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 | 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.

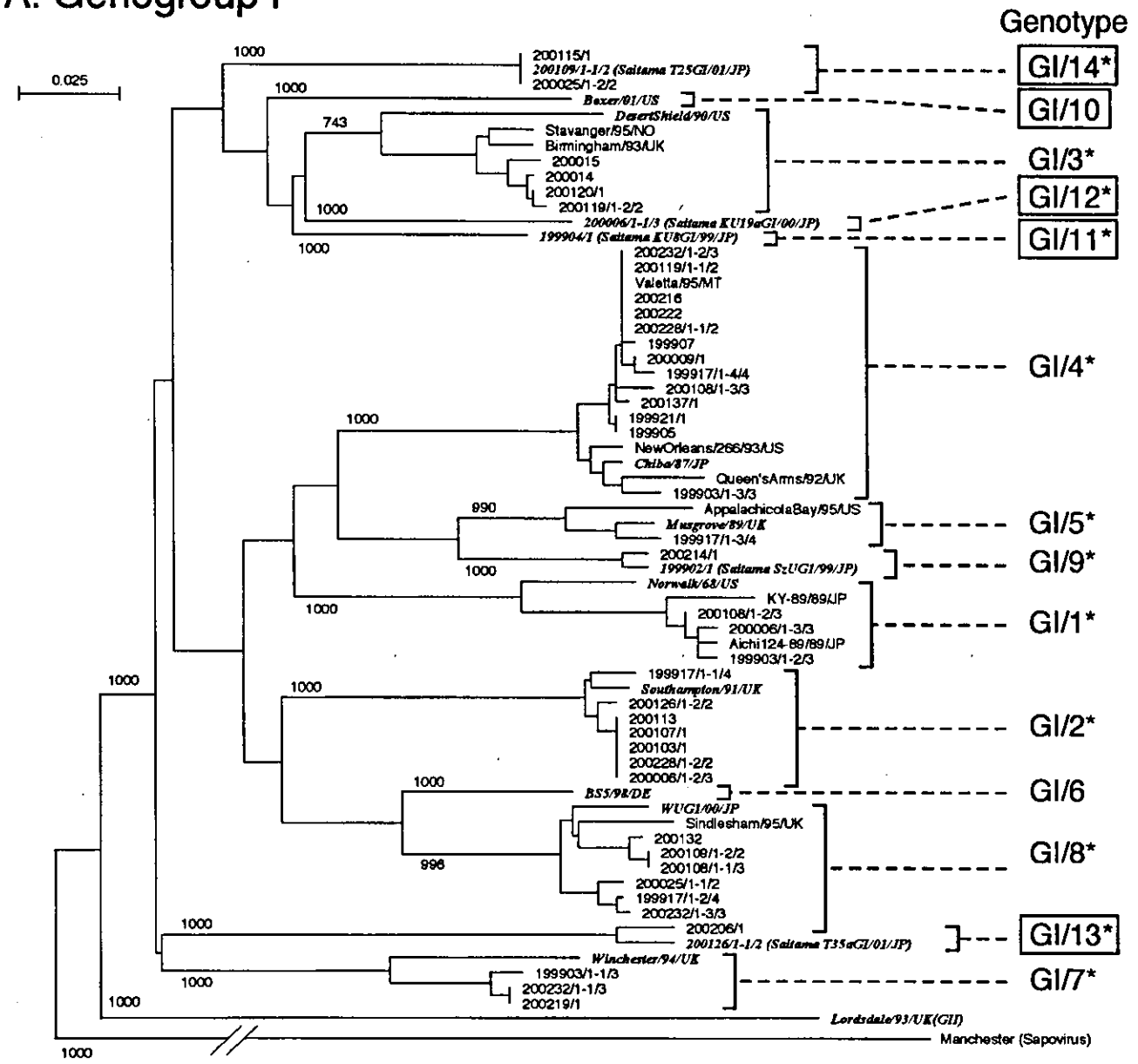
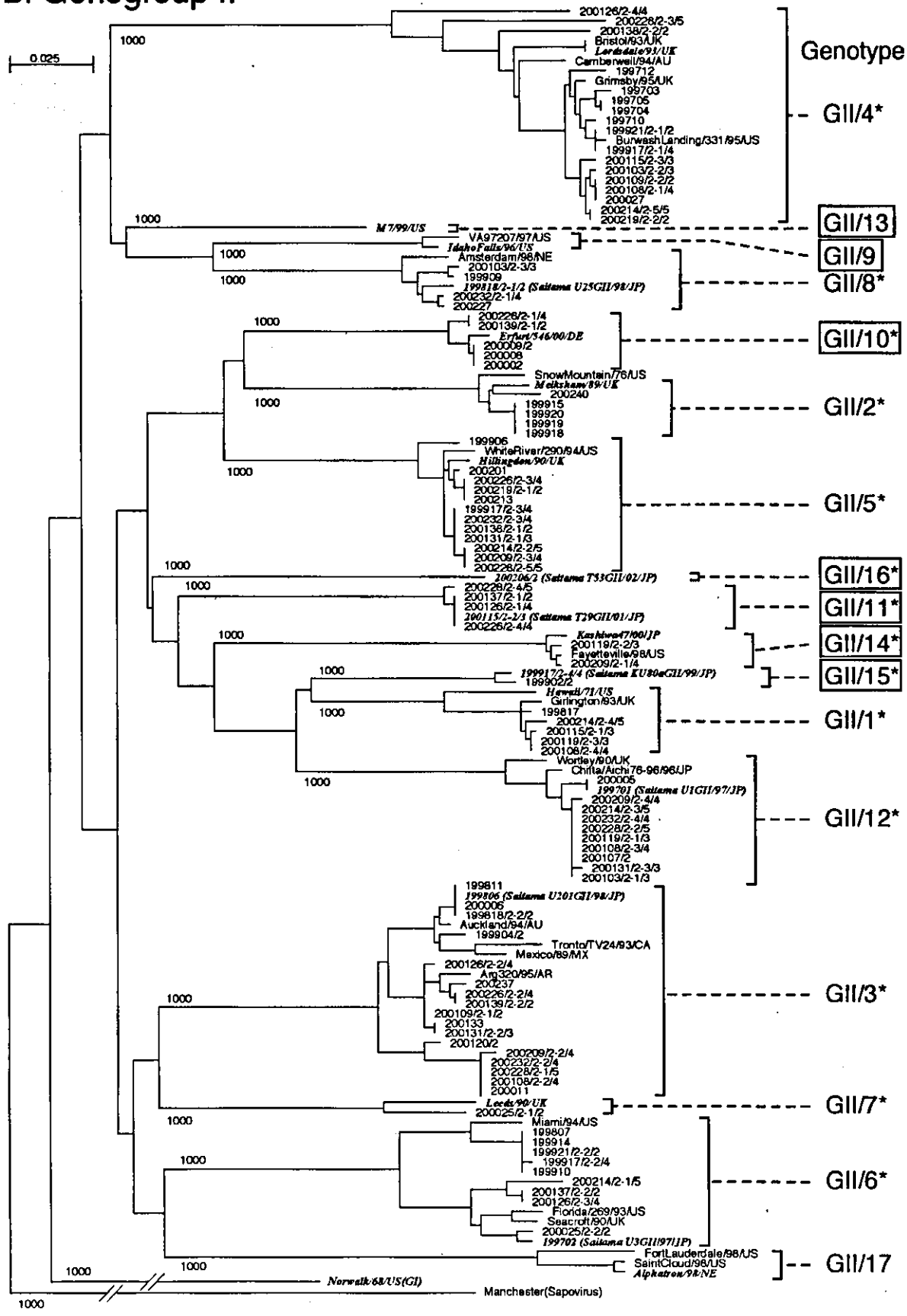



FIG. 1. Phylogenetic dendrograms based on the capsid N/S domain gene of NV. Phylogenetic dendrograms were generated separately for GI (A) and GII (B). The numbers on each branch indicate the bootstrap values for the clusters supported by that branch. Letters in italics designate the reference strains. 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



and GII/15). A third contained two genotypes (i.e., GI/5 and GII/15), and the last also contained two genotypes (i.e., GII/5 and GII/15). GII15 was a common genotype. As many as eight genotypes were detected in those outbreaks.

