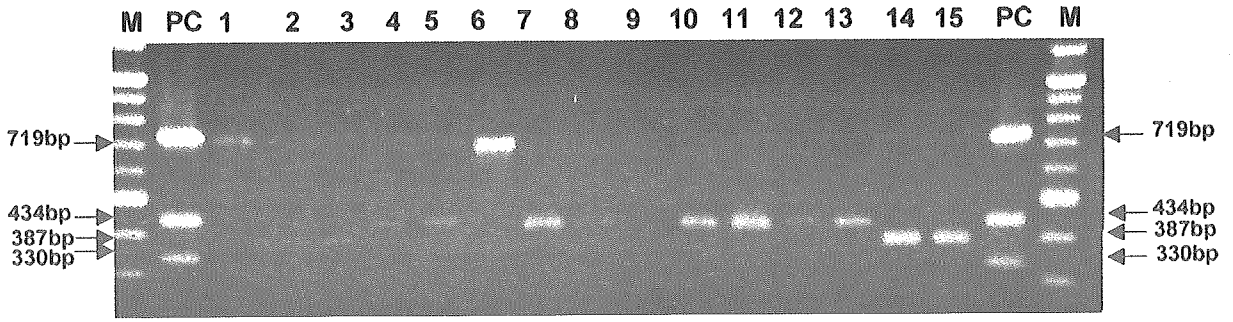

Fig. 5. Typical agarose gel containing Norovirus GII, Sapovirus and astrovirus PCR products from positive samples by using RT-smPCR. Lanes: (2, 3, 9, 14 and 15) Norovirus GII; (4, 5, 7, 8 and 10–13) Sapovirus; (1 and 6) astrovirus; (PC) the mixture of Norovirus GI and GII, Sapovirus and cultured astrovirus, which product sizes were 330 bp for Norovirus GI, 387 bp for Norovirus GII, 434 bp for Sapovirus, and 719 bp for astrovirus, respectively; (M) DNA molecular size markers.

in three months period, November, December and January. Mixed infection was not found in these samples.

4. Discussion

This study has developed a rapid assay to detect simultaneously Norovirus GI and GII, Sapovirus and astrovirus in stool samples. Similar studies on multiplex PCR have been reported previously, e.g. for the detection of all serotypes of human astrovirus and Norovirus (Sakon et al., 2000), the detection of hepatitis A virus, Norovirus, and poliovirus type 1 (Rosenfield et al., 1999), the detection of influenza virus types A, B and C (Poddar et al., 2002) and other pathogens (Williams et al., 1999; Bertolini et al., 2001). Some of these protocols combine two or three different sets of primers. We designed a multiplex assay in which a total of four primer pairs (each primer at 13.2 μM/25 μl) were used for detecting four viral agents by one step PCR reaction. Novel primers SLV5317 and SLV5749 designed to detect Sapovirus were based on the capsid-coding region ORF1 of Sapovirus genome, which is highly conserved region among those published Sapovirus strains (GenBank database). To confirm the sensitivity of new primer set SLV5317/SLV5749, all 377 specimens were tested by conventional RT-PCR using single Sapovirus-specific primer pair SR80/JV33 (Vinje et al., 2000), and also tested by the monoplex PCR which the thermal cycler program was same as the sm-PCR described above, using SLV5317/SLV5749. In conclusion, the sensitivity of SLV5317/SLV5749 was evaluated with the results of 17 (94.4%) Sapovirus-positive detected by SLV5317/SLV5749 out of the 18 which detected by SR80/JV33 among 377 samples. Although the sensitivity of Sapovirus primer set SR80/JV33 showed slightly higher than that of SLV5317/SLV5749, the amplicon size (320 bp) produced by SR80/JV33 was undistinguishable from Norovirus GI amplicon (330 bp) generated by the smPCR when analyzed on a 1.5% agarose gel. Therefore, we used the new primer set SLV5317/SLV5749 for the smPCR to detect Sapovirus. Thus, primer selection for the four viruses was based on size of the amplicon produced, such that the amplicons could be visualized by agarose gel electrophoresis. The annealing temperature was selected at an appropriate temperature in order to decrease nonspecific priming or other artifacts. Times for annealing and extension were minimized to reduce the possibility of nonspecific amplification.

The specificity of the mixture of four primer sets was verified by testing for the positive controls and also for the negative controls adenovirus F-41, poliovirus type1, group A rotavirus G1 and G2 using the smPCR. Regardless we have not found Norovirus GI, we obtained specific product from positive control Norovirus GI. In experiments, we found that equimolar primer concentrations used for the eight primers mixture (each primer 13.2 μM/25 μl) showed almost the same sensitivity when compare to the concentrations which used in the conventional monoplex protocol (each primer 16.5 μM/25 μl) for the eight primers mixture (data not shown).

To date, RT-PCR assays have been shown to be useful diagnostic procedures for RNA virus detection. It was reported that sensitivity of the conventional PCR with specific primers was higher than that of EM (Vipond et al., 2000) and ELISA with monoclonal antibody (Mitchell et al., 1995; Saito et al., 1995), and sensitivity of the nested PCR was even higher (Green et al., 1998; Saito et al., 1995). However, the sensitivity of multiplex PCR assay could not be determined in absolute terms. In most cases, sensitivity of the multiplex PCR dropped 10–100-folds when compared to those of the monoplex PCR (Jackson et al., 1996; Tsai et al., 1994). In our study, the multiplex PCR showed slightly less sensitive than the monoplex PCR, at which the sensitivity dropped about 10 folds (Fig. 4). The detection limits for the single target of different viruses were similar to other multiplex PCR assays previously reported (Jackson et al., 1996; Tsai et al., 1994). On the other hand, although the positive rate of individual virus may vary by the region and period of sample collection, the total positive rate of Norovirus, Sapovirus and astrovirus in this study was 16.4% (62 out of 377 samples), within it, 11.1% for Norovirus (42 out of 377

samples) and 4.2% for Sapovirus (16 out of 377 samples), which are comparable to those of the previous studies using monoplex PCR assay (Okada et al., 2002; Foley et al., 2000).

By sensitivity testing for positive samples/control, the sensitivity of the multiplex PCR on Sapovirus and astrovirus showed similar results to those determined by monoplex PCR assay, and the sensitivity of the multiplex PCR on Norovirus GI and GII was 10-folds less than those of the monoplex PCR. Thus results seem unlikely to limit the clinical diagnostic role of our multiplex PCR assay. Also, consideration of the primer sets and the reaction conditions of the smPCR are potentially applicable for the detection of the four viruses both in the clinical specimens and the research laboratory. Of note, although RT-smPCR is capable of simultaneously detecting these diarrheal viruses from one specimen, no multiple infection was detected in the present study. However, the possibility of dual infection could not be ruled out. Further work would aim at the selection of suitable primer pairs for nested multiplex PCR in order to maximize the detection sensitivity and specificity, and to avoid the possibility of false-negative results for all targets.

This is the first study to detect simultaneously Norovirus GI and GII, Sapovirus and astrovirus in stool samples by RT multiplex PCR. The assay offers the potential for very rapid detection, within a single protocol, a single clinical specimen, and multiple types of potential pathogens, in about 5–6 h from RNA extraction to PCR products visualized by agarose gel electrophoresis. Although the conventional standard monoplex assay was used in the current clinical evaluation, the shortened protocol would be well efficient. The spectrum of potential application of this new RT multiplex PCR might be extensive, as diarrheal viruses early diagnosis is important for the control of nosocomial outbreaks or the outbreaks in community. In summary, the RT-smPCR assay, which allows identification of multiple viruses by only single reaction, provides significant saving in time and cost in comparison to the separate protocols of monoplex PCR using for the four viruses detection. The application of the developed multiplex PCR would likely improve laborious in the studies of large sample size.

