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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

[a] Values are means ± standard deviations.

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

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

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

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

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

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

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

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

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

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

RESULTS

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

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

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

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

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

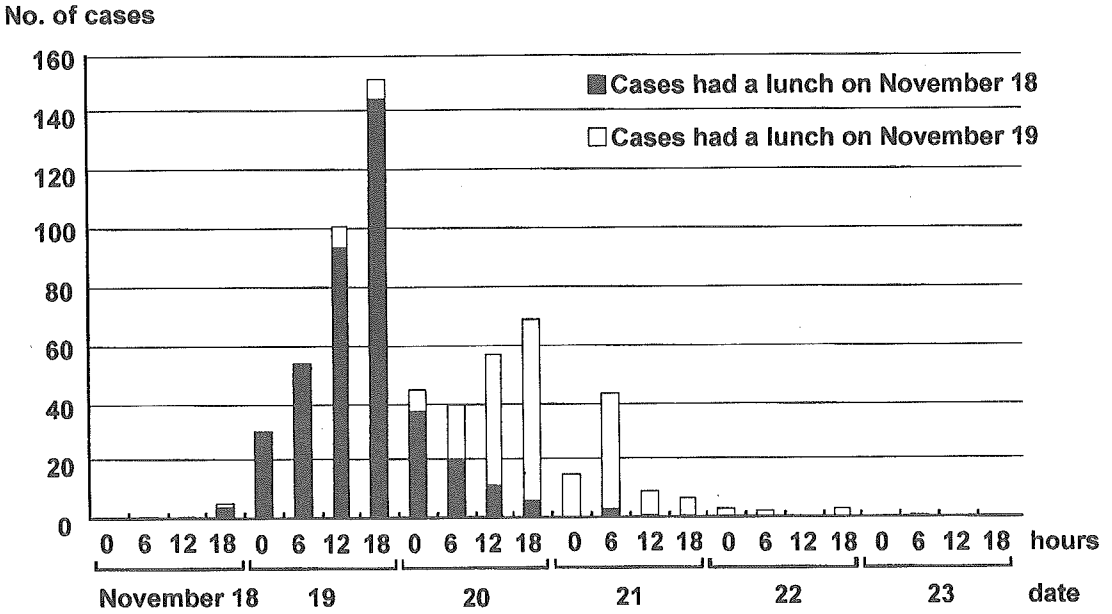

FIG. 1. Epidemic curve of cases, by hours and dates of onset of symptoms. The *x* axis presents the times (in hours) and the days when the onset of symptoms occurred.

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

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

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

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

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

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

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

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

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

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

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

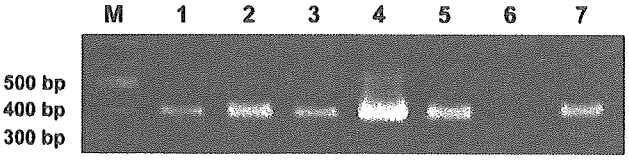

FIG. 2. Detection of NV capsid genes from specimens and samples by RT-PCR with genogroup-specific primers. The PCR products were electrophoresed on a 1.5% agarose gel. Lane M, marker (100-bp ladder; New England BioLabs Inc., Beverly, Mass.); lane 1, fecal specimen from the cook with general fatigue;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