Single genotypes were observed in most outbreaks that occurred in semiclosed communities, such as dormitories, nursing homes, schools, and nursery schools. In outbreak 199906, all five NV-positive stool samples contained a common genotype, GII/5. Similarly, all four specimens in outbreaks 199811 and 199914 contained GII/3 and GII/6, respectively, and all three specimens in 200240 contained GII/2.

Surprisingly, many genotypes were found in the Saitama area in the past 5 years. Although they are from 156 stool specimens within the 66 outbreaks, we identified 26 of 31 genotypes. Only five genotypes (i.e., GI/6, GI/10, GII/9, GII/13, and GII/17) were not observed.

**Genogrouping and genotyping.** Real-time RT-PCR detected and distinguished genogroups GI and GII and has proved itself a useful screening method. Genogrouping based on this method was confirmed in all specimens by genotyping with phylogenetic analysis of RT-PCR products (Table 1). For example, stool specimen KU82 appeared to contain both GI and GII by real-time RT-PCR, and GI/2, GI/4, GI/5, and GII/15 were actually detected by genotyping. In the case of KU4, only GI was detected by real-time RT-PCR, and GI/1 and GI/7 were identified by RT-PCR after sequencing analysis. In the case of T28, only GII was detected, and the specimen contained three genotypes, GII/1, GII/4, and GII/9 (Table 1).

## DISCUSSION

In this study, we used real-time RT-PCR plus electron microscopy to screen NV in stool specimens from 66 outbreaks in the Saitama area. The real-time RT-PCR method greatly saved the time required for selecting stool specimens for further analysis. From 156 NV-positive specimens, we obtained 368 capsid N/S gene sequences after cloning the RT-PCR products that were amplified with primer sets GIFF/GISKR and G2FB/G2SKR (18). Genotyping was performed by phylogenetic analyses according to the scheme described previously (19).

We note that all shellfish-related outbreaks were caused by multiple NV genotypes (Table 1). With their filter-feeding mechanisms, shellfish, such as oysters, can concentrate NV from an environment contaminated by multiple genotypes. In fact, oysters in the markets were found to contain several different genotypes (data not shown). Furthermore, some outbreaks displayed multiple genotypes with relatively high frequencies; the outbreaks occurring in 67% of private homes, 56% of restaurants, 33% of hotels, and 13% of catered lunches were strongly suspected to be due to shellfish. For example, shellfish were the common source for outbreak 199917 (Table 1). In this outbreak, specimens were obtained from four patients, and each specimen contained multiple genotypes, but the genotypes did not coincide with each other except for GII/15. Also, for example, in outbreak 200126, three specimens contained multiple genotype strains, but a common genotype strain did not exist (Table 1).

Among outbreaks which were not directly related to shellfish, there were some in which multiple genotypes were detected. In outbreak 199921 involving a restaurant but not shellfish, multiple genotypes were identified. One specimen (KU105) contained three genotypes (GI/4, GII/4, and GII/6), two specimens (KU109 and KU111) contained two genotypes (GI/4 and GII/6), and another specimen (KU112) contained only one genotype (GI/4). In this outbreak, the four individuals had eaten dinner together. Since no common foods, such as oysters, were identified, the cook, from whom KU115 was collected, was presumed to be the source. KU115 contained one genotype, GI/4, that was a common genotype in this outbreak. Other genotypes were not detected from this specimen. However, the cook was tested for NV long after the other patients were tested. Possibly, in the early stage of the disease, the cook had shed at least all three genotypes (GI/4, GII/4, and GII/6) and transmitted them to the other individuals by poor handling of cooked food. At a later stage, perhaps the virus titers in KU115 were lower, and only the predominant genotype GI/4 was detected. Also, in outbreak 200138, at school, multiple genotypes (GII/4 and GII/5) were also identified in one of three specimens. The other two specimens contained only a common genotype (GII/5) (Table 1).

On the other hand, stool specimens from outbreaks in semiclosed communities contained only single genotypes, with the exception of outbreak 200138. Fourteen of 66 outbreaks occurred in semiclosed communities (schools, nursery schools, nursing homes, and dormitory), and only seven genotypes (i.e., GI/3, GI/4, GII/2, GII/3, GII/4, GII/5, and GII/6) were found. In each outbreak, one genotype was likely transmitted through the fecal-oral route.

In NV infection, individual patients seem to differ in their susceptibility to each genotype. We confirmed that the genotypes which we identified were also antigenically distinct by an antigen enzyme-linked immunosorbent assay with hyperimmune sera against virus-like particles (unpublished data). Thus, susceptibility to each genotype seems to differ in each individual, perhaps due to differences in acquired immunity from previous NV infections. The different susceptibilities may also be due to specific ABO histo-blood group antigens in each individual, as described in recent studies (12-14, 25).

Furthermore, in the case of person-to-person infection with NV, selection of strains may occur during sequential passages in the outbreak due to the factors on the agent side, such as pathogenicity, reproductive rate in the host, and/or stability in the environment. When a person is infected first by multiple genotype strains, a strain that replicates faster and has greater stability may eventually become predominant later in the outbreak. Further epidemiological investigation may be necessary to clarify the mechanism of selection.

In the present study, we classified NV into 31 distinct genotypes (Fig. 1). This analysis added five GI and seven GII genotypes to the previously published list (19), and all of these new genotypes, except for GII/9 and GII/13, were detected in the Saitama area. GII/10 and GII/14 were isolated exclusively in Germany and the United States. In the Saitama area during the study period, a total of 26 of the 31 genotypes, including 10 new genotypes, were found. Saitama Prefecture is only 3,800 $km^2$ and ≈1% of the total area of Japan. It is surprising that this small region contained such a diversity of genotypes, including ones found in North and South America, Europe, Oceania, and Asia (Fig. 1). The extensive diversity in the Saitama area suggests that many genotypes were imported

from and exported to other countries with NV-contaminated foods and travelers afflicted with NV. Various genotypes of NV may be circulating around the world, and more new genotypes are likely to be discovered in the future.

With a combination of screening by real-time RT-PCR and genotyping by phylogenetic analysis, detection of NV in sewage, rivers, seawater, and foods may improve our understanding in the epidemiology of NV and, in turn, help us to prevent and control future NV outbreaks.

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# Novel Recombinant Sapovirus

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We determined the complete genome sequences of two sapovirus strains isolated in Thailand and Japan. One of these strains represented a novel, naturally occurring recombinant sapovirus. Evidence suggested the recombination site was at the polymerase-capsid junction within open reading frame one.

The positive-sense polyadenylated single-stranded RNA virus family *Caliciviridae* contains four genera, *Norovirus*, *Sapovirus*, *Lagovirus*, and *Vesivirus* (1). Human norovirus is the most important cause of outbreaks of gastroenteritis in the United States and infects all age groups (2). Human sapovirus is also a causative agent of gastroenteritis but is more frequent in young children than in adults (3). Most animal caliciviruses are grouped within the other two genera. In 1999, Jiang et al. (4) identified the first naturally occurring human recombinant norovirus, and several other strains were later described as recombinants (5 8). Evidence suggested that the recombination event occurred at the junction of open reading frames one and two (ORF1 and ORF2), but this finding was not proven. Norovirus ORF1 encodes nonstructural proteins, including the RNA-dependent RNA polymerase, ORF2 encodes the capsid protein, and ORF3 encodes a small capsid protein (1). Nucleotide sequence of the polymerase and capsid junction generally is conserved among the human norovirus genotypes (4,6), which likely facilitates a recombination event when nucleic acid sequences of parental strains come into physical contact in infected cells, e.g., during copy choice recombination (9).

