RESULTS

Detection of Viruses Causing Diarrhea

The results shown in Table I revealed that viruses causing diarrhea were detected in 10 out of 18 (55.6%) fecal specimens tested. Group A rotavirus and sapovirus were detected in 5 (3 in D4 and 2 in D7), 5 (1 in D9 and 4 in D11) out of 18 fecal specimens, respectively and this presented 27.8% and 27.8%, respectively. Group B and C rotaviruses, adenovirus, norovirus, and astrovirus were not found in these patients. No virus was identified in the fecal specimen from a member of the staff (Patient no. 9) by multiplex PCR. All of group A rotaviruses were identified as P[4]G2 strains by the serotyping RT-PCR, and confirmed by latex agglutination test (Rotalex kit).

Nucleotide Sequencing and Phylogenetic Analysis of Group A Rotavirus isolates

The rotavirus sequences clustered together in a G2 serotype phylogenetically. The homology of these nucleotide sequences with rotavirus G2 isolates in *VP7* gene were over 96%, among those, homologous to G2 isolates Mvd9707, 9708, 9713, and 9716 (AF480270, AF480273, AF480268, and AF480275, respectively) showed 99%. All of the five isolates of rotavirus demonstrated a high identity (100%) to each other suggested that they probably came from the same source of infection.

Nucleotide Sequencing and Phylogenetic Analysis of Sapovirus Isolates

The sapovirus sequences clustered into only distinct genogroup I (known as the Manchester virus) both in the capsid region (Fig. 2) and the polymerase region (data not shown). However, these sapoviruses did not belong to any of the published clusters and represented a potential novel sapovirus GI genotype in the capsid region according to the classification scheme of Okada et al. [2002]. They had a rather low identity on the nucleotide as well as the amino acid with other reference strains in the same genogroup previously registered in the DDBJ DNA database, ranged from 83% to 89% in the capsid region. Also, the homologies of these sapoviruses with the prototype strain Sapporavirus/82 in the polymerase region was 73% and 77% on the nucleotide and amino acid sequence, respectively. All sapoviruses had a high identity (100%) at the nucleotide as well as the amino acid to each other also suggesting that they probably came from the same source of infection. The nucleotide sequence data for the capsid region from strains Takasaki 5, 7, 8, 11, and 13 has been submitted to the DDBJ DNA database and has been assigned accession number AB180405, AB180406, AB180482, AB180483, AB180484, respectively.

DISCUSSION

The present study describes an outbreak of acute gastroenteritis in a mental health care facility in

TABLE I. Characteristics of 10 Viral Positives in the Outbreak of Acute Gastroenteritis in a Mental Health Care Facility in Takasaki City, Japan During 6th February and 27th March 2002

Patient no.	Dormitory code	Sex	Age (year)	Date of onset	Duration (day)	Symptoms			Laboratory findings			
						Diarrhea	Fever	Nausea	RT-PCR	Rotalex	RV G-typing	RV P-typing
3	D7	F	50	28 February	1	+	+	+	RA	+	G2	P[4]
4	D7	F	61	8 March	1	+	+	+	RA	+	G2	P[4]
5	D9	M	53	3 March	3	+	−	−	SV	−	nd	nd
7	D11	F	44	6 March	1	+	−	−	SV	−	nd	nd
8	D11	F	54	8 March	1	+	+	−	SV	−	nd	nd
11	D11	F	61	9 March	1	+	−	−	SV	−	nd	nd
13	D11	F	67	9 March	1	+	+	+	SV	−	nd	nd
14	D4	M	59	20 March	3	+	+	+	RA	+	G2	P[4]
15	D4	M	47	23 March	2	+	−	−	RA	+	G2	P[4]
17	D4	M	40	27 March	1	+	−	−	RA	+	G2	P[4]

No., number; M, male; F, female; RA, group A rotavirus; SV, sapovirus; nd, not done; +, positive; −, negative.