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Evaluation of a Bedside Immunochromatographic Test for Detection of Adenovirus in Respiratory Samples, by Comparison to Virus Isolation, PCR, and Real-Time PCR

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An immunochromatography (IC) kit for human adenovirus (HAdV) was evaluated with 138 patient nasopharyngeal samples. The samples were collected at a sentinel clinic in Japan from January through June 2003. Patients were diagnosed by clinical manifestation of pharyngoconjunctival fever (n = 38) or exudative tonsillitis (n = 100). The IC kit was positive for 84% (116 of 138) of patients diagnosed at bedside. The remaining extract solution of the IC kit test was transferred into maintenance medium and tested via laboratory diagnoses. The IC kit had 95% sensitivity (116 of 122 patients) with HAdV isolation (isolation) as the standard and 91% sensitivity (116 of 128 patients) with PCR as the standard. All of the IC kit-positive samples were isolation and PCR positive. Similarly, all the isolation-positive samples were PCR positive. Twenty-two IC kit-negative samples were evaluated by real-time PCR. Six samples were IC kit negative and isolation positive and contained 3.8×10^7 to 2.5×10^9 copies of the HAdV genome/ml. Five samples that were only PCR positive contained 3.0×10^4 to 3.8×10^5 copies of the HAdV genome/ml, but one sample was real-time PCR negative. We conclude that the IC kit is a useful bedside diagnostic tool for HAdV infections because it has 95% sensitivity (compared to isolation), but a negative result does not always rule out HAdV infection.

Human adenoviruses (HAdVs) are one of the most commonly isolated viruses and are a significant cause of diseases of the respiratory tract and eye. Pharyngoconjunctival fever (PCF) is a widely known adenoviral disease. HAdVs, particularly types 7 and 3, are also known to cause pneumonia (12, 13). Furthermore, HAdVs are increasingly being recognized as fatal pathogens in immunocompromised patients (1, 2, 5, 18).

Recently, four different kinds of diagnostic methods for HAdV infection became available. The virus isolation technique (isolation) is usually considered the "gold standard" but is time-consuming. PCR is a rapid and sensitive diagnostic technique that can be completed in a few hours, and sequencing the amplified products yields useful data. Real-time PCR for HAdVs has recently become available. Real-time PCR produces results quickly, and quantitative data can be obtained (5, 8, 18). In addition to these laboratory diagnostic techniques, immunochromatography (IC) kits have become available for HAdV diagnosis at the patient's bedside (6, 7, 16, 20, 21).

IC kits for HAdVs have been evaluated in comparison with an isolation technique (6, 20), an enzyme-linked immunosorbent assay kit (16), and PCR (7, 21). However, they have not been evaluated in comparison to the combination of isolation, PCR, and real-time PCR. Quantitative evaluation alone was insufficient; therefore, we evaluated an IC kit in comparison to these three methods. The sensitivity and specificity of the IC kit change according to which method is considered to be the gold standard (15).

The purpose of this study was to evaluate the IC kit, qualitatively and quantitatively, in comparison to multiple, sensitive laboratory diagnostic methods. The reliability and limitations of the IC kit were evaluated. The detection limit of the IC kit was evaluated with HAdV type 1, 2, 3, 5, 6, and 7 isolates.

We found that the IC kit had a higher sensitivity than reported previously, although it was less sensitive than isolation, which was less sensitive than PCR and real-time PCR. However, the IC kit was found to have 95% sensitivity compared to HAdV isolation, the diagnostic technique usually considered the gold standard.

MATERIALS AND METHODS

Patients and clinical samples. A total of 138 nasopharyngeal swabs were collected from patients suffering from pharyngoconjunctival fever (n = 38) or exudative tonsillitis (n = 100) during the period of January through June 2003. The mean age of the patients, who ranged in age from <1 to 10 years, was 3.6 ± 0.19 years (mean ± standard deviation [SD]), except for one adult patient who was 38 years old. There was no significant difference in the sex of the patients (72 males and 66 females). All of the patients had temperatures in the range of 38.0 to 40.7°C; the mean temperature was 39.4 ± 0.05°C (mean ± SD). The clinical picture of patients with typical HAdV-associated exudative tonsillitis includes a high fever (>38°C) which persists for about 5 days, and C-reactive protein is usually strongly positive. All clinical samples were collected at the Okafuji Pediatric Clinic, Himeji City, Japan. The protocol was reviewed and approved by the local institutional review board, and informed consent was obtained from all patients.

Clinical diagnosis at bedside. The IC kit used in this study was the SAS Adeno Test (SA Scientific, Inc., San Antonio, Tex.), which was sold under the name Check Ad (AZWELL, Osaka, Japan). The tonsils and posterior pharynx of each patient were vigorously rubbed with a cotton swab moistened in sterile physiological saline. The swabs were extracted with 500 μl of mucolytic agent provided

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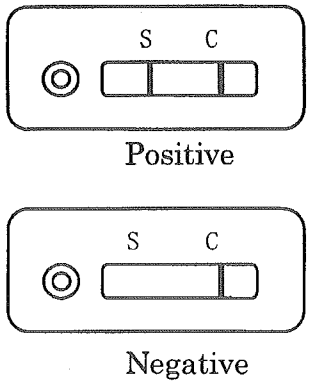

FIG. 1. Detection of adenovirus in a clinical sample by the IC kit. The test is positive if two colored lines appear in the sample (S) and control (C) areas. The test is negative when only one colored line appears in the control area.

by the manufacturer and rubbed in a soft tube. An aliquot of the extract (200 μl) was filtered and dropped into the IC kit device. Both the test tube and filter were provided by the manufacturer. The IC kit indicated an HAdV-positive result when two colored lines appeared in the device. When only one colored line appeared in the control area of the IC kit, the result was HAdV negative (Fig. 1). The remaining extract solution (about 200 μl) was transferred into a test tube containing 2 ml of Dulbecco's modified Eagles's medium (Sigma) and used as a clinical sample for a later laboratory diagnosis. The test tubes were kept at −80°C until use.

Virus isolation and serotyping. Clinical samples were inoculated onto 80% confluent monolayers of HEp-2 cells in duplicate wells of a 24-well plate (Nippon Becton Dickinson, Tokyo, Japan). For cultivation, Dulbecco's modified Eagle's medium (Sigma) supplemented with 2% heat-inactivated fetal calf serum and antibiotics was used as a maintenance medium. The HEp-2 cells were subpassaged three to eight times to allow time for the cytopathic effect (CPE) to develop. When the CPE became evident, the HAdV isolates were serotyped by a neutralization test (NT) by use of antisera purchased from Denkaseiken (Tokyo, Japan). HAdV type 7 (HAdV 7) used in the detection limit test was isolated and identified in 1998 by the same technique. Enteroviruses were isolated and identified in the same way. Isolates which could not be identified by NT using antiserum for HAdV 1 to 7 were identified by a combination of PCR and sequencing techniques.

Viral DNA preparation. HAdV genomes and enterovirus RNAs were prepared directly from the clinical samples with a QIAamp blood kit (QIAGEN) or a High Pure viral nucleic acid kit (Roche Diagnostics). DNAs from HAdV 1, 2, 3, 5, and 7 isolates were extracted with the High Pure kit.

PCR and sequencing. A single-tube multiplex PCR for HAdVs was carried out as reported previously (3). HAdVs were distinguished by the size of the amplified products. HAdVs which amplified to produce 188- and 301-bp DNA fragments were typed as HAdV 3 and non-subgroup B HAdVs, respectively.

Another primer pair for HAdV 3 (and HAdV 7) was designed and used for PCR and sequencing of the hexon region of HAdVs. The sequences of the primers were 5′-AGAATCATGGACTGATACTGATG-3′ (sense) and 5′-AGC CTGTCATTGCCAGGCCAGC-3′ (antisense). Amplification reactions were conducted in 50 μl of reaction mixture containing a 0.5 μM concentration of each of the primers, a 200 μM concentration of each dideoxynucleotide, 1.25 U of *Taq* polymerase (TaKaRa Shuzo, Shiga, Japan), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. The reaction was carried out with a cycle of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min and was continued for 35 cycles. In the first cycle, the denaturation step continued for 5 min at 94°C, and in the last cycle, the extension step continued for 5 min at 72°C. The expected product size was 1,596 bp (from position 591 to 2186 based on the hexon sequence of HAdV3; GenBank accession number X76549).

Two RT-PCR methods for enteroviruses developed by Ishiko et al. (11) and Oberste et al. (14) were used for enterovirus identification. PCR-amplified products were sequenced directly as reported previously (3, 4, 17). PCR and RT-PCR were performed using a Thermal Cycler Dice (TaKaRa Shuzo). The nucleotide sequence was determined with a model 310 genetic analyzer (Applied Biosystems Japan [ABI]).