## The Study

We used genetic analysis to investigate a novel, naturally occurring recombinant sapovirus. Two strains were used for the analysis, Mc10 strain (GenBank accession no. AY237420), isolated from an infant hospitalized with acute gastroenteritis in Chiang Mai, Thailand, in 2000 (5), and C12 strain (AY603425), isolated from an infant with gastroenteritis in Sakai, Japan, in 2001 (unpub. data). Although the original polymerase chain reaction (PCR) primer sets that detected these two strains were different, both were directed toward the conserved 5′ end of the capsid gene and have been shown to detect a broad range of sapovirus sequences in genogroup I (GI) and GII (5,10). For Mc10, primers SV5317 and SV5749 were used; for C12, primers SV-F11 and SV-R1 were used.

The complete genomes for Mc10 and C12 were determined as previously described (6). As shown in Figure 1A, the sapovirus genome has an organization slightly different from that of the norovirus genome. ORF1 encodes nonstructural proteins, polymerase, and the capsid protein, and ORF2 encodes a small protein (1).

Initially, we grouped Mc10 and C12 into two distinct GII clusters (i.e., genotypes), on the basis of their capsid sequences (Figure 2A) and the phylogenetic classification scheme of Okada et al. (10). In addition, the overall genomic nucleotide similarity between Mc10 and C12 was 84.3%, while ORF1 and ORF2 shared 85.5% and 73.3% nucleotide identity, respectively. These results corresponded with the capsid-based grouping shown in Figure 2A. By comparing sequence similarity across the length of the genomes with SimPlot with a window size of 100 (11), we discovered a potential recombination site, where the similarity analysis showed a sudden drop in nucleotide identity after the polymerase region (Figure 1B). Nucleotide sequence analysis of ORF1 less the capsid sequence and the capsid sequence indicated 90.1% and 71.3% nucleotide identity, respectively (Figure 1A). To additionally illustrate the nucleotide identities of ORF1 less the capsid sequence, a phylogenetic tree of polymerase sequences of Mc10, C12, and other available strains was developed (Figure 2B). However, for three strains (Mex14917/00,

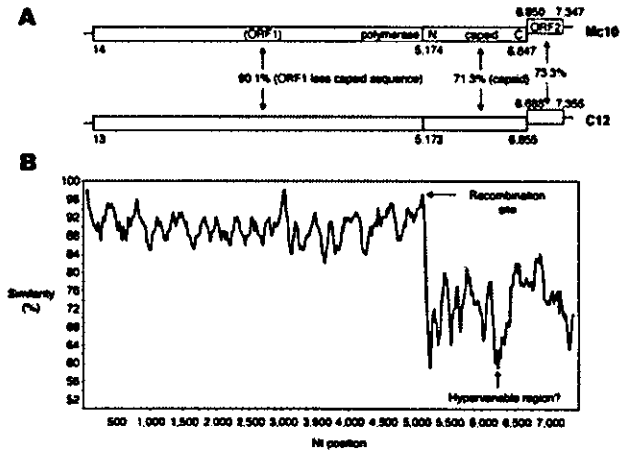


Figure 1. A) The genomic organization of Mc10 and C12 strains. B) the SimPlot analysis of Mc10 and C12. Mc10 genome sequence was compared to C12 by using a window size of 100 bp with an increment of 20 bp. All gaps were removed. The recombination site is suspected to be located between polymerase and capsid genes, as shown by the arrow. The possible hypervariable region for the capsid protein is also shown.

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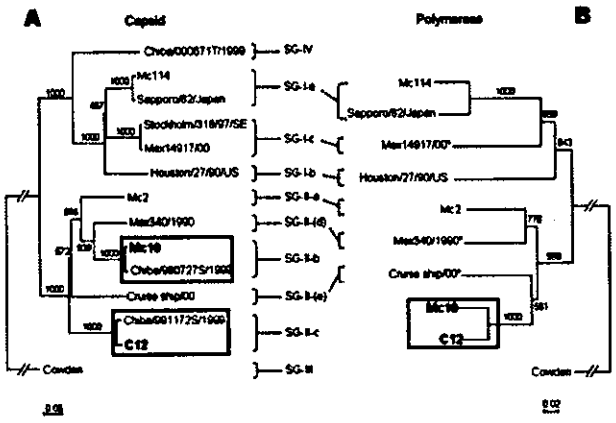


Figure 2. Phylogenetic analysis of (A) capsid (376 nt) and (B) polymerase (289 nt) sequences of Mc10, C12, and additional strains in GenBank. Sapovirus capsid sequences were classified on the basis of the scheme of Okada et al. (10). Two unclassified strains, Mex340/1990 and Cruise ship/00, were assigned SG-II-(d) and SG-II-(e). The asterisks indicate noncontinuous polymerase-capsid sequences. The numbers on each branch indicate the bootstrap values for the genotype. Bootstrap values of >950 were considered statistically significant for the grouping (6). The scale represents nucleotide substitutions per site. GenBank accession no. for the reference strains are as follows: Chiba/000671T/1999, AJ412805; Chiba/990727S/1999, AJ412795; Chiba/991172S/1999, AJ412797; Mc114, AY237422; Cruise ship/00, AY289804 and AY157863; Cowden, AF182760; Houston/27/90/US, U95644; Mc2, AY237419; Mc10, AY237420; Mex340/1990, AF435809 and AF435812; Mex14917/00, AF435813 and AF35810; Sapporo/82/Japan, U65427; and Stockholm/318/97/SE, AF194182.

Mex340/1990, and Cruise ship/00), the polymerase and capsid sequences of ORF1 were not continuous, i.e., they may represent two different strains. Nevertheless, Mc10 and C12 were in the same cluster by polymerase-based grouping but were in distinct clusters by capsid-based grouping (Figure 2). All other strains maintained clusters by polymerase- and capsid-based groupings.

These findings showed Mc10 and C12 had high sequence identity up to the beginning of the capsid region where the sequence identity was considerably lower. These results are easily explained by a recombination event, a single point recombination event occurring at the polymerase-capsid junction. At the end of the polymerase region, there were 44 nt, which included the first 8 nt of the capsid gene and showed 100% homology. After these nucleotides, the identity decreased and was clearly different, as shown in Figure 1B. This conserved region may represent the break and rejoin site for Mc10 and C12 during viral replication, although direct evidence for this event is lacking.

A sudden drop was indicated, followed by a rise in nucleotide identity between nt 6,250 and 6,500 (Figure 1B). Although our initial hypothesis was that another recombination event occurred, closer inspection indicated that this region corresponded to amino acids 358 and 440 for the capsid protein and likely represented the hypervariable region, as described recently in the structural analysis of sapovirus capsid protein (12). For recombinant norovirus strains, we also observed a sudden decrease in nucleotide identity in the related capsid region (13), which represents the outermost protruding domain (P2) and is subject to immune pressure (14). For these reasons, a low homology, even between closely related strains, is generally seen in this region (6), although further studies by sequence analysis with other strains are needed.

In a recent study, we genetically and antigenically analyzed two recombinant norovirus strains (13). When the polymerase-based grouping was performed, these two strains clustered together; when capsid-based grouping was performed, these two strains belonged in two distinct genotypes. When we compared the cross-reactivity of these two viruslike particles (VLPs) and hyperimmune sera against the VLPs, we found distinct antigenic types for the VLPs, although a considerable level of cross-reactivity was found between them. We recently expressed C12 capsid protein that resulted in the formation of VLPs, but we were unsuccessful in expressing Mc10 VLPs (G.S. Hansman, unpub. data); therefore the antigenicity of these two strains remains unknown.

Jiang et al. (4) reported two potential parental norovirus strains that were cocirculating in the same geographic region (Mendoza, Argentina, in 1995), which provides some evidence for where and when the recombination event may have occurred. In addition, Jiang identified the progeny strain from the event, the Arg320 strain. In our study, Mc10 and C12 were isolated from Thailand and Japan, respectively, but we have no evidence for the place and time of the event. While the genetic analysis for Mc10 and C12 identified a possible recombinant sapovirus strain, the analysis does not clarify which of the two strains was the parent strain and which was the progeny strain. Further extensive studies are needed that perform sequence analysis of polymerase and capsid genes and compare results with analysis of other strains. Nevertheless, other strains with capsid sequences that closely match those of Mc10 and C12 are in the public database, which suggests the circulation of other recombinant sapovirus strains.