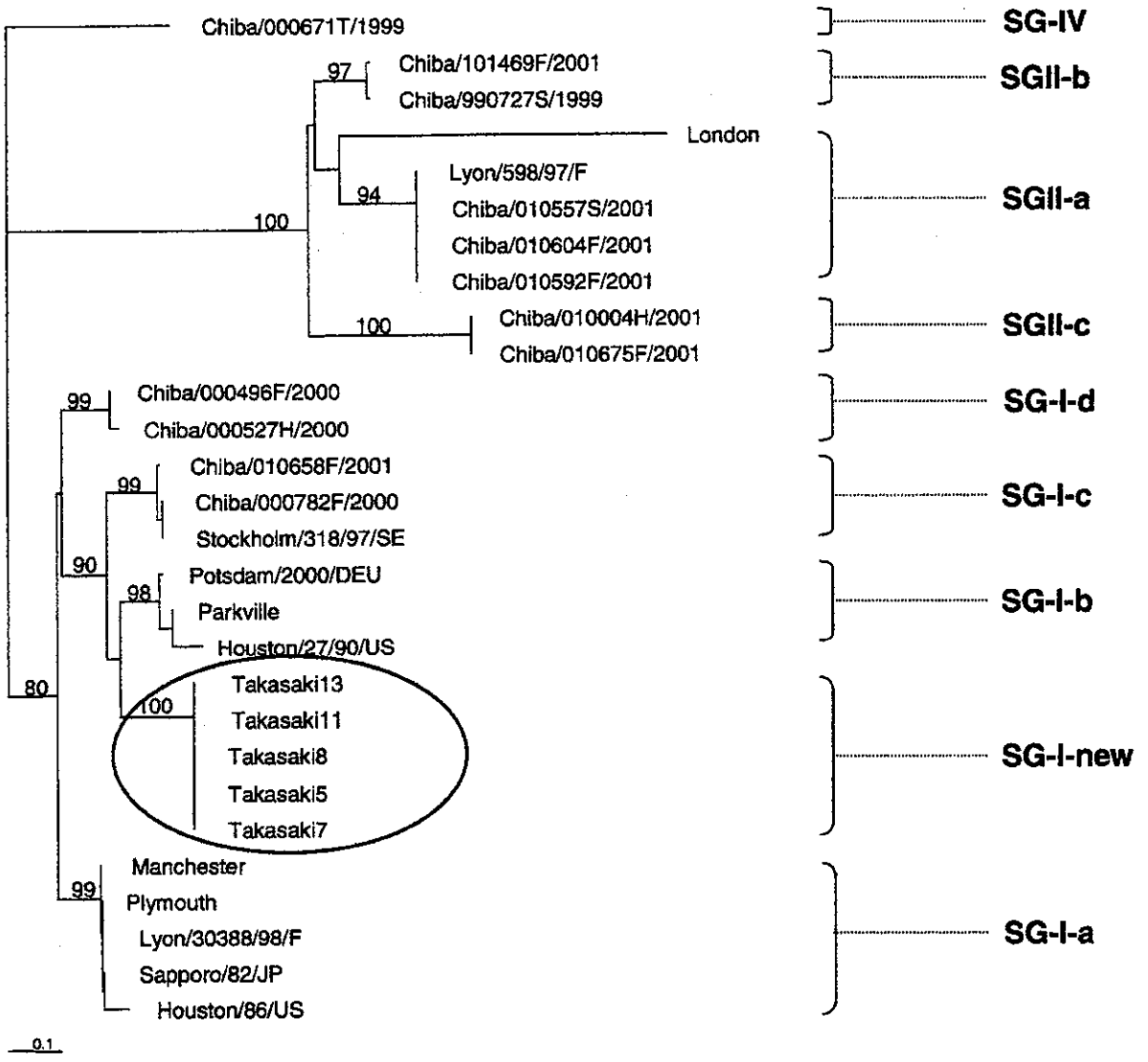

Fig. 2. Phylogenetic tree of nucleotide sequences of five isolates of sapovirus. The tree was constructed from partial amino acid sequences of 5 isolates of sapovirus detected in the outbreak of acute gastroenteritis among adults in mental health care facility, Takasaki city, Japan during 6th February and 27th March 2002. Reference strains of sapovirus selected from DDBJ/GenBank under the accession number indicated in the text. Sapoviruses found in the outbreak were indicated in the oval circle and presented a novel sapovirus GI genotype. The numbers in the branches indicate the bootstrap value.

Takasaki city, Japan during 6th February to 27th March 2002 was caused by group A rotavirus and sapovirus. Out of the 18 patients from whom single fecal specimen was available, about 55.6% might be due to the diarrheal viruses and 44.4% caused by other etiologic agents. The findings indicate that the rotavirus infections observed in this study were unlikely to be due to infection by group A rotavirus. Numerous reports of group A rotavirus infection in infants and children with acute gastroenteritis worldwide have been published, however, it has been also established that group A rotaviruses cause infection in adults and that outbreaks occur commonly particularly in closed institutions [Cubitt and Holzel, 1980; Holzel et al., 1980]. Among gastroenteritis outbreaks in adults in the USA between November 1998 and December 2000, 3 out of 263 outbreaks were linked to rotavirus serotype G2 [Griffin et al., 2002]. In a recent Australian report, 3 out of 7 rotavirus-associated outbreaks mainly in mid-winter to early-spring were identified with G2, among 53 gastroenteritis outbreaks occurred in aged-care facilities [Marshall et al., 2003]. The results of this study were in agreement with previous findings that group A rotavirus serotype G2 was detected mainly during the cold season and accounted for 27.8% (5/18) of acute gastroenteritis in adults patients (Patient nos. 3, 4, 14, 15, and 17). Our findings provide further evidence to support the hypothesis that natural immunity to G2 is inadequate in adults

[Gentsch et al., 1996]. In Japan, the incidence of P[4]G2 was detected to be mostly among school-aged children [Inoue et al., 2003]. The present study is a first report on an acute gastroenteritis outbreak associated with group A rotavirus P[4]G2 among Japanese adults.

To date, there have been several reports of outbreaks of sapovirus that involved adults, e.g., HuCV/Sapporo/Japan, the prototype sapovirus was detected from an outbreak in Japan that affected adults and children. The Parkville strain was isolated from an outbreak involving adults in England [Noel et al., 1997]. Interestingly, our study has demonstrated that infections with sapovirus in a relatively high percentage of 27.8% (5/18) in this outbreak (Patient nos. 5, 7, 8, 11, and 13). Similarity in seasonal pattern of infections among infants and children with acute gastroenteritis, mostly sapovirus was detected during the cold season [Phan et al., 2004]. In our study, sapovirus was subjected to molecular analysis by sequencing in both partial capisid region and polymerase region. A total of five sapovirus amino acid sequences were examined by phylogenetics and grouped using the recent sapovirus capsid region classification scheme of Okada et al. [2002]. It was noteworthy to point out that all sapoviruses demonstrated a high identity (100%) at the nucleotide as well as the amino acid to each other suggesting that they probably came from the same source of infection. Interestingly, as described above, these sapovirus sequences clustered into genogroup I, but did not belong to any of the published clusters and represented a potential novel sapovirus GI genotype. This finding also indicated that the sapovirus infections observed in our study were unlikely to be due to contamination by sapovirus.

Viruses causing diarrhea can be transmitted by a variety of routes, including fecal–oral, foodborne, and waterborne [Brugha et al., 1999; Mead et al., 1999]. In addition, airborne droplet has also been suggested as a route of transmission that might cause gastroenteritis outbreak [Sawyer et al., 1988; Marks et al., 2003]. Some investigators indicated that in long-term care facilities such as long-term hospital ward, elderly nursing home, retirement facilities, viruses causing diarrhea spread easily among residents and staffs through the care process and highly infectious agent contamination in the excretions that remain on environmental surfaces [Caceres et al., 1998; Green et al., 1998; Kuusi et al., 2002]. In the present study, the route of transmission remains unknown, however, the outbreak associated with viruses such as group A rotavirus and sapovirus spread gradually from dormitory to dormitory with a distinctive epidemic curve based on analysis of the prevalence pattern, suggesting a high possibility of spread by person-to-person contact.

The present study demonstrated an outbreak caused by two distinct viruses circulating simultaneously. Although similar findings have been reported [Gray et al., 1987; Lewis et al., 1989], this is the first finding of an outbreak of gastroenteritis associated with co-circulation of group A rotavirus and sapovirus.