TABLE 1. IC kit and typing results of detected viruses (n = 138)

Virus type(s) identified[a]	No. (%) of patients	No. (%) of patients positive by:			
		IC kit	Virus isolation		Multiplex PCR
			HAdV	Enterovirus	
HAdV 1	11 (8.0)	9 (7.8)	11 (9.0)		11 (8.6)
HAdV 2	17 (12)	17 (15)	17 (14)		17 (13)
HAdV 3	86 (62)	80 (69)	84 (69)		86 (67)
HAdV 5	4 (2.9)	4 (3.4)	4 (3.3)		4 (3.1)
HAdV 6	2 (1.4)	2 (1.7)	2 (1.6)		2 (1.6)
HAdV 3 + 5	1 (0.7)	1 (0.9)	1 (0.8)		1 (0.8)
HAdV 3 + CB 2	2 (1.4)	2 (1.7)	2 (1.6)	2 (33)	2 (1.6)
HAdV 3 + CB 5	1 (0.7)	1 (0.9)	1 (0.8)	1 (17)	1 (0.8)
HAdV NI + CB 2	1 (0.7)			1 (17)	1 (0.8)
HAdV NI	3 (2.2)				3 (2.3)
CB 2	2 (1.4)			2 (33)	
Total	138[b]	116	122	6	128

[a] NI, not identified.
[b] Includes eight patients (5.8% of the total number) negative for all viruses.

Real-time PCR. Real-time PCR for a wide range of HAdVs was used in this study. Primers derived from the highly conserved HAdV hexon 3 and 4 genes by Echavarria et al. (1) were used. The sequences of the primers were 5′-GACAT GACTTTCGAGGTCGATCCCATGGA-3′ (Hex3) and 5′-CCGGCTGAGAA GGGTGTGCGCAGGTA-3′ (Hex4). The expected product size was 140 bp (from position 21589 to 21728 based on the complete sequence of HAdV2; GenBank accession number J01917). One microliter of template DNA was added to a final volume of 25 μl containing 1× SYBR green PCR master mix (ABI) and a 160 nM concentration of the primers Hex3 and Hex4. The real-time PCR was carried out with an ABI PRISM 7900 HT sequence detection system for a cycle of 94°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min, which was continued for 40 cycles. During thermal cycling, the emissions from each sample were recorded and SDS (sequence detection system) software processed the raw fluorescence data to produce threshold cycle (C_T) values for each sample. The SDS software then computed a standard curve from the C_T value of the diluted standards and extrapolated absolute quantities for the unknown samples based on their C_T values. The DNA of the prototype HAdV 2 was amplified by the PCR system of Echavarria et al. (1) as described above. The product (140 bp) was cloned into a pCR2.1 vector and used as a standard.

Detection limit of the IC kit. HAdV 1, 2, 3, 5, and 6 isolated in this study and HAdV 7 isolated in 1998 were diluted in phosphate-buffered saline and tested with the IC kit. Diluted solutions showing slight but clear positivity by the IC kit were used to determine the detection limits of the IC kit. The detection limits were evaluated by determining the 50% tissue culture infective dose ($TCID_{50}$) per milliliter by use of HEp-2 cells (10) and real-time PCR. Additionally, repeated IC kit detection limit tests were performed with a twofold serially diluted HAdV 3 solution to check the reproducibility. At each concentration, 10 IC kits were tested.

RESULTS

Virus isolation and identification. All the IC kit-positive samples were isolation positive. Six IC kit-negative samples were isolation positive and typed HAdV 1 or 3. Enteroviruses were also isolated individually or as a mixture with HAdVs (Table 1). The IC kit was able to detect HAdVs in coinfected samples. Because enteroviruses grow faster than HAdVs in HEp-2 cells, the CPE of coinfected samples was indistinguishable from that of enterovirus-infected samples. However, the supernatant of coinfected samples was IC kit positive. The IC kit was also useful for confirming HAdV isolation when the HAdV CPE was not clear.

TABLE 2. Results of real-time PCR and typing of the IC kit-negative samples (n = 22)

Test results[a] (n)	Virus type identified in each sample[b]	Copies of genome/ml[c]
PCR pos.; isolation pos. (6)	HAdV 1	3.8×10^7
	HAdV 3	9.0×10^7
	HAdV 3	4.0×10^8
	HAdV 1	4.5×10^8
	HAdV 3	1.3×10^9
	HAdV 3	2.5×10^9
PCR pos.; isolation neg. (6)	HAdV 3	ND
	HAdV 3	3.0×10^4
	HAdV NI	7.1×10^4
	HAdV NI	1.1×10^5
	HAdV NI + CB 2[d]	2.1×10^5
	HAdV NI	3.8×10^5
PCR neg.; isolation neg. (10)	Neg. (n = 9)	ND (n = 9)
	Neg.[e] (n = 1)	3.3×10^5

[a] pos., positive; neg., negative.
[b] NI, not identified.
[c] ND, not detected (the multiplex PCR was positive [188 bp] but the band was weak).
[d] The isolate was neutralized by anti-CB 2 serum.
[e] This sample was recorded as PCR negative because only a very weak band (301 bp) was observed.

PCR, RT-PCR, and real-time PCR. All isolation-positive samples, including IC kit-positive samples, were positive by PCR. However, all HAdV PCR-positive and isolation-negative samples were IC kit negative (Table 1). Samples positive by isolation but IC kit negative had higher concentrations of HAdVs than PCR-positive but isolation-negative samples (Table 2). One isolation-positive sample was coinfected with HAdV (2.1×10^5 copies/ml), and only coxsackievirus type B2 (CB 2) was isolated. Two PCR-amplified products were not clear when stained with ethidium bromide. The two samples did not give concordant results with PCR and real-time PCR. A sample containing HAdV (3.3×10^5 copies/ml) was recorded as PCR negative because only a very weak signal was observed after multiplex PCR. Similarly, one real-time-PCR-negative sample was multiplex PCR positive, but the product band was weak.

IC kit for HAdV. The detection limit of the IC kit was $10^{1.6}$ to $10^{4.0}$ $TCID_{50}$/ml and $10^{4.6}$ to $10^{5.8}$ copies of the HAdV genome/ml. The IC kit was not less sensitive in detecting HAdV 3 and 7 than in detecting HAdV 1, 2, 5, and 6 (Table 3). In repeated IC kit tests, HAdV 3 was positive 10 of 10 times at detection limit concentrations, 3 of 10 times at a twofold serial dilution, and 0 of 10 times at a fourfold serial dilution. A high reproducibility for the IC kit was ascertained.

Five clinical samples that were IC kit and isolation negative but PCR positive contained 3.0×10^4 to 3.8×10^5 copies of the HAdV genome/ml. However, six clinical samples were IC kit negative in spite of the presence of 3.8×10^7 to 2.5×10^9 copies of the HAdV genome/ml. These samples were all isolation positive, and the IC kit had sensitivity to the isolates.

At the bedside, 40 patient samples were recorded as strongly positive, since in each case a positive colored line appeared before the control line appeared. Seventy-three samples were positive within 10 min. Only three samples required 20 min.

TABLE 3. Detection limit of IC kit

Virus type	Detection limit	
	$TCID_{50}$/ml (10^n)	Copies of HAdV genome/ml (10^n)
HAdV 1	2.9	4.6
HAdV 2	3.2	5.4
HAdV 3	1.9	5.8
HAdV 5	3.9	5.8
HAdV 6	4.0	5.3
HAdV 7[a]	1.6	5.6
Mean ± SD (CV)[b]	2.9 ± 1.0 (0.34)	5.4 ± 0.4 (0.08)

[a] This strain was isolated in 1998. Other serotypes were isolated in this study.
[b] CV, coefficient of variation.

DISCUSSION

The IC kit has proven to be a useful method for bedside diagnosis of HAdV infections. HAdV infection is difficult to diagnose by the presence of symptoms alone. The sensitivity of the IC kit was 95% in comparison to HAdV isolation in HEp-2 cells. This percentage is higher than that reported earlier by Tsutsumi et al. (20) (72.6%; 69/95). As stated in the manufacturer's guide for the IC kit, the test is highly dependent on the technique of sample collection. We took a great deal of care in sample collection, transportation, and preservation. Additionally, we had more than 10 years of experience in sampling HAdV for isolation before the IC kit became available, which may explain the high detection level. Hara (7) reported a sensitivity of 94.2% (97 of 103 samples) for the same kit in comparison to isolation and that the high sensitivity is dependent on the method of sample collection.