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

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

DISCUSSION

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

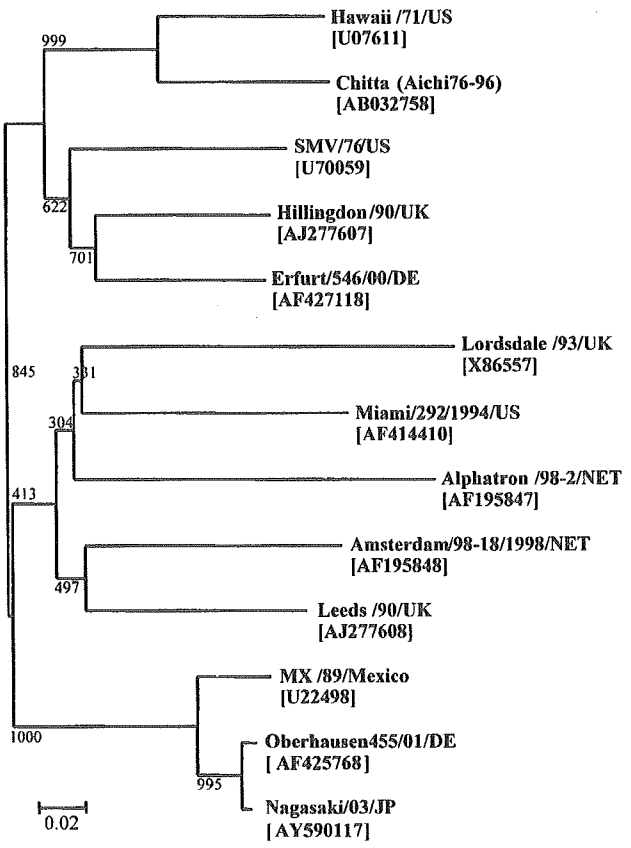

FIG. 3. Phylogenetic tree constructed on the basis of the sequences of a part of the capsid gene of GII NVs from the present outbreak and known strains from the GenBank database. GenBank accession numbers for the strains are indicated in the parenthesis. The causative viral strain of the present outbreak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

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

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

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

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

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

cases is continuously needed. It is also essential that food samplers not work when they are ill and that good handwashing facilities be provided in all restaurants.