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We sequenced a 1,012-bp fragment encompassing the first two thirds of the 16S rDNA of ROG140 (accession no. AY692224). The sequence was compared with those of type strains of all members of the former genus *Micrococcus*, and a phylogenetic tree was deduced by the neighbor–joining method (Figure). The sequences of ROG140 and the *K. schroeteri* type strain only differ by an A-to-G substitution at position 747 (*E. coli* numbering). Among the 21 nucleotide differences between the sequences of *K. schroeteri* and the closely related species *K. sedentarius*, 10 are located on a 30-base stretch and constitute a convenient *K. schroeteri* signature (Figure)

Antimicrobial susceptibility testing performed with the disk diffusion method and Etests (AB Biodisk, Solna, Sweden) indicated that the isolate was resistant to penicillins, cephalosporins, kanamycin, tobramycin, erythromycin, clindamycin, sulfonamides, and fusidic acid, but susceptible to imipenem (MIC, 0.25 µg/mL), gentamicin (MIC, 1 µg/mL), trimethoprim (MIC, 0.25 µg/mL), tetracycline (MIC, 0.12 µg/mL), linezolid (MIC, 0.25 µg/mL), vancomycin (MIC, 0.125 µg/mL), teicoplanin (MIC, 0.06 µg/mL), and rifampicin (MIC, <0.002 µg/mL). Unlike the original isolate reported by Becker et al. (1), isolate ROG140 was resistant to ofloxacin and ciprofloxacin (MICs, 8 µg/mL). Conversely, moxifloxacin displayed excellent in vitro activity (MIC, 0.05 µg/mL). As moxifloxacin was more rapidly microbicidal than vancomycin in an animal model of *Staphylococcus aureus* prosthetic valve endocarditis (4), it might present a potential advantage against infections caused by *K. schroeteri*, especially when the oral route is favored.

The natural habitat of *K. schroeteri* remains unknown. The only isolates of *K. schroeteri* identified so far originated from blood or cardiac material, although *Kytococcus* literally means "a coccus from the skin." Our attempts to recover *K. schroeteri* from the mouth, nose, or skin of our patient were unsuccessful. In a recent study, Szczerba et al. were able to isolate most micrococcal species, including *K. sedentarius* but not *K. schroeteri*, from human skin and mucosa (5). However, at that time the authors may not have been aware of this newly described species. The mode of contamination also remains unclear. In the original description (1), *K. schroeteri* endocarditis had developed in the patient <3 months after she underwent cardiac surgery, which suggested perioperative contamination. Here, we describe a late onset, subacute infection 3 years after surgery, which is more likely to have been caused by hematogenous spread.

Although *Micrococcus*-like organisms cause endocarditis infrequently (6), the description of 2 independent infections due to a new species in a short period is intriguing and suggests a specific pathogenicity, at least on prosthetic heart devices. By demonstrating the presence of the bacteria in the infected site, this report establishes *K. schroeteri* as a genuine pathogen in this clinical setting and should prompt further investigations to identify its natural habitat and virulence determinants. At present, commercial systems are not able to identify *K. schroeteri*. However, gram-positive cocci that are strictly aerobic, oxacillin-resistant, and arginine dihydrolase-positive should be recognized as potential *Kytococcus* species and taken into account when endocarditis is suspected.

Cécile Le Brun,* Julien Bouet,* Philippe Gautier,* Jean-Loup Avril,* and Olivier Gaillot*

*Centre Hospitalier Universitaire de Rennes, Rennes, France

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Address for correspondence: Olivier Gaillot, Laboratoire de Bactériologie-Virologie, Faculté de Médecine-Université de Rennes 1, 35033 Rennes, France; fax: +33 2 99 28 41 59; email: Olivier.Gaillot@univ-rennes1.fr

Viral Gastroenteritis in Mongolian Infants

To the Editor: Viral agents of gastroenteritis affect millions of persons of all ages worldwide (1). The major agents include rotavirus, norovirus, sapovirus, astrovirus, and enteric adenovirus. Rotavirus is the most frequent cause of acute sporadic childhood gastroenteritis (1), whereas norovirus infects both adults and children and is mainly associated with

outbreaks of acute gastroenteritis (1). These viruses are commonly transmitted by foodborne, person-to-person, fecal-oral, and environmental routes.

In 1999, the infant death rate was 37.3 per 1,000 live births in Mongolia (2). Bacterial pathogens, such as *Shigella flexneri* and *Salmonella*, are commonly detected in hospitalized patients with gastroenteritis, but no data exist concerning viral agents of gastroenteritis in hospitalized patients or in the general community (2).

This preliminary community-based molecular epidemiologic study was the first to report viral agents of gastroenteritis in Mongolian infants. Stool specimens collected from July to August 2003 from 36 infants belonging to 25 different households from 2 areas in Mongolia were screened for rotavirus, norovirus, sapovirus, astrovirus, and adenovirus. The 2 areas were Tov Province, which included Zuun Mod (provincial center) and Bayanchandmani (provincial district center), and Ulaanbaatar area (capital city), which included Chingeltei, Bayangol, Songinok-harikhan, and Bayanzurkh. A total of 48 stool specimens, which were randomly selected from negative-enterovirus specimens (poliovirus and nonpolio enterovirus (Minako Kuramitsu, unpub. data), were screened. Of the 36 infants in the study, 2 specimens were collected 3 weeks apart from each of 12 infants, and 1 specimen was collected from each of 24 infants. In 10 households, specimens were collected from 2 or 3 siblings. Clinical symptoms were recorded when available.

RNA extraction, cDNA synthesis, and polymerase chain reaction (PCR) were performed as described elsewhere (3); for norovirus genogroup (G) I (GI), PCR, G1SKF, and G1SKR primers were used, and for norovirus GII PCR, G2SKF, and G2SKR primers were used (4). For sapovirus, a nested PCR approach was used for all human genogroups (5). For the first sapovirus PCR, SV-F11 and SV-R1 primers were used, while for the nested PCR, SV-F21, and SV-R2 primers were used. For astrovirus PCR, Mon244, and 82b primers were used (6). All PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. For rotavirus and adenovirus screening, a rapid dry-spot latex agglutination test, Diarlex Rota-Adeno (Orion Diagnostica, Espo, Finland) was used.

Reverse transcription (RT)–PCR products were excised from the gel and purified by the 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 (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were aligned with Clustal X and the distances were calculated by Kimura's 2-parameter method (3). The nucleotide sequence data determined in this study have been deposited in GenBank under accession no. AY590250–AY590262.