## Conclusions

Recombination and evolution are important survival events for all living creatures as well as viruses. These events in viruses are not completely understood, but they can be potentially dangerous for host species, and they likely influence vaccine designs (15). From our studies, the human sapovirus and norovirus recombination appears limited to the intragenogroup because no intergenogroup

or intergenus recombination has yet been identified and recombination only occurs at the polymerase-capsid junction. Finally, the results of this study have increased our awareness of recombination in the *Sapovirus* genus and may have an influence on the future phylogenetic classification of sapovirus strains.

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**Short Communication**

# Norovirus and Sapovirus Infections in Thailand

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**SUMMARY**: Stool specimens collected between November 2002 and April 2003 from hospitalized infants with acute gastroenteritis from four distinct geographical regions in Thailand were examined for norovirus (NoV) and sapovirus (SaV) by reverse transcription-PCR and sequence analysis. Of the 80 specimens examined, we identified 11 NoV and 9 SaV single infections, and 3 NoV/ SaV mixed infections. The majority of NoV strains (64%) belonged to genogroup II/genotype 4 (GII/4; Lordsdale cluster). Other NoV strains co-circulating belonged to GII/1, GII/3, GII/6, and one new genotype cluster (GII/New). The majority of SaV strains (83%) were from the Manchester cluster. One isolated SaV strain represented a recently discovered novel genogroup within the SaV genus (SG-V), and another isolated SaV strain represented a novel SaV genogroup II cluster.

Norovirus (NoV) is the most important cause of outbreaks of gastroenteritis in the United States, infecting all age groups (1). Human NoVs have been divided into two genogroups, genogroup I (GI) and genogroup II (GII). A recent study indicated that NoV GI and GII strains consist of 14 and 17 genotypes, respectively (2). Sapovirus (SaV) is also a causative agent of gastroenteritis, though more frequent in young children than in adults (3). SaVs can be divided into four genogroups (SG-I to SG-IV) (4), though only SG-I, SG-II, and SG-IV are known to infect humans, whereas SG-III affects pigs. Human SaV genogroups tentatively comprise four SG-I clusters, three SG-II clusters, and one SG-IV cluster (4). Other viruses causing gastroenteritis include rotavirus, astrovirus, and enteric adenovirus. These viruses can be transmitted by the fecal-oral route through person-to-person contact, and food- and water-borne infections (5-7).

We recently reported the genetic diversity of NoV and SaV in hospitalized infants in the Northeastern region of Thailand (8) and identified a diversity of NoV and SaV strains, including one strain that belonged to a new NoV genotype (GII/10).

In this study, 80 of 321 stool specimens from hospitalized infants with sporadic cases of acute gastroenteritis, all of which had previously been found negative for rotavirus, astrovirus, enteric adenovirus, and bacterial agents (unpublished data), were examined for NoV and SaV using RT-PCR and sequence analysis (Table 1). Specimens were collected between November 2002 and April 2003 from four distinct geographical regions in Thailand: Northern, Tak province; Northeastern, Nong Khai province; Central, Sa Kaeo and Chanthaburi provinces; and Southern, Songkhla province. RNA was extracted and purified as described elsewhere (8). The purified RNA (12.5 $\mu$l) was added to 2.5 $\mu$l of the reaction mixture containing DNase I buffer and 1 unit of RQ1 DNase (Promega, Madison, Wis., USA). This reaction mixture was incubated for 30 min at 37°C, then for 5 min at 75°C to inactivate RQ1 DNase. The reaction mixture was then added to 15 $\mu$l of a mixture containing 100 mM Tris-HCl (pH 8.3), 150 mM KCl, 6 mM $MgCl_2$, 1 mM of each dNTPs, 10 mM DTT, 75 pmol of random hexamers (pdN6; Amersham, Life Science, Buckinghamshire, England), 30 units of RNase inhibitor (Promega) and 200 units of SuperScript II RNaseH (−) reverse transcriptase (Invitrogen, Carlsbad, Calif., USA). Reverse transcription was performed for 1 h at 37°C, and inactivation of the enzyme was performed for 5 min at 94°C. The cDNA was kept at −20°C until used in PCR. The NoV PCR was carried out according to the method described by Kojima et al. (9). For NoV GI, G1SKF and G1SKR primers were used. For NoV GII, G2SKF and G2SKR primers were used. The SaV PCR was performed according to the method described by Okada et al. (4). For the first PCR, SV-F11 and SV-R1 primers were used. For the nested PCR, SV-F21 and SV-R2 primers were used. The PCR products were analyzed with 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The PCR-generated amplicons of either the first or nested PCR were excised from the gel and purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany). Nucleotide sequences were prepared with the terminator cycle sequence kit (version 3.1) and determined with the ABI 3100 avant sequencer (Perkin-Elmer ABI, Boston, Mass., USA). Nucleotide sequences were aligned with Clustal X and the distances were calculated by Kimura's two-parameter method (10). Phylogenetic trees with bootstrap analysis from 1,000 replicas were generated by the neighbor-joining method as described elsewhere (10).

In total, 23 of 80 infants (29%) were positive for NoV and/ or SaV (Table 1). We identified 11 (14%) NoV and 9 (11%) SaV single infections, and 3 (4%) NoV/SaV mixed infections.

Fourteen NoV sequences were used for phylogenetic analy-

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Table 1. Details of positive NoV and SaV specimens in Thailand between November 2002 and April 2003

| Province | No. of specimens | | | | | |
|---|---|---|---|---|---|---|
| | Total | selected randomly | Positive[1)] | Single positive[1)] | | Mixed positive[1)] |
| | | | | NoV | SaV | NoV/SaV |
| Sa Kaeo | 48 | 15 | 8 | 5 | 2 | 1 |
| Chanthaburi | 57 | 16 | 5 | 1 | 4 | 0 |
| Songkhla | 97 | 23 | 4 | 3 | 1 | 0 |
| Nong Khai | 69 | 13 | 4 | 1 | 2 | 1 |
| Tak | 50 | 13 | 2 | 1 | 0 | 1 |
| Total | 321 | 80 | 23 (29%) | 11 (14%) | 9 (11%) | 3 (4%) |

[1)]: from the randomly selected specimens.