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REFERENCES

1. **Ando, T., J. S. Noel, and R. L. Fankhauser.** 2000. Genetic classification of "Norwalk-like viruses." J. Infect. Dis. **181**(Suppl. 2):S336–S348.
2. **Beller, M., A. Ellis, S. H. Lee, M. A. Drebot, S. A. Jenkerson, E. Funk, M. D. Sobsey, O. D. Simmons III, S. S. Monroe, T. Ando, J. Noel, M. Petric, J. P. Middaugh, and J. S. Spika.** 1997. Outbreak of viral gastroenteritis due to a contaminated well. International consequences. JAMA **278**:563–568.
3. **Dedman, D., H. Laurichesse, E. O. Caul, and P. G. Wall.** 1998. Surveillance of small round structured virus (SRSV) infection in England and Wales, 1990-5. Epidemiol. Infect. **121**:139–149.
4. **de Wit, M. A., M. P. Koopmans, L. M. Kortbeek, W. J. Wannet, J. Vinje, F. van Leusden, A. I. Bartelds, and Y. T. van Duynhoven.** 2001. Sensor, a population-based cohort study on gastroenteritis in The Netherlands: incidence and etiology. Am. J. Epidemiol. **154**:666–674.
5. **Dippold, L., R. Lee, C. Selman, S. Monroe, and C. Henry.** 2003. A gastroenteritis outbreak due to norovirus associated with a Colorado hotel. J. Environ. Health **66**:13–17, 26.
6. **Fankhauser, R. L., J. S. Noel, S. S. Monroe, T. Ando, and R. I. Glass.** 1998. Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States. J. Infect. Dis. **178**:1571–1578.
7. **Froggatt, P. C., I. B. Vipond, C. R. Ashley, P. R. Lambden, I. N. Clarke, and E. O. Caul.** 2004. Surveillance of norovirus infection in a study of sporadic childhood gastroenteritis in South West England and South Wales, during one winter season (1999-2000). J. Med. Virol. **72**:307–311.
8. **Glass, R. I., J. Noel, T. Ando, R. Fankhauser, G. Belliot, A. Mounts, U. D. Parashar, J. S. Bresee, and S. S. Monroe.** 2000. The epidemiology of enteric caliciviruses from humans: a reassessment using new diagnostics. J. Infect. Dis. **181**(Suppl. 2):S254–S261.
9. **Hohne, M., and E. Schreier.** 2004. Detection and characterization of norovirus outbreaks in Germany: application of a one-tube RT-PCR using a fluorogenic real-time detection system. J. Med. Virol. **72**:312–319.
10. **Inouye, S., K. Yamashita, S. Yamadera, M. Yoshikawa, N. Kato, and N. Okabe.** 2000. Surveillance of viral gastroenteritis in Japan: pediatric cases and outbreak incidents. J. Infect. Dis. **181**(Suppl. 2):S270–S274.
11. **Iritani, N., Y. Seto, H. Kubo, T. Murakami, K. Haruki, M. Ayata, and H. Ogura.** 2003. Prevalence of Norwalk-like virus infections in cases of viral gastroenteritis among children in Osaka City, Japan. J. Clin. Microbiol. **41**:1756–1759.
12. **Johansson, P. J., M. Torven, A. C. Hammarlund, U. Bjorne, K. O. Hedlund, and L. Svensson.** 2002. Food-borne outbreak of gastroenteritis associated with genogroup I calicivirus. J. Clin. Microbiol. **40**:794–798.
13. **Kageyama, T., S. Kojima, M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, N. Takeda, and K. Katayama.** 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J. Clin. Microbiol. **41**:1548–1557.
14. **Katayama, K., H. Shirato-Horikoshi, S. Kojima, T. Kageyama, T. Oka, F. Hoshino, S. Fukushi, M. Shinohara, K. Uchida, Y. Suzuki, T. Gojobori, and N. Takeda.** 2002. Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. Virology **299**:225–239.
15. **Kobayashi, S., T. Morishita, T. Yamashita, K. Sakae, O. Nishio, T. Miyake, Y. Ishihara, and S. Isomura.** 1991. A large outbreak of gastroenteritis associated with a small round structured virus among schoolchildren and teachers in Japan. Epidemiol. Infect. **107**:81–86.
16. **Kojima, S., T. Kageyama, S. Fukushi, F. B. Hoshino, M. Shinohara, K. Uchida, K. Natori, N. Takeda, and K. Katayama.** 2002. Genogroup-specific PCR primers for detection of Norwalk-like viruses. J. Virol. Methods **100**: 107–114.
17. **Koopmans, M., J. Vinje, M. de Wit, I. Leenen, W. van der Poel, and Y. van Duynhoven.** 2000. Molecular epidemiology of human enteric caliciviruses in The Netherlands. J. Infect. Dis. **181**(Suppl. 2):S262–S269.
18. **Lopman, B., H. Vennema, E. Kohli, P. Pothier, A. Sanchez, A. Negredo, J. Buesa, E. Schreier, M. Reacher, D. Brown, J. Gray, M. Iturriza, C. Gallimore, B. Bottiger, K. O. Hedlund, M. Torven, C. H. von Bonsdorff, L. Maunula, M. Poljsak-Prijatelj, J. Zimsek, G. Reuter, G. Szucs, B. Melegh, L. Svennson, Y. van Duijnhoven, and M. Koopmans.** 2004. Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. Lancet **363**:682–688.
19. **Lopman, B. A., G. K. Adak, M. H. Reacher, and D. W. Brown.** 2003. Two epidemiologic patterns of norovirus outbreaks: surveillance in England and Wales, 1992-2000. Emerg. Infect. Dis. **9**:71–77.
20. **Lopman, B. A., D. W. Brown, and M. Koopmans.** 2002. Human caliciviruses in Europe. J. Clin. Virol. **24**:137–160.
21. **Lopman, B. A., M. H. Reacher, Y. Van Duijnhoven, F. X. Hanon, D. Brown, and M. Koopmans.** 2003. Viral gastroenteritis outbreaks in Europe, 1995-2000. Emerg. Infect. Dis. **9**:90–96.
22. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5**:607–625.
23. **Nicollier-Jamot, B., V. Pico, P. Pothier, E. Kohli, B. K. Bohnker, and S. Thornton.** 2003. Molecular cloning, expression, self-assembly, antigenicity, and seroepidemiology of a genogroup II norovirus isolated in France. J. Clin. Microbiol. **41**:3901–3904.
24. **Nishida, T., H. Kimura, M. Saitoh, M. Shinohara, M. Kato, S. Fukuda, T. Munemura, T. Mikami, A. Kawamoto, M. Akiyama, Y. Kato, K. Nishi, K. Kozawa, and O. Nishio.** 2003. Detection, quantitation, and phylogenetic analysis of noroviruses in Japanese oysters. Appl. Environ. Microbiol. **69**: 5782–5786.
25. **Noel, J. S., R. L. Fankhauser, T. Ando, S. S. Monroe, and R. I. Glass.** 1999. Identification of a distinct common strain of 'Norwalk-like viruses' having a global distribution. J. Infect. Dis. **179**:1334–1344.
26. **Otsu, R., A. Ishikawa, K. Mukae, H. Nakayama, and M. Sarashi.** 2003. Molecular epidemiology of Norwalk-like virus (NLV) outbreaks occurring in Kyushu Japan between 1988 and 1993. Eur. J. Epidemiol. **18**:369–372.
27. **Pang, X. L., S. Honma, S. Nakata, and T. Vesikari.** 2000. Human caliciviruses in acute gastroenteritis of young children in the community. J. Infect. Dis. **181**(Suppl. 2):S288–S294.
28. **Tompkins, D. S., M. J. Hudson, H. R. Smith, R. P. Eglin, J. G. Wheeler, M. M. Brett, R. J. Owen, J. S. Brazier, P. Cumberland, V. King, and P. E. Cook.** 1999. A study of infectious intestinal disease in England: microbiological findings in cases and controls. Commun. Dis. Public Health **2**:108–113.
29. **Vinje, J., J. Green, D. C. Lewis, C. I. Gallimore, D. W. Brown, and M. P. Koopmans.** 2000. Genetic polymorphism across regions of the three open reading frames of "Norwalk-like viruses." Arch. Virol. **145**:223–241.
30. **Vinje, J., H. Vennema, L. Maunula, C. H. von Bonsdorff, M. Hoehne, E. Schreier, A. Richards, J. Green, D. Brown, S. S. Beard, S. S. Monroe, E. de Bruin, L. Svensson, and M. P. Koopmans.** 2003. International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses. J. Clin. Microbiol. **41**:1423–1433.
31. **Wang, J., X. Jiang, H. P. Madore, J. Gray, U. Desselberger, T. Ando, Y. Seto, I. Oishi, J. F. Lew, K. Y. Green, et al.** 1994. Sequence diversity of small, round-structured viruses in the Norwalk virus group. J. Virol. **68**:5982–5990.
32. **Widdowson, M. A., E. H. Cramer, L. Hadley, J. S. Bresee, R. S. Beard, S. N. Bulens, M. Charles, W. Chege, E. Isakbaeva, J. G. Wright, E. Mintz, D. Forney, J. Massey, R. I. Glass, and S. S. Monroe.** 2004. Outbreaks of acute gastroenteritis on cruise ships and on land: identification of a predominant circulating strain of norovirus—United States, 2002. J. Infect. Dis. **190**:27–36.
33. **Yan, H., F. Yagyu, S. Okitsu, O. Nishio, and H. Ushijima.** 2003. Detection of norovirus (GI, GII), sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR. J. Virol. Methods **114**:37–44.