Specimens from 12 (33%) of 36 infants were positive for viral agents of gastroenteritis. Specimens from 9 infants were positive for noroviruses, specimens from 2 infants were positive for astroviruses, and a specimen from 1 infant was positive for sapovirus. All specimens were negative for rotavirus and adenovirus. Ten isolated norovirus sequences (9 persons) were classified according to the recent capsid-based sequence scheme of Kageyama et al. (7). Two norovirus sequences belonged to genogroup I/genotype 11 (GI/11), 4 sequences belonged to GII/3, 1 sequence belonged to GII/7, and 3 sequences belonged to GII/6 (Table).

In 1 household, 2 female infants (isolates 213-3 and 214-3, respectively) were infected with a norovirus GI/11 strain that shared 100% nucleotide identity. This strain was

Table. Mongolian infants positive for viral agents of gastroenteritis

Virus	Genogroup/genotype	Specimen*	Symptom†	Age (mo.)	Sex
Norovirus	GI/11	213-3‡	NA	4	F
Norovirus	GI/11	214-3‡	NA	24	F
Norovirus	GII/6	101-1	None	5	F
Norovirus	GII/3	109-1	Diarrhea	6	F
Norovirus	GII/6	205-3	NA	5	F
Norovirus	GII/3	209-1	Diarrhea	3	M
Norovirus	GII/3	317-1§	NA	24	M
Norovirus	GII/6	613-1	None	5	M
Norovirus	GII/7	613-3	NA	5	M
Norovirus	GII/3	609-3§	NA	5	M
Astrovirus	GI	121-3	NA	4	M
Astrovirus	GI	201-3	NA	5	M
Sapovirus	GI	217-1	Diarrhea	1	F

*First 3 numbers before the hyphen refer to the infant; number after the hyphen refers to the week the specimen was collected.
†NA, not available.
‡Two siblings from the same household.
§Only 1 of the siblings from this household was infected.
¶Astrovirus GI = serotype 1.

likely the same and suggests a common source of contamination or person-to-person transmission. Strains belonging to this new genotype have only been detected in Japan and Switzerland (7).

In a different household, 2 different norovirus strains were detected 3 weeks apart in a 5-month-old male infant (isolates 613-1 and 613-3, respectively). These 2 isolated norovirus sequences shared 77.5% nucleotide identity and clustered into two different genotypes, GII/6 (isolate 613-1) and GII/7 (isolate 613-3). In spite of this infection, the infant had no symptoms of gastroenteritis during excretion of the first norovirus strain.

In 4 other households, 4 infants (isolates 109-1, 609-3, 317-1, and 209-1) were infected with norovirus strains belonging to GII/3. These 4 isolated sequences shared >98% nucleotide identity to Arg320 sequence (AF190817), which was previously found to be a recombinant norovirus (8). This result suggests these 4 strains are also recombinant noroviruses, though further sequence analyses of other genetic regions are needed to confirm this result.

Astrovirus was detected in 2 male infants from different households. One infant was 4 months of age (isolate 121-3), and the other infant was 5 months of age (isolate 201-3). These 2 isolated astrovirus sequences had 100% nucleotide identity, which suggests a common source of contamination. These isolated astrovirus sequences shared 98% nucleotide identity to astrovirus Oxford virus sequence (genogroup I). Sapovirus was detected in 1 stool specimen (isolate 217-1) from a 1-year-old female with diarrhea. The isolated sapovirus sequence shared 98% nucleotide identity to sapovirus Manchester virus sequence (genogroup I). Rotavirus and adenovirus were not detected in any of these specimens; further studies, including those of hospitalized infants, may be useful since infants with rotavirus infections are commonly admitted to hospitals (9).

Our preliminary findings have shown that norovirus was a common agent of gastroenteritis (9 of 36 persons) in Mongolian infants. In a recent report on norovirus gastroenteritis, the risk of contracting gastroenteritis was high when another household member was infected and slightly higher when that member was a child (10). In our study, we found 2 siblings infected with an identical norovirus strain during the same period. In Mongolia, diarrhea has become a major healthcare problem (2), therefore, general education in sanitation and hygiene practices may help reduce the transmission of these viruses and lessen the frequency of this disease.

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This work was carried out in the Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

Grant Stuart Hansman,*†
Minako Kuramitsu,*
Hiromu Yoshida,†
Kazuhiko Katayama,†
Naokazu Takeda,†
Hiroshi Ushijima,*
Gungaa Surenkhand,‡
Dugerjav Gantulga,‡
and Chushi Kuroiwa*

*University of Tokyo, Tokyo, Japan; †National Institute of Infectious Diseases, Tokyo, Japan; ‡Ministry of Health, Ulaanbaatar, Mongolia

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Address for correspondence: Chushi Kuroiwa, Graduate School of Medicine, International Health Policy and Planning, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, 113-0033, Tokyo, Japan; fax: 81-3-5841-3637; email: ckuroiw@m.u-tokyo.ac.jp

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Cross-reactivity among sapovirus recombinant capsid proteins

G. S. Hansman[1,2], K. Natori[1], T. Oka[1], S. Ogawa[1], K. Tanaka[3], N. Nagata[3], H. Ushijima[2], N. Takeda[1], and K. Katayama[1]

[1]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan
[2]Department of Developmental Medical Sciences, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
[3]Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan

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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 3×10^6 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	GACTAGTTCTAGATCGCGAGCGGCCGCCCTTTTTTTTTTTTTTTTTTTTTTTTTTT	End	−
p+1Mc114	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACC**ATGGAGGGCAATGGCTCCA ACTCAGAGCCAAAG**	1–33	+
p+1C12	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACC**ATGGAGGGTGTACCCCGCC CAGAGGGCCCAAG**	1–33	+
p+1NK24	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACC**ATGGAGGGCAATGGCTCCC GACTTGGGAAC**	1–33	+
attB2TX30SXN	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGACTAGTTCTAGATCGCGAGCGGCCGCCCTTTTTT TTTTTTTTTTTTTTTTTTTTTTTT	End	−
N-terminal-Mc114	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**ATGGAGGGCAATGGCTCCAACTCAGAGCCAAAG**	1–33	+
N-terminal-Mc114	**CTTCTGTTTGCTAAGGCCACCAGGTCAACAG**TAGGACCCAGCTTTCTTGTACAAAGTGGTCCCC	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	**CATTGGTCACAACCCTGGCAGCTCCAATGTC**TAGGACCCAGCTTTCTTGTACAAAGTGGTCCCC	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 2.7×10^6 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,