All IC kit-positive samples were isolation positive. Two enterovirus-infected samples were negative by the IC kit and other HAdV diagnoses; the specificity of the IC kit was 100%, as reported previously (6, 20).

In this study, we were able to identify many HAdV-infected patients within a short period of time because a large outbreak of HAdV occurred in Japan in 2003 (13). This outbreak was the largest of its kind in the previous 10 years. The main causative agent was HAdV 3, which accounted for 69% (84 of 122) of the patients diagnosed by NT. According to previous reports, the IC kit is faster than the enzyme-linked immunosorbent assay kit (which takes at least 40 min) and is more useful for detection of respiratory HAdVs (21). Therefore, the IC kit is the only practical bedside diagnostic tool for respiratory HAdV infections.

The IC kit was useful for detecting HAdV in samples coinfected with HAdV and enterovirus. There was no clinical difference between coinfected patients and other HAdV-infected patients. Therefore, HAdV seemed to be the main causative agent of infection in these patients. Coinfection of HAdV3 and HAdV5 could be identified by PCR-based sequencing (17) and use of the HAdV 3- and 7-specific primers designed in this study.

We found that when the SYBR green real-time PCR method was used with isolated HAdV strains, the detection limit of the IC kit was $10^{4.6}$ to $10^{5.8}$ virus genome copies/ml for HAdV 1, 2, 3, 5, 6, and 7. However, there were clinical samples from which we isolated HAdV 3 (four samples) and HAdV 1 (two samples) that were negative by the IC kit despite concen-

trations of 9.0×10^7 to 2.5×10^9 virus genome copies/ml for HAdV 3 and 3.8×10^7 to 4.5×10^8 virus genome copies/ml for HAdV 1. These clinical samples had virus concentrations within the sensitivity limits of the IC kit; the detection limits for HAdV 3 and HAdV 1 were $10^{5.8}$ and $10^{4.6}$, respectively. According to Hierholzer (9), the bulk of newly synthesized HAdV product is not continuously released but remains cell associated. Only the products that are released into the extraction solution contribute to a positive result by the IC kit. This may explain the 5% false-negative rate.

The IC kit used in this study utilizes a monoclonal antibody made with the HAdV 2 hexon protein. Uchio et al. (21) reported that the IC kit had a lower sensitivity to HAdV 3 and 7 than to HAdV 4, when using serotypes 3, 4, 7, 8, 11, and 37, although these researchers used conjunctival swabs as samples. They reported that the minimum amounts of HAdV 3 and HAdV 8 detected by the IC kit were 4×10^3 and 4×10^4 PFU, respectively. Shimizu et al. (16) reported that the detection limit of an IC kit was $10^{4.45}$ (2.8×10^4) $TCID_{50}$/ml for HAdV 3 and that the sensitivities of other IC kits were similar. In our results, the IC kit was sensitive to $10^{1.9}$ to $10^{4.0}$ $TCID_{50}$/ml for HAdV 1, 2, 3, 5, and 7. Detection limits for the six serotypes were 2.9 ± 1.0 log $TCID_{50}$/ml and 5.4 ± 0.4 log copies of HAdV genome/ml (mean ± SD). The coefficients of variation were 0.34 and 0.08, respectively. Based on the real-time PCR results, the detection limit of the IC kit was approximately $10^{5.4}$ copies of HAdV genome/ml. In repeated IC kit tests, the high reproducibility of the IC kit was confirmed.

In this study period, no clinical samples containing HAdV 7 were obtained. HAdV 7 is known to cause fatal pneumonic infections in children, including nosocomial infections. We confirmed that the detection limits for HAdV 7 were $10^{5.6}$ copies of the HAdV 7 genome/ml and $10^{1.6}$ $TCID_{50}$ of HAdV 7/ml. The sensitivity of the IC kit was not lower for HAdV 7 than for the other serotypes in this study. According to Uchio et al. (21), the detection rate of the IC kit is 31% (8 of 26 patients) for HAdV 3 and 60% (3 of 5 patients) for HAdV 7 compared to that of PCR. We were able to detect the presence of HAdV 3 in respiratory samples with the IC kit 93% of the time (in 80 of 86 samples) in comparison to PCR.

The five samples that were IC kit and isolation negative but multiplex PCR positive contained 3.0×10^4 to 3.8×10^5 copies of the HAdV genome/ml. As isolation requires viable virus, PCR was more sensitive than isolation. It is known that many HAdV particles are not infectious, perhaps because they have genomic defects or lack fiber or some other protein (19).

In conclusion, the IC kit is a useful method for diagnosing HAdV diseases at the bedside because it has 95% sensitivity relative to that of isolation. To prevent nosocomial infections, rapid diagnosis at the bedside is necessary. Furthermore, the IC kit provides pediatricians with information about the prognosis of the respiratory diseases at the patient's first visit. However, because the IC kit test had a 5% false-negative rate compared to isolation, careful interpretation is required in cases where IC kit-negative results are obtained and HAdV infection is suspected.

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Coexistence of Multiple Genotypes, Including Newly Identified Genotypes, in Outbreaks of Gastroenteritis Due to *Norovirus* in Japan

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Norovirus **(NV) (formerly called Norwalk-like virus) is the most common cause of acute nonbacterial gastroenteritis in humans. Recently, we reported an NV genotyping scheme based on variability in the capsid N-terminal/shell (N/S) domain gene (Katayama et al., Virology 299:225-239, 2002). We found 19 genotypes, including nine of genogroup I and 10 of genogroup II. In the present study, we investigated the molecular epidemiology of NV from 66 outbreaks that occurred in Saitama Prefecture, Japan, from 1997 to 2002. We screened 416 stool specimens by a real-time reverse transcription (RT)-PCR method (Kageyama et al., J. Clin. Microbiol. 41:1548-1557, 2003) and detected 156 NV-positive specimens, from which we amplified the capsid N/S domain gene by RT-PCR and then cloned the PCR products. After sequencing these clones, we obtained 368 sequence variants (strains). By applying our classification scheme to the strains from Saitama and other published strains, we identified a total of 31 genotypes, including an additional five genotypes for genogroup I and seven for genogroup II. Of the 31 genotypes, 26 were present in the Saitama area during that time period. These results provide additional evidence for the great diversity of human NV genotypes. Specimens from all shellfish-related infections contained multiple genotypes, including several new genotypes. On the other hand, single genotypes were observed mostly in outbreaks that originated in semiclosed communities. Thus, the number of NV genotypes in each outbreak depended on the route of transmission.**

Norovirus (NV) (formerly called Norwalk-like virus) is a member of the family *Caliciviridae* and causes acute nonbacterial gastroenteritis in humans worldwide (8, 15, 29). NV is highly infectious and spreads by ingestion of contaminated food, such as oysters and water. NV also spreads by person-to-person transmission through the fecal-oral route in semiclosed communities, such as hospitals, schools, nursing homes, and cruise ships (8). These characteristics make NV a major public health concern.

The lack of a tissue culture system for propagation of NV has been a significant obstacle to the study of this group, but recent advances in cloning and sequencing of NV have enabled their genomic characterization. NV contains an ≈7.5-kb positive single-stranded RNA with a poly(A) tail at the 3′ end. The genome contains three open reading frames (ORFs). ORF1, the largest, encodes a polyprotein precursor for several nonstructural proteins (23), including NTPase, proteinase, and RNA-dependent RNA polymerase (RdRp). ORF2 encodes the capsid protein (17). ORF3, the smallest, encodes a protein of unknown function that has been suggested to be a minor component of the virion (6).

A recently developed reverse transcription-PCR (RT-PCR) assay that targets the RdRp (1, 2, 10, 16, 26, 27) or capsid gene (4, 7, 9, 11, 21, 22, 28, 31, 33) and phylogenetic analysis revealed that NV is classified into two genogroups, genogroup I (GI) and genogroup II (GII). In a previous study, we proposed a genotyping scheme for NV based on diversity in the capsid N terminus/shell (N/S) gene and reported nine genotypes in GI and 10 genotypes in GII (19).