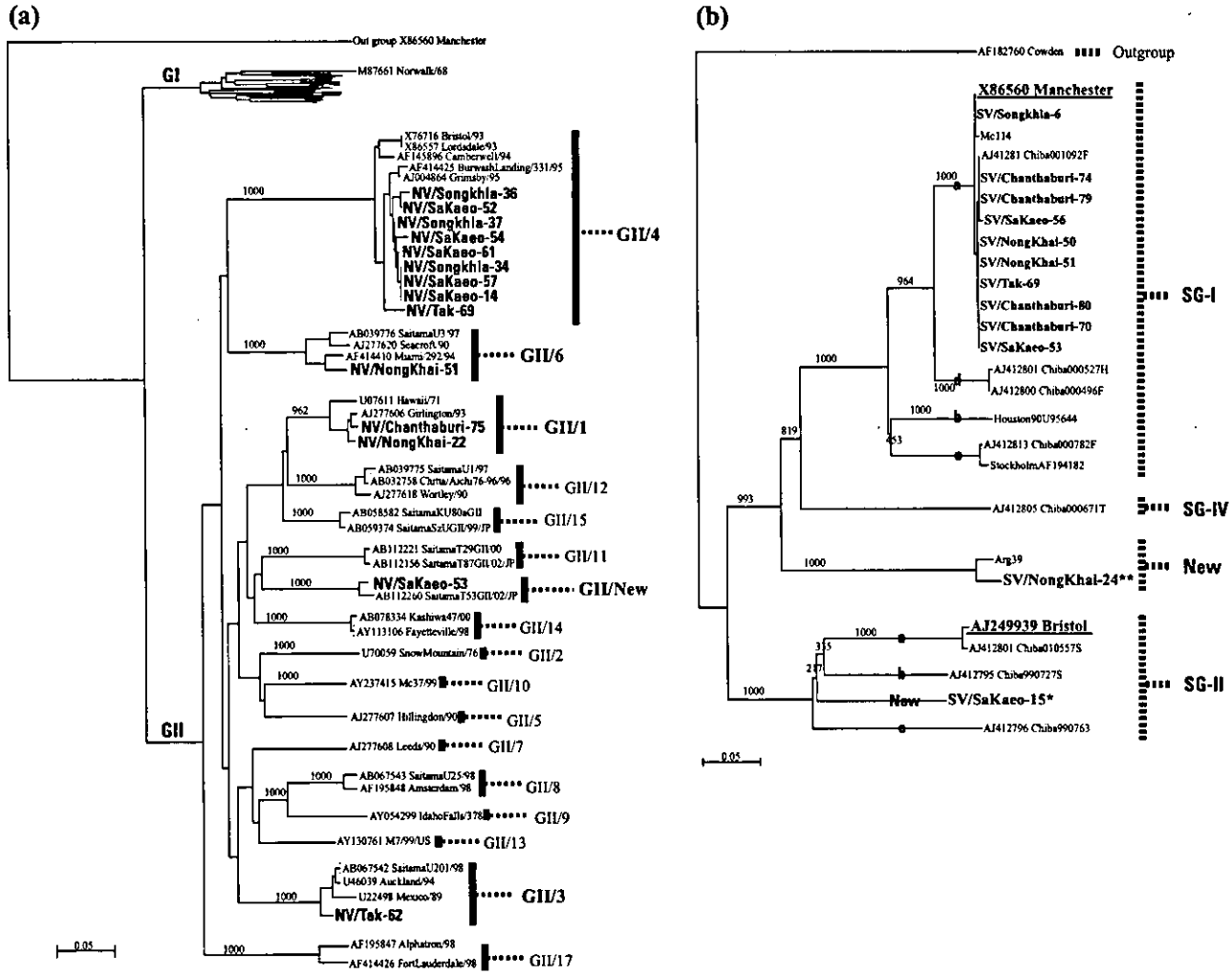


Fig. 1. Phylogenetic trees of the Thai sequences isolated in this study (represented in bold). The numbers on the branches indicate the bootstrap values for the clusters. Bootstrap values of 950 or higher were considered statistically significant for the grouping (10). The scale represents nucleotide substitutions per site. (a) The NoV sequences were constructed with the partial N/S capsid region, using SaV Manchester strain as an outgroup. The genotypes are indicated on each genotype cluster according to the method described by Kageyama et al. (2). (b) The SaV sequences were constructed with the partial N-terminal capsid region, using SaV Cowden strain as an outgroup. The genogroups and genotypes are indicated on each branch according to the method described by Okada et al. (4). Underlined bold letters showed SG-I-a and SG-II-a reference strains. The asterisks indicate new strains.

sis and classified according to the method described by Kageyama et al. (2). All NoV sequences were grouped in GII (Fig. 1a). Nine of 14 NoV sequences (64%) were clustered in GII/4. These nine isolates were derived from each distinct geographical region. These results not only showed that GII/4 strains were dominant but that they were circulating throughout Thailand. Strains belonging to GII/4 have been reported as a major cause of global outbreaks (Fig. 1a) (11-13). Several other NoV strains were also found to be co-circulating in these four regions. We detected one GII/3 strain (isolate NV/Tak-62), two GII/1 strains (isolates NV/NongKhai-22 and NV/Chanthaburi-75), one GII/6 strain (isolate NV/NongKhai-

51), and one newly identified genotype strain (GII/New; isolate NV/SaKaeo-53) (2). All genotype clusters, including the newly found GI/new cluster were statistically supported by the bootstrap value (Fig. 1a).

Twelve SaV sequences were used for phylogenetic analysis and classified according to Okada et al. (4). Ten of 12 SaV sequences (83%) were grouped in genogroup-I-a cluster (SG-I-a) (Fig. 1b). The remaining two SaV sequences (isolates SV/NongKhai-24 and SV/SaKaeo-15) were grouped in two novel SaV clusters (Fig. 1b). NongKhai-24 represented a novel genogroup within the SaV genus (new cluster), whereas SaKaeo-15 represented a novel SG-II genetic cluster (SG-II-new cluster). The NongKhai-24 cluster was statistically supported by the bootstrap value (bootstrap value = 1,000). The bootstrap value of SaKaeo-15 was low (bootstrap value = 217) (Fig. 1b), though the branch length (distance) between the Bristol strain and the SaKaeo-15 strain was the same as that between the Bristol strain and the Chiba990727S strain. Therefore, SaKaeo-15 was considered a distinct genetic cluster. Arg39 (AF405715) containing similar sequence to NongKhai-24 (97% nucleotide identity) was recently detected in the US. This finding suggests the emergence and circulation of a novel human SaV genogroup.

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# Cross-reactivity among sapovirus recombinant capsid proteins

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**Summary.** Sapovirus (SaV), a member of the genus *Sapovirus* in the family *Caliciviridae*, is an agent of human and porcine gastroenteritis. SaV strains are divided into five genogroups (GI–GV) based on their capsid (VP1) sequences. Human SaV strains are noncultivable, but expression of the recombinant capsid protein (rVP1) in a baculovirus expression system results in the self-assembly of virus-like particles (VLPs) that are morphologically similar to native SaV. In this study, rVP1 constructs of SaV GI, GII, and GV strains were expressed in a baculovirus expression system. The structures of the GI, GII, and GV VLPs, with diameters of 41–48 nm, were morphologically similar to those of native SaV. However a fraction of GV VLPs were smaller, with diameters of 26–31 nm and spikes on the outline. This is the first report of GII and GV VLP formation and the first identification of small VLPs. To examine the cross-reactivities among GI, GII, and GV rVP1, hyperimmune rabbit antisera were raised against *Escherichia coli*-expressed GI, GII, and GV N- and C-terminal VP1. Western blotting showed the GI antisera cross-reacted with GV rVP1 but not GII rVP1; GII antisera cross-reacted weakly with GI rVP1 but did not cross-react with GV rVP1; and GV antisera reacted only with GV rVP1. Also, hyperimmune rabbit and guinea pig antisera raised against purified GI VLPs were used to examine the cross-reactivities among GI, GII, and GV VLPs by an antigen enzyme-linked immunosorbent assay (ELISA). The ELISA showed that the GI VLPs were antigenically distinct from GII and GV VLPs.

## Introduction

Human sapovirus (SaV) is a member of the genus *Sapovirus* in the family *Caliciviridae*. The prototype strain of human SaV, the Sapporo virus, was originally discovered from an outbreak in an orphanage in Sapporo, Japan, in October 1977 [5]. Chiba et al. identified viruses with the typical animal calicivirus morphology, called the "Star of David" structure, by electron microscopy (EM). In addition, SaV strains typically feature a diameter of 41–48 nm, cup-shaped depressions, and ten spikes on the outline.

Human SaV strains infect all age groups and can cause outbreaks of gastroenteritis and sporadic infections requiring hospitalization [7, 13, 18, 19, 21, 27]. Several groups have purified native SaV particles from stool specimens and produced antisera against them for use in immunoassays, including immune-EM and enzyme-linked immunosorbent assays (ELISAs) [14, 18, 20, 25]. However, the most widely used method to detect SaV is reverse transcription-polymerase chain reaction (RT-PCR), which has a high sensitivity [23]. SaV strains were recently divided into five genogroups (GI–GV), of which GI, GII, GIV, and GV strains infect humans, while GIII strains infect porcine species [6]. The SaV GI, GIV, and GV genomes are predicted to each contain three main open reading frames (ORFs), whereas SaV GII and GIII genomes each have only two main ORFs [6, 8, 17, 24]. The SaV ORF1 encodes the nonstructural proteins and the major capsid protein (VP1), while ORF2 and ORF3 encode proteins of yet-unknown functions. Therefore, the VP1 may be produced by either of two pathways: (i) translated as part of ORF1 and then cleaved, or (ii) translated from subgenomic RNA, although the subgenomic RNA of human SaV has not yet been identified.