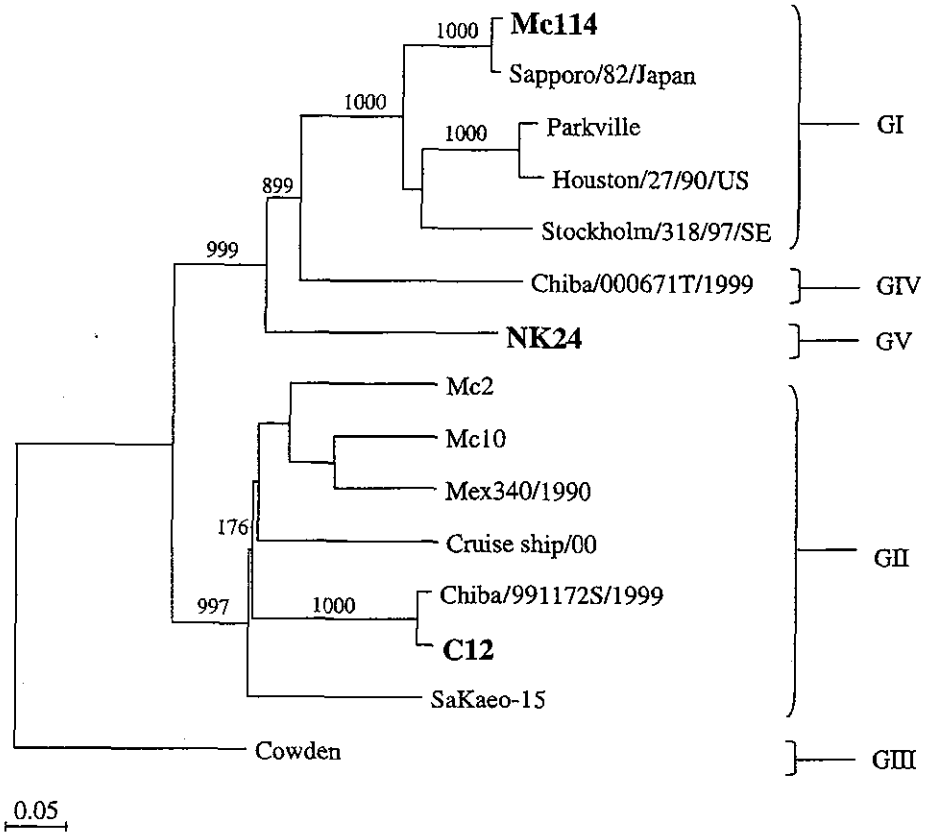

Fig. 1. Phylogenetic analysis of Mc114, C12, and NK24 strains (bold) based on capsid sequences (376 bp). SaV were classified based on the scheme of Farkas et al. [6], using Cowden (SaV GIII) as an outgroup. The number on each branch indicates the bootstrap value for the genotype. Bootstrap values of 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

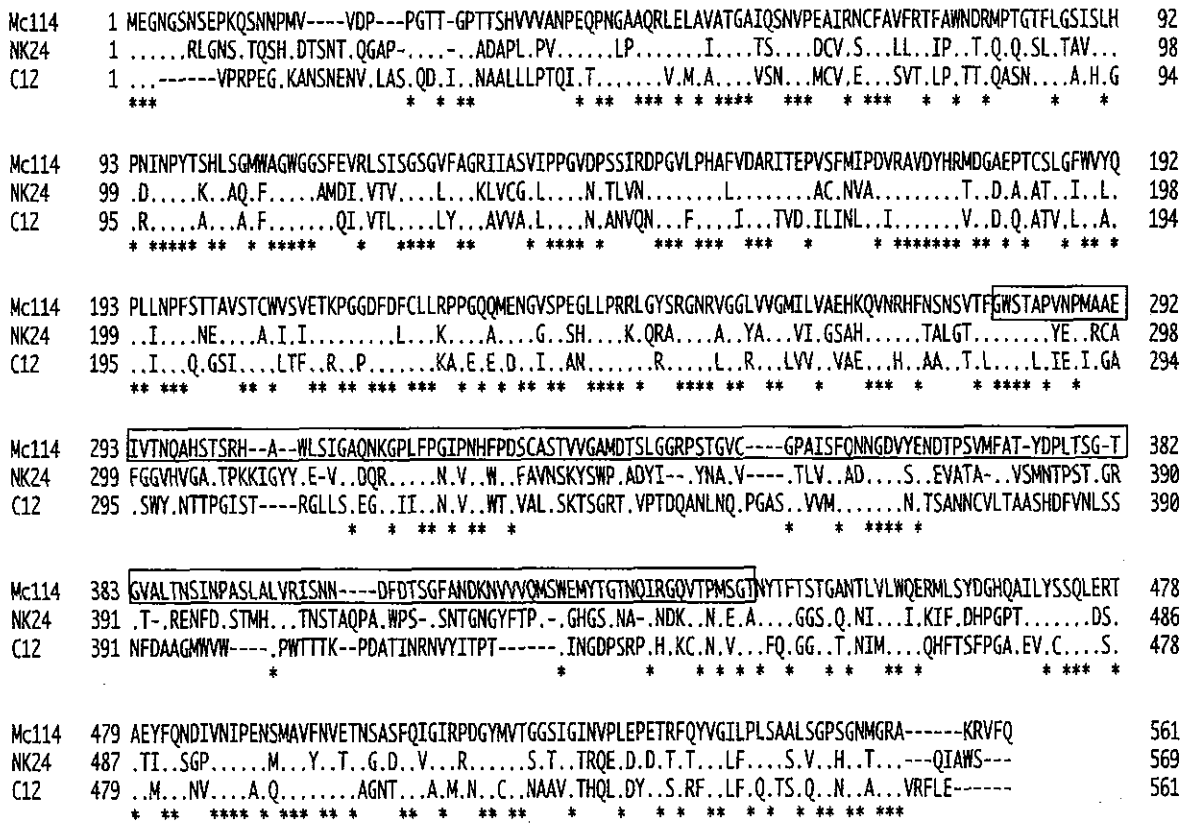

Fig. 2. An amino acid alignment of VP1 sequences of Mc114 (GI), C12 (GII), and NK24 (GV) strains. The sequences with a rectangular box represent the predicted P2 domain [4]. The asterisks indicate conserved amino acids among these three VP1 sequences