We also established a real-time RT-PCR system for the routine detection of NV GI- and GII-specific RNAs (18). This detection system is highly sensitive and broadly reactive and rapid. Using this system, we reported that many stool specimens contain both GI and GII strains, suggesting coinfection by multiple strains (18). Coinfection was found in many food-borne outbreaks, but epidemiologic studies of these outbreaks lacked a detailed molecular analysis, including sequencing and genotyping.

In this study, we screened 416 stool specimens collected from 66 outbreaks in Saitama Prefecture, Japan, between January 1997 and May 2002 by real-time RT-PCR. With RT-PCR and GI- and GII-specific primer sets and sequencing, we were able to phylogenetically analyze the strains in each outbreak. Our results offer a more detailed study of the molecular epidemiology of this significant public health concern.

MATERIALS AND METHODS

Screening of NV-positive stool specimens. We used real-time RT-PCR and/or electron microscopy (3) to screen 416 stool samples from patients with nonbacterial acute gastroenteritis. The samples were from 66 outbreaks in Saitama Prefecture, Japan, between January 1997 and May 2002. These NV outbreaks occurred in a variety of epidemiological settings, including restaurants, schools,

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nursery schools, a nursing home, hotels, catered lunch businesses, a private home, and a dormitory (Table 1).

Real-time RT-PCR was performed as described previously (18) with slight modifications, which facilitated the detection of the GII/17 strain (19), such as Alphatron. In brief, RNA extraction from 10% stool suspensions and cDNA synthesis were carried out as described previously (18). Real-time RT-PCR was carried out in a 50-μl reaction volume containing 5 μl of cDNA solution, 25 μl of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, Calif.), a set of primers, and probes. In the detection of NV GI, a 400 nM concentration of each of the primers COG1F and COG1R and a mixture of fluorogenic probes [15 pmol of RING1(a)-TP and 5 pmol of RING1(b)-TP] were used. To detect NV GII, a 400 nM concentration (each) of a modified forward primer, COG2Fex (5′-MRSTGGATGMGRTTYTCWGA-3′), and of the reverse primer, COG2R, and a modified probe mixture of 15 pmol of RING2(a) (5′-FAM-TGGGAGG GYGATCGCAATCT-TAMRA-3′) and 5 pmol of RING2(b) (5′-FAM-TGGG AGGGGGATCGCGATCT-TAMRA-3′) were used. PCR amplification was performed with the ABI Prism 7700 sequence detector (Applied Biosystems), and amplification data were collected and analyzed with Sequence Detector software version 1.7 (Applied Biosystems) as described previously (18).

PCR amplification, cloning, and sequencing of capsid N/S domain. To amplify a 597-bp NV GI gene, including the capsid N/S domain, RT-PCR was carried out with a mixture of three forward primers, G1FF (5′-ATHGAACGYCAAATYT TCTGGAC-3′, 5′-ATHGAAAGACAAATCTACTGGAC-3′, and 5′-ATHGA RAGRCARCTNTGGTGGAC-3′, corresponding to nucleotides 5075 to 5671 in Norwalk/68) (18), and a reverse primer, G1SKR (5′-CCAACCCARCCATTRT ACA-3) (22). To obtain a 468-bp NV GII gene, including the capsid N/S domain, PCR amplification was also performed with another mixture of three forward primers, G2FB (5′-GGHCCMBMDTTYTACAGCAA-3′, 5′-GGHCCMBMDT TYTACAAGAA-3′, and 5′-GGHCCMBMDTTYTACARNAA-3′, corresponding to nucleotides 4922 to 5389 of Lordsdale) (18), and a reverse primer, G2SKR (5′-CCRCCNGCATRHCCRTTRTACAT-3′) (22). The PCR products were cloned into a PCR cloning vector, pT7 Blue (Novagen, Madison, Wis.). DNA sequences were determined with at least three clones with the BigDye terminator cycle sequence kit and ABI 377A sequencer (Applied Biosystems). The accession numbers used in this study are AB058511 to AB058598, AB059374 to AB059393, AB059635 to AB059641, and AB059682 (18).

Phylogenetic analysis. The nucleotide sequences of the capsid N/S domain gene starting at nucleotide 295 of GI (corresponding to nucleotides 5385 to 5652 in Norwalk/68) and nucleotide 282 of GII (corresponding to nucleotides 5084 to 5366 of Lordsdale) from its initiation codon were aligned with Clustal X (32). Genetic distances were calculated by Kimura's two-parameter method (20), and a distance matrix file was created as described previously (19). The phylogenetic dendrogram was constructed by the neighbor-joining method (30) with the capsid N/S domain gene and 1,000 bootstrap resamplings (5) as described previously (19).

Genome sequences. The complete genome sequences of the nine Saitama strains were deposited in the DNA Data Bank of Japan (DDBJ) (19). The accession numbers were AB039774 to AB039782. The following partial and complete genome sequences were also used in this study: Aichi124-89 (Seto), GenBank accession no. AB031013; Alphatron, AF195847; Amsterdam, AF195848; Appalachicola, AF414406; Arg320, AF190817; Auckland, U46039; M7, AY130761; Birmingham, AJ277612; Boxer, AF538679; Bristol, X76716; BS5, AF093797; Burwash Landing, AF414425; Camberwell, AF145896; Chiba, AB022679; Chitta, AB032758; Desert Shield, U04469; Erfurt, AF427118; Fayetteville, AY113106; Florida, AF414407; Fort Lauderdale, AF414426; Girlington, AJ277606; Grimsby, AJ004864; Hawaii, U07611; Hillingdon, AJ277607; Idaho Falls, AY054299; Kashiwa47, AB078334; KY-89, L23828; Leeds, AJ277608; Lordsdale, X86557; Manchester, X86560; Mexico, U22498; Melksham, X81879; Miami, AF414410; Musgrove, AJ277614; New Orleans, AF414422; Norwalk/68, M87661; Queensarms, AJ313030; Saint Cloud, AF414427; Seacroft, AJ277620; Sindlesham, AJ277615; Snow Mountain, U70059; Southampton, L07418; Stavanger, AF145709; Toronto, U02030; VA97207, AY038599; Valetta, AJ277616; White River, AF414423; Winchester, AJ277609; Wortley, AJ277618; and WUG1, AB081723.

Nucleotide sequence accession numbers. The nucleotide sequences between the C terminus of RdRp and the capsid N/S domain determined in this study were submitted to the DDBJ and given accession numbers AB112084 to AB112335.

RESULTS

Screening for NV and genogrouping by real-time RT-PCR. Stool specimens from 66 acute gastroenteritis outbreaks in the Saitama area were examined, and 256 of 416 specimens were positive for NV by real-time RT-PCR and/or electron microscopy (Table 1). The real-time RT-PCR used in this study detects NV in a genogroup-specific manner. Nine (14%) outbreaks contained only GI strains, and 36 (55%) outbreaks contained only GII strains. Both GI and GII strains were identified in 21 (31%) outbreaks. Although NV-related outbreaks occurred in a variety of settings, 32, or nearly half of them (49%), occurred in restaurants, 14 (21%) in semiclosed communities (schools, nursery schools, a nursing home, and a dormitory), 8 (12%) in catered lunches, 6 (9%) in hotels, and 6 (9%) in private homes (Table 1). In outbreaks from which both genogroups were detected, each stool specimen contained either one genogroup or both. In outbreak 200107, two specimens contained both GI and GII strains, and one specimen contained only a GI strain, whereas two other specimens contained only a GII strain. Both GI and GII strains were found frequently in restaurants, private homes, and catered lunch settings.

Cloning and sequencing of NV capsid N/S domain gene. With 156 NV-positive stool specimens, the sequences including the capsid N/S domain were amplified by RT-PCR with primer sets G1FF/G1SKR and G2FB/G2SKR for NV GI and GII strains, respectively. These primer sets were designed to amplify a broad spectrum of NV strains from the C terminus of RdRp to the capsid N/S domain region (18). The PCR product was then cloned into PCR cloning vector pT7 Blue, and the sequences were determined with at least three clones. Of the 156 NV-positive stool specimens, 368 (100 GI and 268 GII) sequences were obtained, which have been submitted to the DDBJ (see Materials and Methods).