Human SaV strains are noncultivable, but expression of the SaV recombinant VP1 (rVP1) in a baculovirus expression system results in the self-assembly of virus-like particles (VLPs) that are morphologically similar to native SaV [22]. There are four reports to date of the successful assembly of SaV VLPs [4, 9, 14, 22]. In three of these reports, however, the formation of VLPs was observed in rVP1 constructs that included short sequences upstream from the predicted rVP1 start AUG codon. Jiang et al. found that an upstream sequence of 73 nucleotides from the predicted VP1 start AUG codon was crucial for VLP formation [14], whereas the authors in two of the other three reports included 9 and 39 nucleotides upstream, respectively [9, 22]. The predicted human SaV VP1 start contains an amino acid motif, MEG, which is conserved in all human SaV strains and as such is considered the putative VP1 start. Recently, structural analysis of SaV VLPs predicted the shell (S) and protruding domains (subdomains P1 and P2) [4].

In the current study, we report the self-assembly of SaV GI, GII, and GV VLPs using constructs that began exactly from the predicted VP1 start AUG codon in a baculovirus expression system. More importantly, we describe for the first time the cross-reactivities among SaV GI, GII, and GV rVP1 by Western blotting and the cross-reactivities among SaV GI, GII, and GV VLPs by an antigen ELISA.

## Materials and methods

### *Viruses*

SaV Mc114 strain (GenBank accession number AY237422) was isolated from an infant hospitalized with acute gastroenteritis in Chiang Mai, Thailand, in 2001 [11]. SaV C12 strain (AY603425) was isolated from an infant with gastroenteritis in Sakai, Japan, in 2001 (in press). SaV NK24 strain (AY646856) was isolated from an infant with gastroenteritis in Nong Khai, Thailand, in 2003 (manuscript in press).

### *RT-PCR, sequencing, phylogenetic analysis*

RNA extraction, RT-PCR, sequencing, and phylogenetic analysis were performed as previously described [15]. SaV sequences were phylogenetically classified based on the scheme of Farkas et al. [6].

### *Cloning of viral cDNA to produce recombinant bacmids*

For the expression of rVP1 in insect cells, SaV constructs were designed to begin from the predicted VP1 start AUG codon, and included the VP2 and poly(A) sequences. PCR-amplified fragments were cloned according to the protocol of the Baculovirus Expression system using Gateway Technology (Invitrogen, USA). For the Mc114 strain, primers p+1Mc114 and attB2TX30SXN were used. For the C12 strain, primers p+1C12 and attB2TX30SXN were used. For the NK24 strain, primers p+1NK24 and attB2TX30SXN were used. PCR fragments were cut and purified from a 0.8% agarose gel. These were cloned into a donor vector pDONR201 (Invitrogen, USA) and then transferred into a baculovirus transfer vector pDEST8 (Invitrogen, USA). The recombinant pDEST8 was purified and used to transform DH10Bac-competent cells (Invitrogen, USA), producing recombinant bacmids containing the VP1 gene.

### *Expression in insect cells*

Recombinant bacmids were transfected into Sf9 cells (Riken Cell Bank, Japan) and the recombinant baculoviruses were collected as previously described [10]. The recombinant baculoviruses were used to infect approximately $3 \times 10^6$ confluent Tn5 cells (Invitrogen, USA) at a multiplicity of infection (MOI) of 5–10 in 1.5 ml of Ex-Cell 405 medium (JRH Biosciences, USA), and the infected cells were incubated at 26 °C. The culture medium was harvested 5–6 days post-infection (dpi), centrifuged for 10 min at 3,000 × *g*, and further centrifuged for 30 min at 10,000 × *g*. The VLPs were concentrated by ultracentrifugation for 2 h at 45,000 rpm at 4 °C (Beckman TLA-55 rotor), and then resuspended in 30 μl of Grace's medium. Samples were examined for VLP formation by electron microscopy (EM).

### *EM*

The harvested culture medium was examined for VLPs by negative-stain EM. Briefly, the samples (diluted 1:10 in distilled water) were applied to a carbon-coated 300-mesh EM grid and stained with 4% uranyl acetate (pH 4). Grids were examined in an electron microscope (JEM-1220; JEOL, Japan) operating at 80 kV. VLP images were of CsCl purified culture medium as described previously [10].

### *Cloning of viral cDNA to produce the 5′ and 3′ halves of VP1 and ORF2*

The Mc114, C12, and NK24 N- and C-terminal regions of VP1 were constructed in order to raise antibodies for the cross-reactivity study. Mc114 recombinant ORF2 (rVP2) was constructed in a similar manner. The primer sequences used to amplify these regions are listed in Table 1. Briefly, the PCR-amplified fragments (using N-terminal sense and antisense

**Table 1.** Names, sequences, nucleotide positions, and polarity of primers used in this study

| Primer | Sequence (5′ to 3′)[a] | Position (nt)[b] | Sense |
|---|---|---|---|
| TX30SXN | GACTAGTTCTAGATCGCGAGCGGCCGCCCTTTTTTTTTTTTTTTTTTTTTTTTTTTT | End | − |
| $p^+$1Mc114 | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACC**ATGGAGGGCAATGGCTCCA ACTCAGAGCCAAAG** | 1–33 | + |
| $p^+$1C12 | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACC**ATGGAGGGTGTACCCCGCC CAGAGGGCCCAAG** | 1–33 | + |
| $p^+$1NK24 | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACC**ATGGAGGGCAATGGCTCCC GACTTGGGAAC** | 1–33 | + |
| attB2TX30SXN | GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGACTAGTTCTAGATCGCGAGCGGCCGCCCTTTTTT TTTTTTTTTTTTTTTTTTTTTTT | End | − |
| N-terminal-Mc114 | GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**ATGGAGGGCAATGGCTCCAACTCAGAGCCAAAG** | 1–33 | + |
| N-terminal-Mc114 | **CTTCTGTTTGCTAAGGCCACCAGGTCAACAGT**AGGACCCAGCTTTCTTGTACAAAGTGGTCCCC | 657–687 | − |
| C-terminal-Mc114 | GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**ATGGAAAATGGGGTTTCACCAGAAGGCTTATTG** | 688–720 | + |
| C-terminal-Mc114 | **GAACATGGGAAGGGCCAAACGGGTGTTCCAA**TAGGACCCAGCTTTCTTGTACAAAGTGGTCCCC | 1653–1683 | − |
| N-terminal-C12 | GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**ATGGAGGGTGTACCCCGCCCAGAG** | 1–24 | + |
| N-terminal-C12 | **CTAATTCCACCACGCTCGGTTGGTCG**GACCCAGCTTTCTTGTACAAAGTGGTCCCC | 827–852 | − |
| C-terminal-C12 | GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**ACACTCCCAATTGAGCCCATTGCTG** | 853–877 | + |
| C-terminal-C12 | **GACGGGCCGTCAGATTCCTTG**AATAAGACCCAGCTTTCTTGTACAAAGTGGTCCCC | 1661–1683 | − |
| N-terminal-NK24 | GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**CTCCTTCGCACGATTCCTTGGAACAC** | 226–251 | + |
| N-terminal-NK24 | **GATGGTGATAGTTGGCTCAGCACAC**GACCCAGCTTTCTTGTACAAAGTGGTCCCC | 792–816 | − |
| C-terminal-NK24 | GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**CAAGTTAACCGCCATTTCACCGCCCTTG** | 817–844 | + |
| C-terminal-NK24 | **CTGGCCGGGCCCAAATTGCTTGGTCA**GACCCAGCTTTCTTGTACAAAGTGGTCCCC | 1682–1706 | − |
| ORF2-Mc114 | GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**ATGAGTTGGTTAGTGGGTGCCCTCCAGACC** | 1683–1712 | + |
| ORF2-Mc114 | **CATTGGTCACAACCCTGGCAGCTCCAATGTCT**AGGACCCAGCTTTCTTGTACAAAGTGGTCCCC | 2147–2177 | − |

[a]The bold nucleotides represent the virus sequence

[b]The numbering is relative to the strain, where nucleotide one is the first nucleotide of the VP1 gene

primers, C-terminal sense and antisense primers, or ORF2 sense and ORF2 antisense primers) were cloned into vector pDONOR201 (Invitrogen, USA), and then transferred to vector pDEST17 (Invitrogen, USA) according to the manufacturer's protocol.