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Genetic diversity of sapovirus in fecal specimens from infants and children with acute gastroenteritis in Pakistan

Brief Report

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Summary. A total of 517 fecal specimens collected from infants and children with acute gastroenteritis in Karachi city, Pakistan during 1990–1994 were examined for the presence of sapovirus by RT-PCR and sequence analysis methods. Sapovirus was identified in 17 of 517 (3.2%) specimens. Sapovirus was further clustered into three distinct genogroups (I, II and IV) and these presented 70.6%, 23.5% and 5.9%, respectively. Our results clearly indicated that sapovirus could be classified into 7 GI and 4 GII genotypes. It was noteworthy to point out that sapovirus detected among Pakistani infants and children with acute gastroenteritis demonstrated the great genetic diversity and presented novel sapovirus genotypes.

*

Viral gastroenteritis is a common disease with a high morbidity reported worldwide especially in infants and the elderly. The mortality among children due to acute gastroenteritis is greater in developing than in developed countries. Acute gastroenteritis consistently ranks as one of the top six causes of all deaths. Apart from rotavirus as the major etiologic agent of gastroenteritis in children and young animals, sapovirus is considered to be a significant global enteropathogen [6, 7].

Sapovirus was first named after its discovery in an outbreak of acute gastroenteritis in a home for infants in Sapporo, Japan in October 1977 [1]. They have a typical "Star of David" configuration by EM and are antigenically identical to each other by immune EM [2]. Sapovirus contains a positive sense single-strand RNA genome surrounded by an icosahedral capsid. Sapovirus genome contains

two ORFs (ORF1 and 2); ORF 1 encodes the non-structural as well as the capsid proteins. Based on the sequence analysis of the capsid gene, sapovirus is divided into three genogroups (I, II and IV) and currently genogroup V as known to infect human [3, 9, 15]. Moreover, porcine enteric calicivirus (PEC) is reported to be a member of genogroup III [4].

Etiologic studies of acute gastroenteritis in infants conducted in Japan with sensitive reverse transcription–PCR methods showed that sapovirus was one of the most causes of outbreaks of viral gastroenteritis among infants in Sapporo [1, 2]. This virus also is associated with sporadic outbreaks of gastroenteritis worldwide and is recognized just as important as rotavirus infecting children less than 2 years of age in Finland [10, 11]. Recently, seroepidemiologic studies have indicated a worldwide distribution of sapovirus [6, 7]. The aged-related prevalence of antibody against this virus also has shown that infections commonly occur in children less than 5 years old. The pattern of acquisition of the antibody is similar to that of other common virus infection [14].