Phylogenetic analysis. With 48 reference strains from the database, including ones reported previously (19), the operational taxonomic units of 368 capsid N/S domain sequences were calculated from the frequency distributions of the pairwise distances, and the genotype clusters were identified as described previously (19). The frequency distributions of intergenotype distances ranged from 0.122 to 0.356 (mean ± 3 SD, 0.239 ± 0.117) for GI and from 0.118 to 0.464 (0.291 ± 0.173) for GII.

With these sequences, phylogenetic dendrograms were constructed by the neighbor-joining method with the Manchester strain of sapoviruses (24) as an outgroup. Sequences from the same outbreak that branched to "the same cluster" were grouped as one strain (Fig. 1). The strains were further grouped into the same genotype when pairwise distances were less than 0.121 for GI and less than 0.117 for GII.

In the phylogenetic dendrograms, NV GI strains were separated into 14 genotypes. This analysis added five new GI genotypes (GI/10 to GI/14) to the previous nine (19) (Fig. 1A). The numbering of genotypes, GI/1 to GI/7, was changed from the previously published list (19) and is based on *Fields Virology*, 4th edition (8). All genotype clusters were statistically supported by the bootstrap value with the exception of genotype GI/3 (bootstrap value = 743) (Fig. 1A). Although this value was <950, the distances between the other strains of the same genogroup indicated that their pairwise distances were within the range of NV GI distances of the mean ± 3 SD. Therefore, each operational taxonomic unit within this cluster was considered a distinct genotype cluster (19). In the previous nine GI genotypes, Norwalk/68 was a typical strain of GI/1.

TABLE 1. Description of NV gastroenteritis in Saitama area, Japan, from 1997 to 2002

Outbreak no.	Mo-yr	Setting	No. of persons		Attack rate (%)	No. of NV-positive stool specimens/no. tested[a]	Stool code(s) for sequencing analysis	Genogroup(s)[b]	Genotype(s)
			Ill	At risk					
199818	Dec.-98	Restaurant[c]	3	4	75	2/2	U25	GII	GII/8
							U26	GII	GII/3
199902	Feb.-99	Restaurant[c]	2	3	67	1/1	SzU	GI + GII	GI/9, GII/15
199904	Apr.-99	Restaurant[c]	5	5	100	2/2	KU8	GI + GII	GI/11, GII/3
							KU9	GII	GII/3
199917	Nov.-99	Restaurant[c]	9	13	69	5/7	KU80	GI + GII	GI/8, GII/4, 6, 15
							KU82	GI + GII	GI/2, 4, 5, GII/15
							KU83	GI + GII	GI/5, GII/15
							KU84	GII	GII/5, 15
200006	Jan.-00	Restaurant[c]	7	9	78	7/7	KU18, KU26, KU27	GII	GII/3
							KU19	GI + GII	GI/1, 2, 12, GII/3
200009	Mar.-00	Restaurant[c]	6	34	18	6/9	KU35, KU37	GII	GII/10
							KU36	GI + GII	GI/4, GII/10
200025	Dec.-00	Restaurant[c]	11	14	79	5/9	T3, T5	GI + GII	GI/8, GII/7
							T4	GI	GI/8
							T6	GII	GII/6
							T7	GI	GI/14
200103	Jan.-01	Restaurant[c]	7	18	39	7/7	T9, T12	GII	GII/12
							T10, T13	GI + GII	GI/2, GII/12
							T11	GII	GII/4, 12
							T14	GI + GII	GI/2, GII/8
							T15	GII	GII/12
200107	Jan.-01	Restaurant[c]	13	29	45	8/11	T16, T17	GI	GI/2
							T18, T19	GII	GII/12
							T20	GI + GII	GI/2, GII/12
200115	Feb.-01	Restaurant[c]	2	2	100	2/2	T28	GII	GII/1, 4, 9
							T29	GI + GII	GI/14, GII/11
200119	Apr.-01	Restaurant[c]	7	17	41	7/7	T30	GI + GII	GI/4, GII/12
							T31	GII	GII/14
							T32	GI + GII	GI/3, GII/1
200126	Jun.-01	Restaurant[c]	5	9	56	5/5	T35	GI + GII	GI/2, 13, GII/11
							T36	GI + GII	GI/13, GII/3, 6
							T37	GII	GII/3, 4, 11
200206	Jan.-02	Restaurant[c]	2	5	40	1/1	T53	GI + GII	GI/13, GII/16
200209	Jan.-02	Restaurant[c]	7	10	70	4/4	T80	GII	GII/14
							T85	GII	GII/3, 5, 12
200219	Feb.-02	Restaurant[c]	32	86	37	6/7	T59	GI + GII	GI/7, GII/4, 5
							T61	GI + GII	GI/7, GII/5
							T60, T86	GII	GII/5
							T62	GI	GI/7
200228	Mar.-02	Restaurant[c]	14	24	58	3/3	T66	GI + GII	GI/4, GII/3, 12
							T82	GI + GII	GI/2, 4, GII/3, 4, 5, 12
							T87	GII	GII/11
200232	Mar.-02	Restaurant[c]	15	53	28	8/8	T67	GI + GII	GI/7, GII/8
							T68	GI + GII	GI/4, GII/3, 5
							T69, T70	GI	GI/4
							T83	GI + GII	GI/7, GII/12
							T88	GI	GI/8
200108	Jan.-01	Private home[c]	7	15	47	5/6	T21	GI + GII	GI/8, GII/4
							T22	GI + GII	GI/8, GII/3
							T23	GI + GII	GI/1, GII/1, 3, 4
							T24	GI + GII	GI/1, 4, GII/3, 4, 12
							T84	GII	GII/4
200109	Jan.-01	Private home[c]	2	2	100	2/2	T25	GI + GII	GI/14, GII/3
							T26	GI + GII	GI/8, GII/4
200137	Nov.-01	Private home[c]	12	23	52	3/6	T46	GI + GII	GI/4, GII/6, 11
200214	Feb.-02	Private home[c]	2	2	100	2/2	T56	GI + GII	GI/9, GII/1, 5, 6, 12
							T57	GII	GII/4, 5
199701	Jan.-97	Restaurant	12	37	32	5/8	U1, U2	GII	GII/12
199710	Dec.-97	Restaurant	13	15	87	2/4	U10, U11	GII	GII/4
199712	Dec.-97	Restaurant	4	4	100	2/4	U12, U13	GII	GII/4
199817	Dec.-98	Restaurant	11	15	73	8/11	U22, U23, U24	GII	GII/1
199905	Apr.-99	Restaurant	3	12	25	1/1	KU10	GI	GI/4
199920	Dec.-99	Restaurant	15	27	56	3/8	KU98, KU99, KU101	GII	GII/2
199921	Dec.-99	Restaurant	17	28	61	8/12	KU105	GI + GII	GI/4, GII/4, 6