*Expression in Escherichia coli* (E. coli)

pDEST17 plasmids containing N- and C-terminal VP1 and VP2 were transformed into BL21-AI (Invitrogen, USA). Expression was induced by adding 0.2% (w/v) arabinose, followed by incubation at 37 °C for 3 h. The cells were centrifuged for 10 min at 10,000 × *g* at 4 °C, and were resuspended in extraction buffer (BD Clontech, USA) supplemented with 8 M urea. The supernatant was separated from the cell suspension, and the $His_6$-tagged recombinant protein was purified in TALON resin (BD Clontech, USA) and finally eluted in buffer containing 250 mM imidazole (BD Clontech, USA). The quantity of protein was estimated using the Protein Assay Kit (BioRad Laboratories, USA).

*Time-course expression of Mc114 in insect cells*

The expression of Mc114 in the culture medium was analyzed by infecting Mc114 recombinant baculoviruses at a MOI of 14.5 in $2.7 \times 10^6$ confluent Tn5 cells in 1.5 ml of Ex-Cell 405 medium followed by incubation at 26 °C. The culture medium was harvested 1, 2, 3, 4, 5, 6, 7, and 8 dpi, centrifuged for 10 min at 3,000 × *g*, and further centrifuged for 30 min at 10,000 × *g*. The VLPs were concentrated by ultracentrifugation for 2 h at 45,000 rpm at 4 °C (Beckman TLA-55 rotor) and resuspended in 20 μl of Grace's medium. Western blotting, EM, and an antigen ELISA were used to monitor the expression levels.

*SDS-PAGE*

We examined the rVP1 and rVP2 expression using SDS-PAGE with a 5–20% gradient polyacrylamide gel (ATTO, Japan). The concentrated culture medium and cell lysate (diluted 1:10 in distilled water) were mixed with a 1/4 volume of buffer solution containing 62.5 mM Tris-HCl (pH 6.8), 25% (w/v) glycerol, 2% (w/v) SDS, and 0.01% Bromophenol Blue with 5% (v/v) 2-mercaptoethanol and then boiled for 5 min. Electrophoresis was performed in 25 mM Tris/192 mM glycine/0.1% SDS buffer at 20 mA for 1.5 h.

*Western blotting*

The proteins were separated by SDS-PAGE and electrotransferred to PVDF with transfer buffer (25 mM Tris/192 mM glycine/5% methanol) at 100 mA for 1 h and blocked with 5% (w/v) skim milk/PBS for 1 h. Proteins were detected with hyperimmune rabbit antiserum at a dilution of 1:3,000 (as determined previously), then following the manufacturer's instructions were developed by chemiluminescence using ECL detection reagent (Amersham Biosciences, England).

*Antibody production*

Rabbits and guinea pigs were immunized subcutaneously with 10 μg of CsCl-purified Mc114 VLPs as described elsewhere [10]. For *E. coli*-expressed proteins, 500 μg of each recombinant protein was used to immunize rabbits at two-week intervals. The serum was collected one week after the last injection.

*ELISA*

The wells of 96-well microtiter plates (Maxisorp, Nunc, Denmark) were each coated with 100 μl of a 1:8,000 dilution (determined previously) of either Mc114 VLP hyperimmune

rabbit antiserum (P) or preimmune rabbit antiserum (N) diluted in PBS. The plates were incubated overnight at 4 °C. The wells were washed three times with PBS containing 0.1% Tween 20 (PBS-T) and then were blocked with PBS containing 5% skim milk (PBS-SM) for 1 h at room temperature. The wells were washed four times with PBS-T. Five micro-liters of VLPs (see expression in insect cells section) were mixed in 400 µl of PBS-T containing 1% SM (PBS-T-SM), and then 100 µl of this mixture was added to duplicate wells. The plates were then incubated for 1 h at 37 °C. After the wells were washed four times with PBS-T, 100 µl of a 1:8,000 dilution of Mc114 VLP hyperimmune guinea pig antiserum diluted in PBS-T-SM was added to each well, and the plates were incubated for 1 h at 37 °C. The wells were washed four times with PBS-T, and then 100 µl of a 1:1,000 dilution of horseradish peroxidase (HRPO)-conjugated rabbit anti-guinea pig immunoglobulin G (IgG) diluted in PBS-T-SM was added to each well. The plates were then incubated for 1 h at 37 °C. The wells were washed four times with PBS-T, and then 100 µl of substrate *o*-phenylenediamine and $H_2O_2$ was added to each well and left in the dark for 30 min at room temperature. The reaction was stopped by the addition of 50 µl of 2 M $H_2SO_4$ to each well, and the absorbance was measured at 492 nm ($A_{492}$). For this experiment, we included Tn5 cell lysate and native baculovirus as negative controls. We determined the mean P/N ratio of ELISA was 0.94, with a standard deviation of 0.09 (manuscript in review). The cutoff value of ELISA was defined as the mean plus 3 standard deviations, hence a sample with an $A_{492}$ (P–N) of >0.1 and a P/N ratio of >1.2 was considered significantly positive. For the antigen ELISA, CsCl-purified Mc114 VLPs were used as the positive control at concentrations ranging from 500 to 0.24 ng (data not shown).

## Results

### *Genetic analysis*

The nucleotide sequence of the 3′ end of the genome (containing the VP1 gene) was determined for each of the strains, Mc114, C12, and NK24. Based on the recent SaV classification [6], these strains belonged respectively to the genogroups GI, GII, and GV (Fig. 1). The Mc114 VP1 encoded 561 amino acids and had an apparent molecular weight of approximately 60,100 (60K protein); the C12 VP1 encoded 561 amino acids and had an apparent molecular weight of approximately 60,100 (60K protein); and NK24 VP1 encoded 569 amino acids and had an apparent molecular weight of approximately 60,500 (60K protein). Figure 2 shows the VP1 amino acid alignments of these strains. All of the sequences contained the predicted VP1 start amino acid motif, MEG (Fig. 2). Based on the recent structural analysis of SaV GI VLPs [4], the Mc114, C12, and NK24 VP1 amino acids were predicted to be more conserved between the 5′ to P2 and P2 to 3′ domains than in the P2 domain (Table 2). Mc114 and NK24 ORF2 (VP2) encoded 161 and 155 amino acids, respectively, and shared 41% amino acid similarity.