The objectives of this study were: to analyze the epidemiology of sapovirus among Pakistani pediatric population with acute gastroenteritis, to characterize the detected sapovirus according to genogroup and genotype, and to describe the genetic diversity of sapovirus.

Five hundreds and seventeen fecal specimens were collected from infants and children with acute gastroenteritis in the Civil Karachi Hospital, Dow Medical College, Karachi city, Pakistan during the period of January 1990 to December 1994. All fecal specimens were determined previously to be negative for rotavirus and adenovirus [8]. These specimens were diluted with Eagle's minimum essential medium to 10% suspensions, and clarified by centrifugation at 5000 g for 20 min. The supernatants were collected and stored at −30 °C until use for the detection of sapovirus. The viral genome was extracted from 140 µl of a 10% fecal suspension using a spin column technique according to the instructions in the QIAamp® Viral RNA Mini Kit Handbook, Germany. Sapovirus was detected by RT-PCR analysis of extracted viral RNA with specific primers previously published [19]. Briefly, a pair of published primers (SLV5317 and SLV5749) for amplifying capsid region of sapovirus was used. The PCR was performed at 94 °C for 3 min followed by 35 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 60 s, and a final extension at 72 °C for 7 min, and then held at 4 °C [19]. PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (EtBr, 5 mg/ml) for 20 min then visualized under ultraviolet (UV) light, and the results were recorded by photography. The nucleotide sequences of PCR products (DNA) positive for sapovirus were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc.). Sequence analysis was performed using E-CLUSTAL W (Version 1.6). Reference strains and accession numbers used in this study are as follows: Southampton (L07418), PEC (AF182760), Bristol/89 (AJ249939), Lyon/598/97/F (AJ271056), London/92 (U95645), Mex340/90 (AF435812), Cruise ship/00 (AY289804), Hou7-1181/90 (AF435814), Arg39 (AY289803), Stockholm/97 (AF194182), Mex14917/00 (AF435813), Houston/90 (U95644), Parkville/94 (U73124),

Houston/86 (U95643), Sapporo/82 (U65427), Manchester/93 (X86560), Plymouth/92 (X86559), Lyon/30388/98 (AJ251991), Southampton (L07418).

Out of 517 fecal specimens collected from infants and children with acute gastroenteritis in Karachi city, Pakistan, 168 were collected in 1990; 86 in 1991; 76 in 1992; 99 in 1993; and 88 in 1994. For the pediatric population, the lowest age was under 1 month, the highest was 5 years, and the average age was 1 year (12 months). Moreover, the number of male (60.9%) was higher than that of female (39.1%). All fecal specimens were tested for the presence of sapovirus by RT-PCR. The results shown in Table 1 revealed that sapovirus was detected in 17 out of 517 (3.2%) specimens tested. Sapovirus was identified in each of the five years. The highest incidence fell into 1991 (29.4%, 5 of 17) and the lowest in 1993 (5.9%, 1 of 17). The age at sapovirus infection ranged from 5 months to 3 years. The viral infectious rate was highest in the 6–11 months old group (41.2%) and lowest in children over 35 months or less than 6 months of age (5.9%). Moreover, the number of male infected sapovirus with acute gastroenteritis (58.8%) was higher than that of female.

A total of 17 sapovirus amino acid sequences were analyzed by phylogenetics and grouped using the recent sapovirus capsid region classification scheme of Farkas et al. (2004). Majority of the sapovirus sequences clustered into two distinct genogroupes I and II (GI, GII). Interestingly, one of the sequenced specimens positive for sapovirus, Karachi/730/1992, turned out to belong to a cluster called

Table 1. Characteristics of seventeen sapovirus infections among infants and children with acute gastroenteritis in Karachi city, Pakistan during 1990 and 1994