Continued on following page

TABLE 1—*Continued*

Outbreak no.	Mo-yr	Setting	No. of persons		Attack rate (%)	No. of NV-positive stool specimens/no. tested[a]	Stool code(s) for sequencing analysis	Genogroup(s)[b]	Genotype(s)
			Ill	At risk					
							KU109, KU111	GI + GII	GI/4, GII/4
							KU112, KU115	GI	GI/4
200008	Mar.-00	Restaurant	5	8	63	3/5	KU31, KU32	GII	GII/10
200027	Dec.-00	Restaurant	22	45	49	3/5	T8	GII	GII/4
200113	Feb.-01	Restaurant	12	36	33	1/3	T27	GI	GI/2
200139	Dec.-01	Restaurant	12	23	52	11/12	T50	GII	GII/3, 10
200213	Feb.-02	Restaurant	20	55	36	1/2	T55	GII	GII/5
200216	Feb.-02	Restaurant	4	10	40	2/3	T58	GI	GI/4
200222	Feb.-02	Restaurant	2	2	100	1/1	T63	GI	GI/4
200227	Mar.-02	Restaurant	2	2	100	1/1	T81	GII	GII/8
200132	Oct.-01	Private home	3	6	50	3/3	T44	GI	GI/8
200237	May.-02	Private home	6	6	100	5/6	T75	GII	GII/3
199811	May.-98	School	53	212	25	4/6	U18, U19, U20, U21	GII	GII/3
199906	May.-99	School	40	60	67	11/16	KU17, E10, E11, E12, E13	GII	GII/5
199907	May.-99	School	12	34	35	2/5	KU24	GI	GI/4
199915	Nov.-99	School	21	Unknown		1/5	KU68	GII	GII/2
199919	Dec.-99	School	Unknown	Unknown		2/8	KU93, E24	GII	GII/2
200014	Apr.-00	School	14	33	42	7/9	T1	GI	GI/3
200015	Apr.-00	School	13	38	34	4/6	T2	GI	GI/3
200138	Dec.-01	School	56	217	26	7/14	T47, T49	GII	GII/5
							T48	GII	GII/4, 5
200240	May-02	School	3	3	100	3/3	T76, T78, T79	GII	GII/2
199703	Oct.-97	Nursery school	12	20	60	6/7	U5, U6	GII	GII/4
199914	Oct.-99	Nursery school	50	103	49	8/16	KU62, KU63, KU64, KU66	GII	GII/6
200133	Nov.-01	Nursery school	19	128	15	5/5	T45	GII	GII/3
199704	Nov.-97	Nursing home	Unknown	Unknown		3/4	U7, U8	GII	GII/4
199807	Feb.-98	Dormitory	6	49	12	2/2	U16, U17	GII	GII/6
199702	Feb.-97	Catered lunch	19	20	95	3/4	U3, U4	GII	GII/6
199705	Nov.-97	Catered lunch	19	20	95	3/4	U9	GII	GII/4
199910	Jun.-99	Catered lunch	16	33	48	1/7	KU44	GII	GII/6
199918	Dec.-99	Catered lunch	10	35	29	5/9	KU85, KU88, KU89, E22, E23	GII	GII/2
200002	Jan.-00	Catered lunch	2	2	100	1/1	KU5	GII	GII/10
200005	Jan.-00	Catered lunch	3	3	100	2/2	KU16	GII	GII/12
200120	Apr.-01	Catered lunch	12	19	63	8/8	T33	GII	GII/3
							T34	GI + GII	GI/3, GII/3
200131	Oct.-01	Catered lunch	19	37	51	13/19	T39	GII	GII/3, 5, 12
							T42	GII	GII/3, 12
							T43	GII	GII/12
199806[c]	Jan.-98	Hotel	27	52	52	5/5	U201, U15	GII	GII/3
199903	Mar.-99	Hotel	10	11	91	3/4	KU4	GI	GI/1, 7
							KU6	GI	GI/1
							KU7	GI	GI/4
199909	Jun.-99	Hotel	16	264	6	1/7	KU34	GII	GII/8
200011	Mar.-00	Hotel	34	139	24	9/22	KU49, KU53	GII	GII/3
200201	Jan.-02	Hotel	21	50	42	13/17	T52	GII	GII/5.
200226	Feb.-02	Hotel	Unknown	Unknown		3/4	T64	GII	GII/3, 5, 10
							T65	GII	GII/10, 11
Total						256/416	156		

[a] NV positive by real-time RT-PCR and/or electron microscopy (3).
[b] GI, GI detected; GII, GII detected by real-time RT-PCR (see Materials and Methods).
[c] Shellfish-related outbreak.

Others are indicated in Fig. 1A. In the five new GI genotypes, GI/10, represented by Boxer/01/US, was not found in stool specimens from the Saitama area, whereas the representative strains of GI/11, GI/12, GI/13, and GI/14 (KU8GI/99, KU19aGI/00, T35aGI/01, and T25GI/01, respectively) found in Saitama have not been reported in other parts of the world.

Similarly, NV GII strains were separated into 17 genotypes (Fig. 1B), including seven new genotypes (GII/9 to GII/11 and GII/13 to GII/16). The numbering of genotypes GII/1 to GII/7 was changed from the previously published list (19) and is based on *Fields Virology* (8). All genotype clusters were statistically supported by bootstrap values (Fig. 1B). In the previous 10 GII genotypes, Lordsdale/93/UK was a typical strain of GII/4. Others are indicated in Fig. 1B. In the seven new GII genotypes, GII/9, GII/10, GII/13, and GII/14 were represented by Idaho Falls/96/US, Erfurt/546/00/DE, M7/99/US, and Kashiwa47/00/JP, respectively, and GII/9 and GII/13 were not found in stool specimens from Saitama. In contrast, typical strains of GII/11, GII/15, and GI/16 (T29GII/01, KU80aGII/99, and T53GII/02, respectively) found in Saitama have not been reported in other parts of the world.