### *EM analysis*

The insect cell culture medium was harvested at 5–6 dpi and examined for VLPs by negative-stain EM. Mc114, C12, and NK24 rVP1 all formed VLPs with diameters of 41–48 nm, while NK24 rVP1 also formed smaller VLPs with diameters of 26–31 nm, though these made up a smaller proportion than the 41–48 nm VLPs (Fig. 3). The 41–48 nm VLPs were morphologically similar to native SaV,

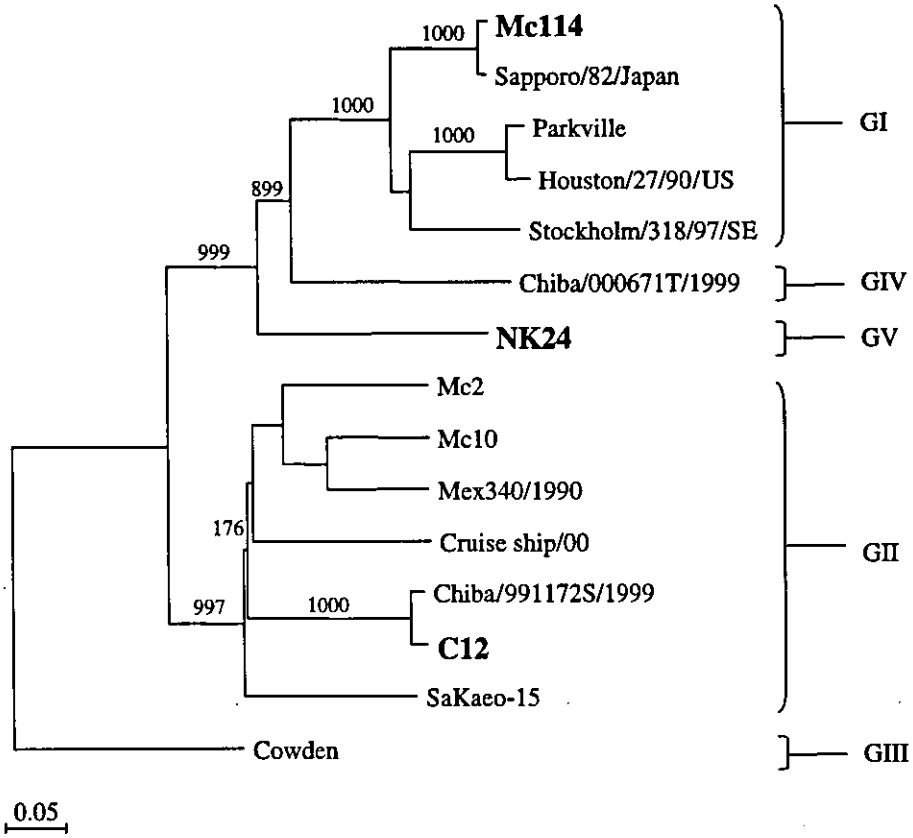


**Fig. 1.** Phylogenetic analysis of Mc114, C12, and NK24 strains (bold) based on capsid sequences (376 bp). SaV were classified based on the scheme of Farkas et al. [6], using Cowden (SaV GIII) as an outgroup. The number on each branch indicates the bootstrap value for the genotype. Bootstrap values of 950 or higher were considered statistically significant for the grouping [15]. The scale represents nucleotide substitutions per site. GenBank accession numbers for the reference strains are as follows: Chiba/000671T/1999, AJ412805; Chiba/991172S/1999, AJ412797; Cruise ship/00, AY289804; Houston/27/90/US, U95644; Mc2, AY237419; Mc10, AY237420; Mex340/1990, AF435812; Cowden, AF182760; Parkville, U73124; Sakaeo-15, AY646855; Sapporo/82/Japan, U65427; and Stockholm/318/97/SE, AF194182

including the typical Star of David structure, cup-shaped surface depressions, and ten spikes on the outline. However, the Star of David structure was visible only when the samples were stained with uranyl acetate, and not with phosphotungstic acid (data not shown). The 26–31 nm VLPs had spikes on the outline, but neither the Star of David structure nor cup-shaped surface depressions were visible.

### *Time-course expression analysis of Mc114 rVP1 in insect cells*

Three tests were used to monitor the time-course expression of Mc114 rVP1 in insect cells. The antigen ELISA first detected VLPs in the culture medium at 3 dpi (Fig. 4A). The Western blot with hyperimmune rabbit Mc114 VLP antiserum first detected rVP1 (60K band) at 4 dpi (Fig. 4B). And VLPs were first detected by EM at

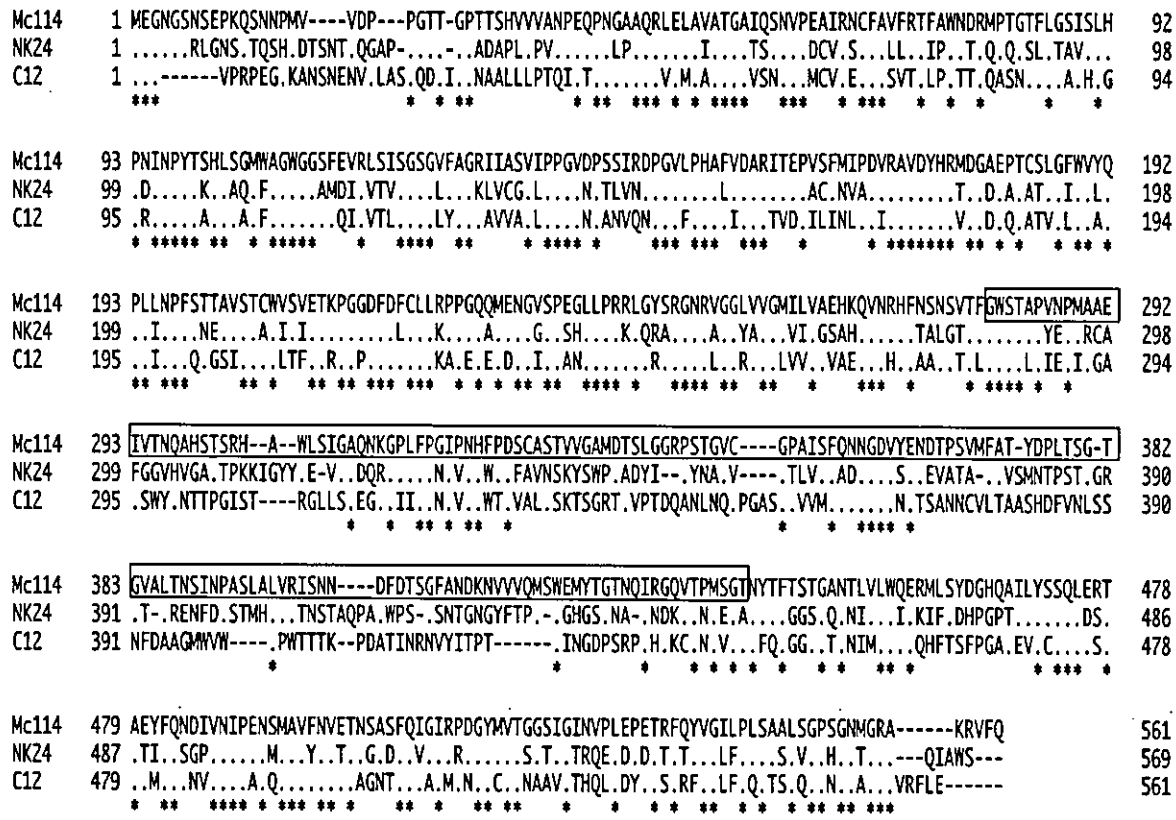


**Fig. 2.** An amino acid alignment of VP1 sequences of Mc114 (GI), C12 (GII), and NK24 (GV) strains. The sequences with a rectangular box represent the predicted P2 domain [4]. The asterisks indicate conserved amino acids among these three VP1 sequences

**Table 2.** VP1[a] amino acid (%) identities of SaV Mc114, C12, and NK24

| | Mc114 | | | | C12 | | | |
|---|---|---|---|---|---|---|---|---|
| | VP1 | 5′ to P2 | P2 | P2 to 3′ | VP1 | 5′ to P2 | P2 | P2 to 3′ |
| C12 | 46.0 | 55.0 | 26.7 | 51.6 | | | | |
| NK24 | 51.0 | 60.0 | 29.9 | 59.0 | 50.1 | 59.8 | 31.8 | 55.0 |

[a]Domains and regions were based on Fig. 2

4 dpi. The expression level of VLPs increased each day thereafter, peaking at 6 dpi before decreasing at 7 and 8 dpi (Fig. 4A). A thin band of approximately 55K was also detected by Western blotting at 4 dpi, which increased each day thereafter as evidenced by an increase in band intensity (Fig. 4B). The 55K band may have represented truncated form(s) of rVP1 as observed with norovirus expression studies [2], though direct evidence is lacking. All bands were absent in the cell controls (the wild-type baculovirus and the mock-infected Tn5 cells) (data not