No.	Patient	Year	Month	Sex	Age	Isolate	Genogroup	Genotype	Accession No.
1	877	1990	August	M	3 y	Karachi/877/1990	GI	5*	AB181133
2	878	1990	August	F	5 m	Karachi/878/1990	GI	6*	AB181228
3	953	1990	September	M	1 y	Karachi/953/1990	GII	1	AB181131
4	1017	1990	October	F	2 y	Karachi/1017/1990	GI	5*	AB181227
5	824	1991	January	M	1 y	Karachi/824/1991	GII	1	AB181130
6	842	1991	May	F	8 m	Karachi/842/1991	GI	1	AB181232
7	872	1991	July	F	6 m	Karachi/872/1991	GI	4*	AB181231
8	934	1991	October	M	9 m	Karachi/934/1991	GI	1	AB181247
9	937	1991	October	M	2 y	Karachi/937/1991	GI	6*	AB181229
10	874	1992	March	M	2 y	Karachi/874/1992	GII	4*	AB181129
11	730	1992	August	M	10 m	Karachi/730/1992	GIV#	N/A	AB126249
12	1021	1992	August	F	9 m	Karachi/1021/1992	GI	4*	AB181230
13	1026	1992	September	F	9 m	Karachi/1026/1992	GI	5*	AB181134
14	876	1993	September	F	1 y	Karachi/876/1993	GI	7*	AB181132
15	928	1994	March	M	1 y	Karachi/928/1994	GII	4*	AB181128
16	938	1994	April	M	9 m	Karachi/938/1994	GI	1	AB181248
17	997	1994	July	M	1 y	Karachi/997/1994	GI	1	AB181233

Note. *No.*, Number; *M*, Male; *F*, Female; *y*, Year; *m*, Month; #Rare genogroup
*New genotype; *N/A*, Not available