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

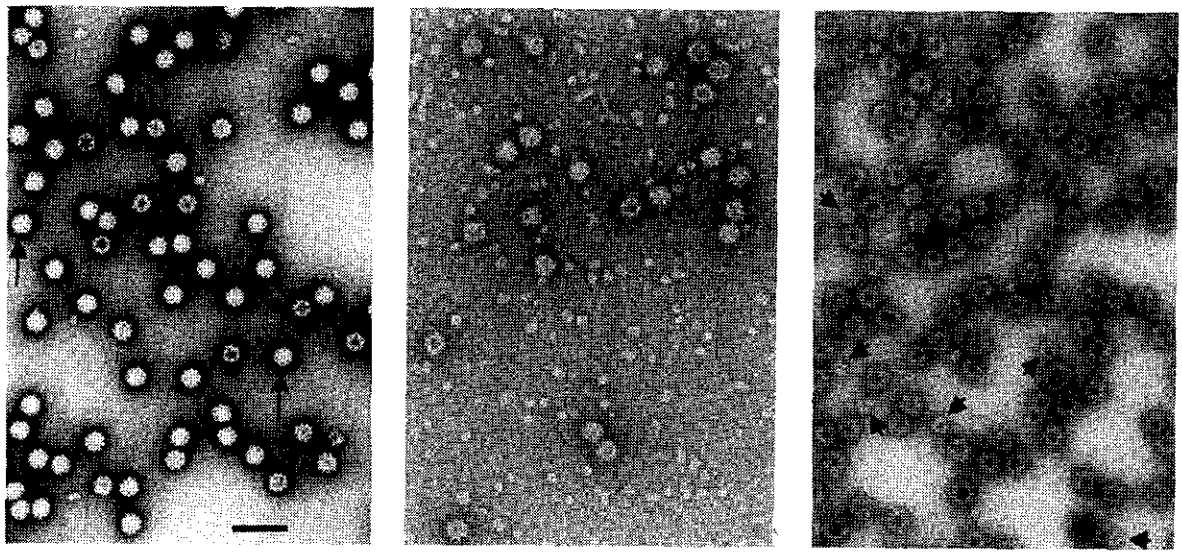

Fig. 3. Electron-microscopic images of CsCl purified (**A**) Mc114, (**B**) C12, and (**C**) NK24 VLPs negative-stained with 4% uranyl acetate (pH 4). The long arrows show the Star of David structure and the short arrows show the small VLPs. The bar indicates 100 nm

shown). Our results indicated that the optimal time to harvest Mc114 VLPs in culture medium was 6 dpi (Fig. 4A).

We also analyzed the time-course expression of Mc114 rVP2 in insect cells by Western blotting with antiserum raised against *E. coli*-expressed Mc114 VP2. However, no bands were detected in either the culture medium or the cell lysate (data not shown).

Western blot cross-reactivity analysis

Western blotting was used to investigate the cross-reactivities among SaV Mc114, C12, and NK24 rVP1 expressed in insect cells. Hyperimmune rabbit antiserum was raised against *E. coli*-expressed Mc114, C12, and NK24 N- and C-terminal VP1. In addition, hyperimmune rabbit antiserum raised against Mc114 VLPs was used. As shown in Fig. 5 and summarized in Table 3, Mc114 (VLP, N- and C-terminal) antisera were reactive with Mc114 and NK24 rVP1 (Mc114 VLP antiserum weakly with NK24 rVP1) but not with C12 rVP1. On the other hand, C12 (N- and C-terminal) antisera were reactive with C12 rVP1 and weakly reactive with Mc114 rVP1, but were not reactive with NK24 rVP1, whereas NK24 (N- and C-terminal) antisera reacted with NK24 rVP1 only.

The three kinds of Mc114 antisera reacted in manner similar to that of Mc114 rVP1, revealing bands at 60K and 55K (Fig. 5), as observed in the time-course analysis (Fig. 4B). These two bands were also detected by C12 N-terminal antiserum with Mc114 rVP1 but not with the C12 C-terminal antiserum. One band was detected by the C12 N-terminal antiserum with C12 rVP1, whereas two bands of