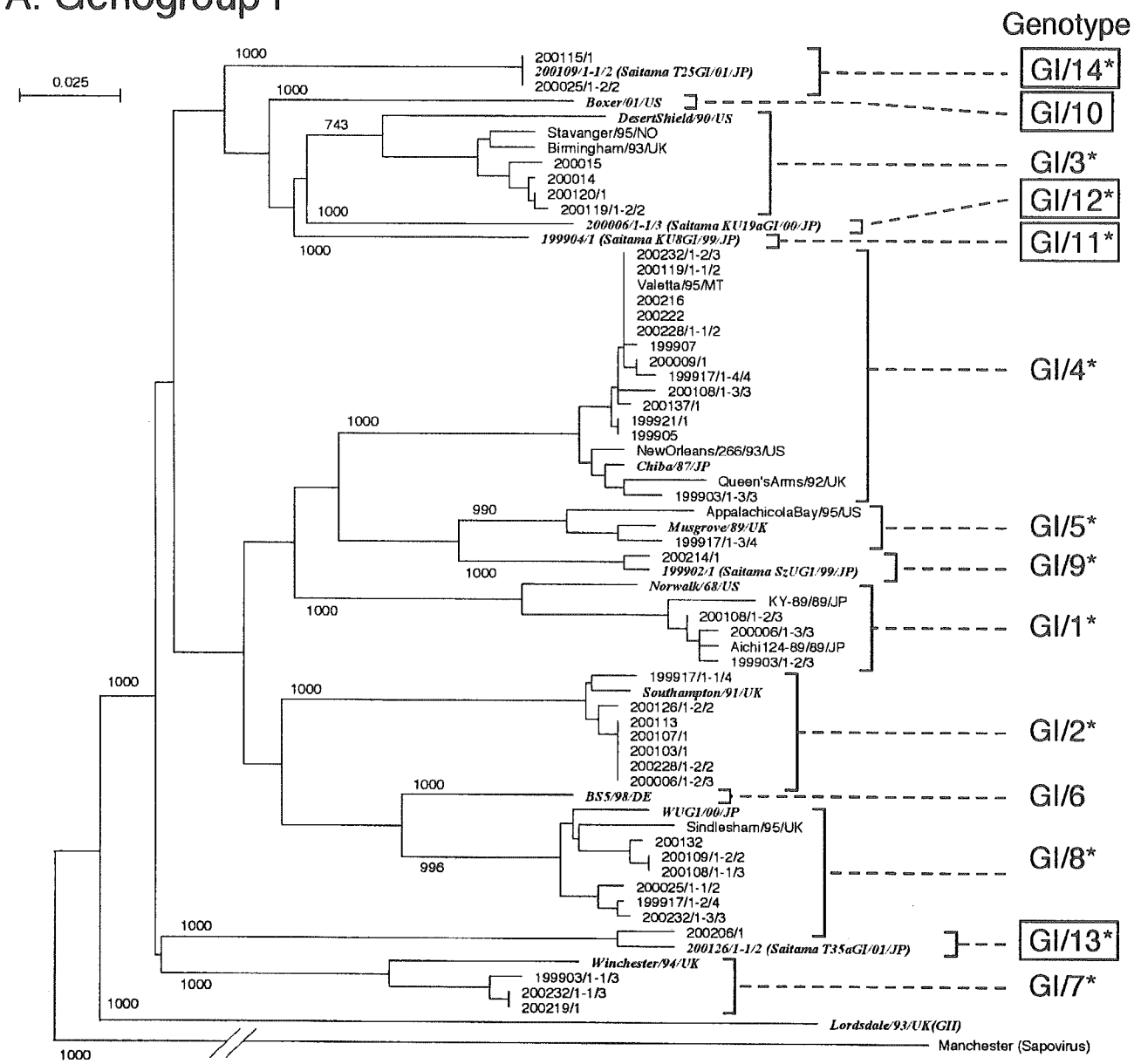
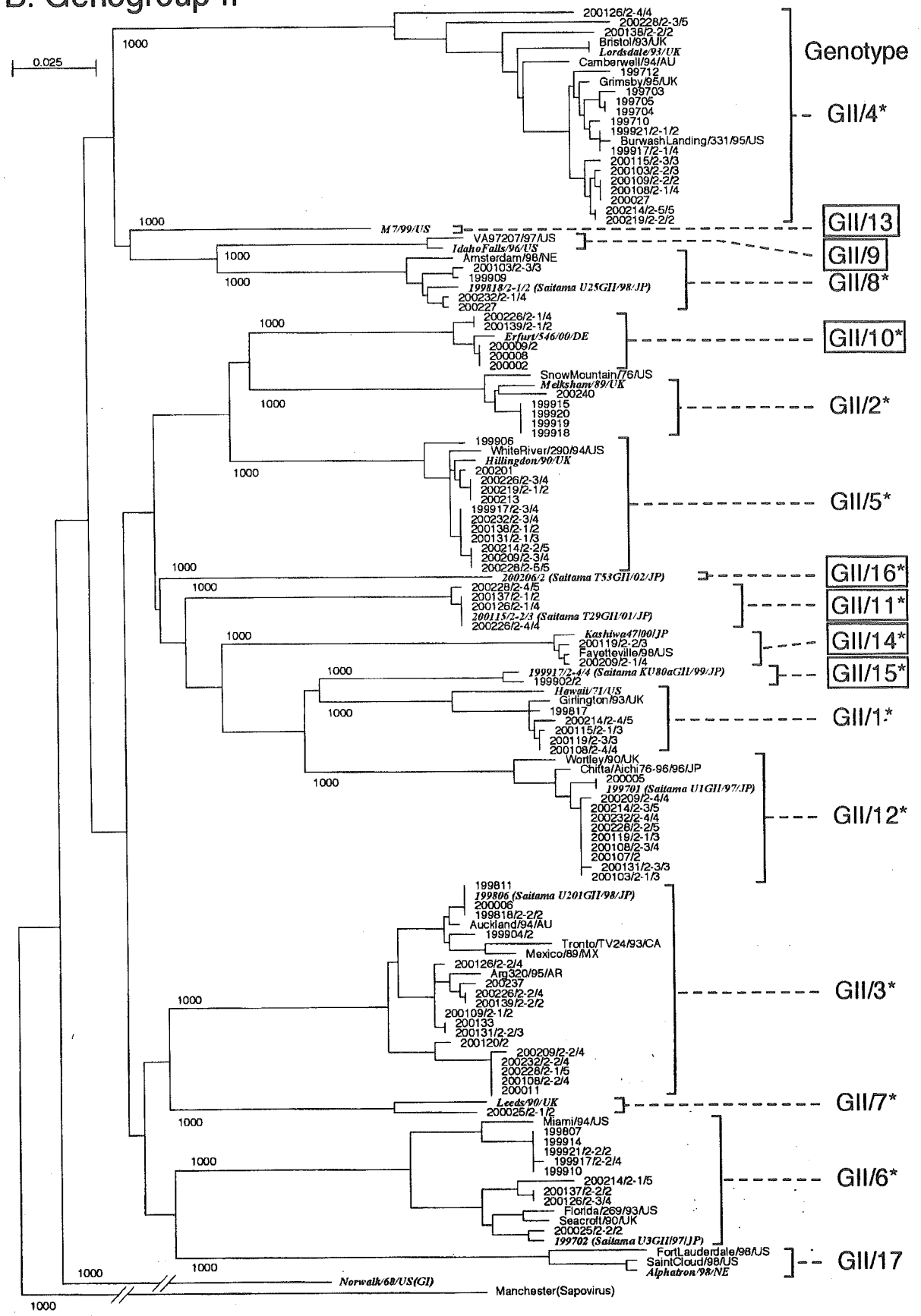


FIG. 1. Phylogenetic dendrograms based on the capsid N/S domain gene of NV. Phylogenetic dendrograms were generated separately for GI (A) and GII (B). The numbers on each branch indicate the bootstrap values for the clusters supported by that branch. Letters in italics designate the reference strains. Cryptograms indicating the location or strain name/(isolate)/year/country are given for key strains. Putative genotypes are indicated for each cluster. The numbering of genotypes GI/1 to GI/7 and GII/1 to GII/7 was changed from the previously published list (19) and is based on *Fields Virology* (8). GI/10 to GI/14, GII/9 to GII/11, and GII/13 to GII/16 (boxed) are genotypes newly identified in this study. Genotypes with an asterisk were present in the Saitama area in 1997 to 2002.

Molecular epidemiology. All genotypes identified in stool specimens from 66 outbreaks are listed in Table 1. A large number of stool specimens, 51 of 156, contained two to six genotypes each. For example, seven specimens (KU80, KU82, KU19, T23, T24, T56, and T82) contained more than four genotypes; T82 contained six genotypes (GI/2, GI/4, GII/3, GII/4, GII/5, and GII/12), including both NV GI and GII genotypes.

Multiple genotypes were observed in the same outbreaks, in four of six (67%) outbreaks at private homes, 18 of 32 (56%) at restaurants, two of six (33%) at hotels, one of eight (13%) at catered lunches, and one of nine (11%) at schools. In outbreak 200107, stool specimens were collected from five patients. Of these, two specimens contained GI/2, another two contained GII/12, and one contained both genotypes. In outbreak 199917, specimens were obtained from four patients. One contained four genotypes (i.e., GI/8, GII/4, GII/6, and GII/15). Another also contained four genotypes (i.e., GI/2, GI/4, GI/5,


B. Genogroup II
0.025
Genotype
GII/4*
GII/13
GII/9
GII/8*
GII/10*
GII/2*
GII/5*
GII/16*
GII/11*
GII/14*
GII/15*
GII/1*
GII/12*
GII/3*
GII/7*
GII/6*
GII/17
200126/2-4/4
200228/2-3/5
200138/2-2/2
Bristol/93/UK
Lordsdale/93/UK
Camberwell/94/AU
199712
Grimsby/95/UK
199703
199705
199704
199710
199921/2-1/2
BurwashLanding/331/95/US
199917/2-1/4
200115/2-3/3
200103/2-2/3
200109/2-2/2
200108/2-1/4
200027
200214/2-5/5
200219/2-2/2
M7/99/US
VA97207/97/US
IdahoFalls/96/US
Amsterdam/98/NE
200103/2-3/3
199909
199818/2-1/2 (Saitama U25GII/98/JP)
200232/2-1/4
200227
200226/2-1/4
200139/2-1/2
Erfurt/546/00/DE
200009/2
200008
200002
SnowMountain/76/US
Melksham/89/UK
200240
199915
199920
199919
199918
199906
WhiteRiver/290/94/US
Hillingdon/90/UK
200201
200226/2-3/4
200219/2-1/2
200213
199917/2-3/4
200232/2-3/4
200138/2-1/2
200131/2-1/3
200214/2-2/5
200209/2-3/4
200228/2-5/5
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200228/2-4/5
200137/2-1/2
200126/2-1/4
200115/2-2/3 (Saitama T29GII/01/JP)
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200119/2-2/3
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200209/2-1/4
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Hawaii/71/US
Girlington/93/UK
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200115/2-1/3
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200232/2-4/4
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200131/2-3/3
200103/2-1/3
199811
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Auckland/94/AU
199904/2
Tronto/TV24/93/CA
Mexico/89/MX
200126/2-2/4
Arg320/95/AR
200237
200226/2-2/4
200139/2-2/2
200109/2-1/2
200133
200131/2-2/3
200120/2
200209/2-2/4
200232/2-2/4
200228/2-1/5
200108/2-2/4
200011
Leeds/90/UK
200025/2-1/2
Miami/94/US
199807
199914
199921/2-2/2
199917/2-2/4
199910
200214/2-1/5
200137/2-2/2
200126/2-3/4
Florida/269/93/US
Seacroft/90/UK
200025/2-2/2
199702 (Saitama U3GII/97/JP)
FortLauderdale/98/US
SaintCloud/98/US
Alphatron/98/NE
Norwalk/68/US(GI)
Manchester(Sapovirus)
1000