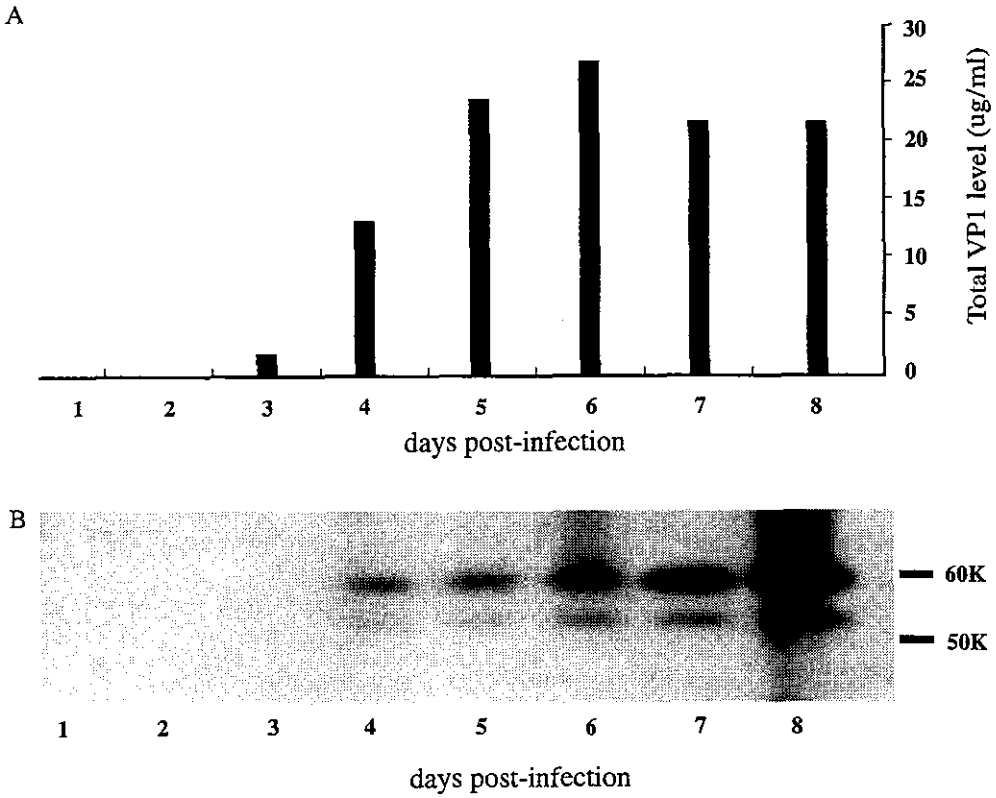

Fig. 4. Time-course expression of the Mc114 rVP1. Confluent Tn5 cells were infected with Mc114 recombinant baculoviruses at MOI of 14.5 and incubated at 26 °C. The culture medium was harvested at the indicated day (days post-infection) and concentrated as described in the Materials and methods. Using the same samples, the expressed proteins were analyzed by (**A**) antigen ELISA with hyperimmune rabbit (capture) and guinea pig (detector) antisera raised against Mc114 VLPs and (**B**) Western blotting with hyperimmune rabbit antiserum raised against Mc114 VLPs. For the antigen ELISA, purified Mc114 VLPs were used as the positive control at concentrations ranging from 500 ng to 0.24 ng

approximately 60K and 58K were detected by the C12 C-terminal antiserum. Two bands of approximately 60K and 55K were detected by NK24 (N- and C-terminal) antisera with NK24 rVP1, and an additional weak band of approximately 45K was detected by the NK24 C-terminal antiserum. Two bands of approximately 60K and 55K were detected by Mc114 VLP and N-terminal antisera with NK24 rVP1, but only a single band of 60K was detected by Mc114 C-terminal antiserum. The significance of these lower bands has not yet been determined.

ELISA cross-reactivity analysis

An antigen ELISA was used to examine the cross-reactivities among Mc114, C12, and NK24 VLPs expressed in insect cells. The ELISA incorporated hyperimmune rabbit (capture) antiserum and guinea pig (detector) antiserum raised against purified Mc114 VLPs. Samples were added to duplicate wells and averaged.

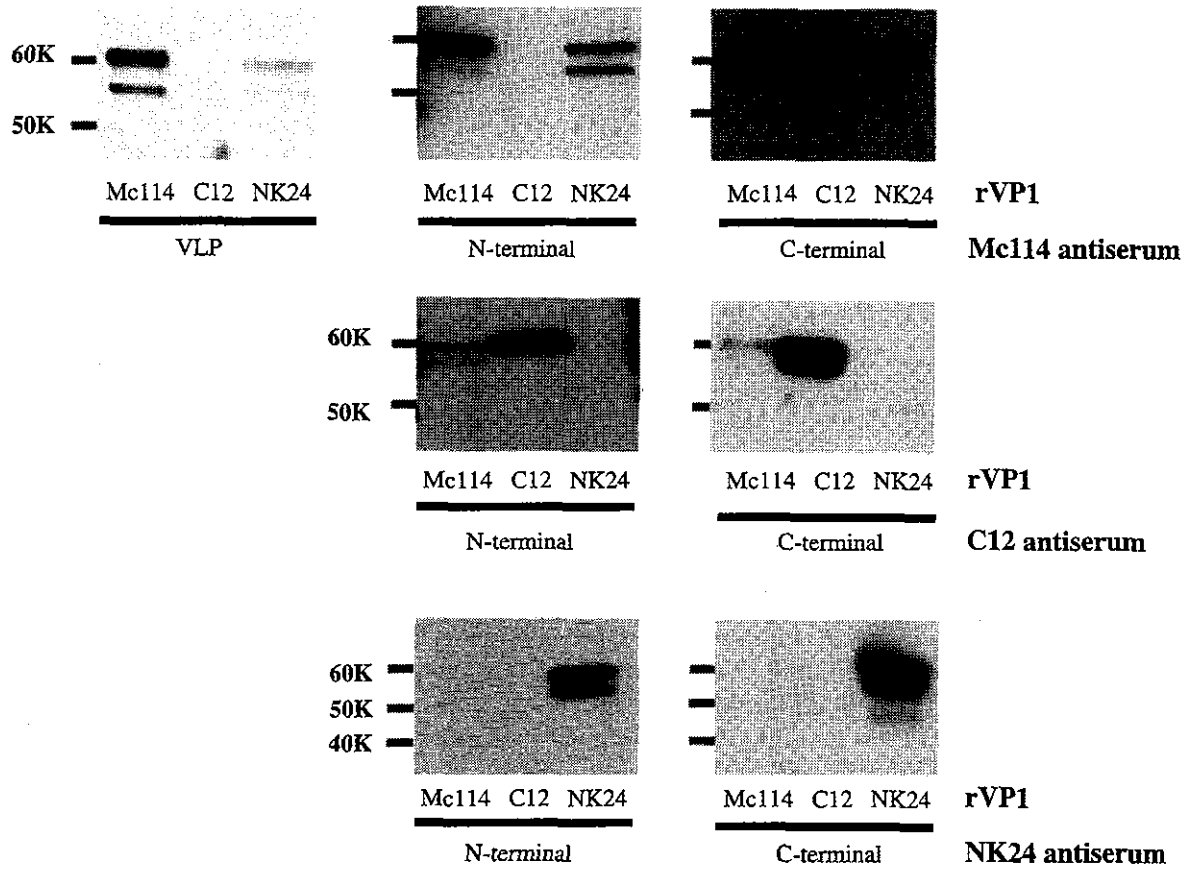

Fig. 5. Western blots of Mc114, C12, and NK24 rVP1 expression in insect cells. Culture medium was harvested at 6 dpi. Seven different hyperimmune rabbit antisera were used, including antisera raised against *E. coli*-expressed Mc114, C12, and NK24 N- and C-terminal VP1, and antisera raised against Mc114 VLPs. The band intensities are relative to each antiserum

Table 3. Reactivities of antiserum with SaV GI, GII, and GV rVP1

rVP1	ELISA	Western blot						
	Mc114 antisera[a]	Mc114 antiserum			C12 antiserum		NK24 antiserum	
	VLP	VLP	N-terminal	C-terminal	N-terminal	C-terminal	N-terminal	C-terminal
Mc114 (GI)	0.46 (9.23)[b]	+	+	+	+[c]	+[c]	−	−
C12 (GII)	0.00	−	−	−	+	+	−	−
NK24 (GV)	0.00	+[c]	+	+	−	−	+	+

[a]The ELISA uses Mc114 VLP (rabbit) antiserum as capture and Mc114 VLP (guinea pig) antiserum as detector
[b]P–N and (P/N ratio) values measured at 492 nm
[c]Weakly reactive

As shown in Table 3, the ELISA detected Mc114 VLPs (A_{492} P–N = 0.46 and P/N ratio = 9.23) but neither C12 VLPs nor NK24 VLPs (A_{492} P–N = 0.00).

Discussion

Human SaV VP1 start contains an amino acid motif, MEG, which is conserved in all human SaV strains and as such is considered to be the putative VP1 start. In this study, we re-examined an earlier suggestion that an upstream sequence from the VP1 start AUG codon is crucial to the formation of human SaV VLP [14]. In addition, we examined 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 antigen ELISA. Our results show the following: (i) SaV GI, GII, and GV rVP1 constructs beginning exactly from the predicted VP1 start AUG codon self-assembled VLPs; (ii) an upstream sequence of the predicted VP1 start AUG codon was not an essential element for SaV GI, GII, or GV VLP formation; (iii) the morphological features of the predominant SaV GI, GII, and GV VLPs were similar to those of native SaV, except that GV VP1 also expressed small VLPs; (iv) by Western blot, GI antisera cross-reacted with GV rVP1 but not with GII rVP1, whereas GII antisera cross-reacted weakly with GI rVP1 but did not cross-react with GV rVP1, while GV antisera reacted with GV rVP1 only; and (v) by antigen ELISA, GI VLPs were antigenically distinct from GII and GV VLPs.

Only three other expression studies of human SaV VLP formation have been reported [4, 14, 22]. In one of those studies, an upstream sequence of 73 nucleotides from the predicted VP1 start AUG codon (construct pHou/90-3, Houston/27/90/US strain) was a crucial element for VLP formation [14], whereas one of the other two reports included 39 nucleotides upstream (Sapporo/82/Japan strain) [22]. Recently, the Parkville strain (U73124) was reported to form empty VLPs with a construct that began exactly from the predicted VP1 start. Surprisingly, the Parkville strain had 97.5% and 100% nucleotide identity to pHou/90-3 VP1 and the 73-nucleotide-upstream sequences, respectively. On the other hand, Mc114 had 94.4% and 94.9% nucleotide identity to the Sapporo/82/Japan VP1 and the 39-nucleotide-upstream sequences, respectively. These results suggest that an upstream sequence may not be an essential element for SaV VLP formation, but that some other factor(s) are necessary.

Porcine enteric calicivirus (PEC) of SaV GIII was also expressed using a baculovirus expression system that resulted in the formation of PEC VLPs [9]. The PEC rVP1 construct contained nine nucleotides upstream of the VP1 start AUG codon [9]. That study also included a bacteriophage T7 RNA polymerase promoter of 19 nucleotides (in front of these 9 nucleotides) and a downstream sequence of 98 nucleotides at the 3′ of the VP1 gene. Although the T7 promoter is independent of the baculovirus expression system, Jiang suggested that an element (i.e., an upstream sequence) could be responsible for initiating transcription or translation; alternatively, this element could serve as a spacer between the polyhedrin promoter and the VP1 gene, or as a stabilizer of the RNA after transcription [26]. Interestingly, Jiang also tried to express two other recombinant constructs (pHou/90-1

and pHou/90-2) that included 9 and 29 nucleotides upstream, respectively, but those constructs failed to express rVP1 or form VLPs [14].

Human norovirus (NoV) also belongs to the family *Caliciviridae* and is typically associated with food- and waterborne infections [1]. Human SaV and NoV strains are morphologically, genetically, and antigenically distinct [1, 16]. The NoV genome is organized in a slightly different way than the SaV, since ORF1 encodes all the nonstructural proteins, ORF2 encodes the capsid protein (VP1), and ORF3 encodes a small protein (VP2). In a recent NoV expression study, inclusion of VP2 and poly(A) sequences in NoV rVP1 constructs were found to stabilize VLP formation [3]. In the pHou/90 constructs, both VP2 and poly(A) sequences were absent [14]. Consequently, if SaV VP2 has functions similar to those of NoV VP2, as has been suggested [1], then some crucial factors for SaV VLP formation could be SaV VP2 and poly(A) sequences. Although in a recent report the rVP1 was expressed for a SaV GII strain as determined by SDS-PAGE [24], VLPs were not formed even when an upstream sequence from VP1 start AUG codon, VP2, and poly(A) sequences were included in the construct.

All of our rVP1 constructs formed the typical native SaV Star of David structure and had diameters of 41–48 nm, while NK24 rVP1 also formed smaller VLPs with diameters of 26–31 nm (Fig. 3). The recombinant Sapporo/82/Japan construct had the Star of David form [22], whereas the pHou/90-3 construct did not [14]. Besides the different genogroups and genotypes, the only obvious differences in the constructs that formed this structure were the inclusions of VP2 and poly(A) sequences. These results suggest that VP2 and/or poly(A) sequences are needed to form the Star of David structure, although direct evidence is lacking.

The Mc114 rVP2 was not detected by our Western blotting during the time-course analysis (data not shown), although NoV studies have found that the expression level of NoV rVP2 was low [3]. On the other hand, we did not detect any SaV rVP1 cleavage products either (data not shown), and a similar result was observed in other SaV expression studies [9, 14, 22], whereas for NoV the rVP1 is cleaved at amino acid residue 227, yielding a 32K C-terminal rVP1 product [12, 16]. These data suggest that SaV rVP1 expression could be quite different from that of NoV. Our studies have also found that the yields of purified SaV VLPs were low in comparison with those of NoV VLPs (data not shown), which was also discussed by Guo et al. [9].

Hyperimmune rabbit and guinea pig antisera raised against GI VLPs were used to examine the cross-reactivities among SaV GI, GII, and GV VLPs by an antigen ELISA. As summarized in Table 3, the antigen ELISA was specific only for the homologous GI VLPs. This result was not so unusual, since the amino acid homologies among the VP1 sequences were low (Fig. 2). In the recent structural analysis of SaV VLPs, the outermost domain of VP1, i.e., the P2 domain, was shown to be the most variable region and is likely to confer strain diversity [4]. The predicted P2 domain was the most variable region among these three SaV VP1 sequences, whereas the S and P1 domains were more conserved (Table 3). These data suggest that there were no shared epitopes among GI, GII, and GV VLPs as determined by antigen ELISA, though further studies are needed to confirm this suggestion.