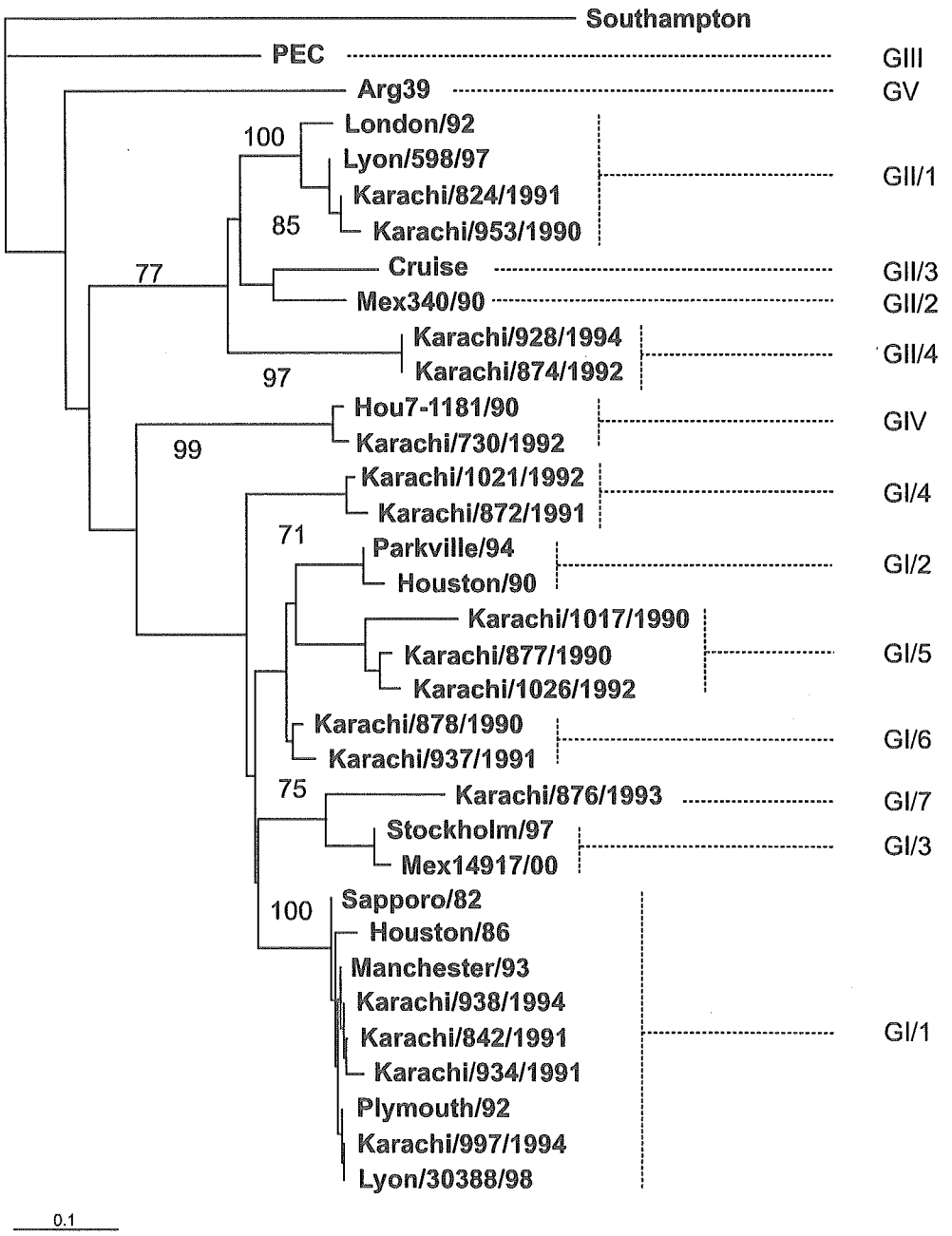

Fig. 1. Phylogentic tree of amino acid sequences of 17 isolates of sapovirus based on the recent sapovirus capsid region classification scheme of Farkas et al. [3]. The tree was constructed from partial amino acid sequences of the capsid region of the seventeen sapoviruses detected in Karachi city, Pakistan during the period of 5 years (1990 through 1994). The numbers in the branches indicate the bootstrap values. Reference strains of sapovirus were selected from DDBJ/GenBank under the accession number indicated in the text. Southampton was used as an out-group strain for phylogenetic analysis

a genogroup IV (known as the Hou7-1181/90 virus). The nucleotide identity between Karachi/730/1992 and Hou7-1181/90 was 96%, and the amino acid identity was 97%.

Most of the sapovirus sequences (70.6%, 12 of 17) belonged to sapovirus GI (known as the Manchester virus cluster). Our results indicated that sapovirus group I was a dominant genogroup. The sapovirus GI sequences clustered into one distinct GI genotype, GI/1 (typified by the Sapporo/82 virus cluster) (33.3%, 4 of 12). In addition we also identified eight isolates (3 in 1990, 2 in 1991, 2 in 1992 and 1 in 1993), forming four novel sapovirus GI genotypes tentatively called GI/4, GI/5, GI/6 and GI/7, respectively (Fig. 1). These sapoviruses had a low identity on the amino acid with other reference strains in the same genogroup ranged from 65% to 86%.

Our findings showed that two (50%) of the sapovirus GII sequences were classified into GII/1 (known as the Lyon/598/97 virus). Interestingly, two isolates named Karachi/928/1994 and Karachi/874/1992 in the present study did not belong to any the genetic clusters and presented a novel sapovirus GII genotype tentatively called GII/4. The sequences with the closest matches to these isolates were from strain isolated in France (Lyon/598/97), showing only 73% amino acid identity.

Sapovirus is one of the leading causes of infantile viral gastroenteritis and also is associated with sporadic outbreaks of gastroenteritis worldwide. In this study, sapovirus was detected in 3.2% fecal specimens tested. These findings suggested that acute gastroenteritis in infants and children in Karachi, Pakistan about 3.2% might be due to sapovirus and 96.8% caused by other etiologic agents. Among all children with acute gastroenteritis due to sapovirus, 94.1% were aged less than 36 months. This result was consistent with previously published reports on epidemiology of sapovirus worldwide in which the prevalence was shown to be 0.3–9.3% [5, 9, 16–18]. It also confirmed sapovirus as one of the enteropathogens responsible for viral gastroenteritis among infants and children worldwide. Furthermore, the highest incidence was the 6–11 month old group, the lowest fell into the infants aged less than 6 months, and the rate of incidence decreased with increasing age over 1 year. Quite possibly, children aged from 6 to 11 months might lack antibody protection to sapovirus, whereas by the time children have reached 1 year old they have begun to acquire viral immunity.

The climate in Karachi city, Pakistan is distinctively seasonal. The summer lasts from April to July, and the hot temperature may reach over 37 °C. The cold season characterized by less rain begins in August and ends in March. The coldest month is January when the temperature may dip as low as 5 °C. In the present study, almost sapoviruses were found in August, September and October. Our results were in line with previous reports of other investigators that sapovirus was observed to prevail in the cold winter months [9, 12, 13, 18].

Up to date, numerous molecular epidemiological studies have revealed a global distribution of sapovirus. However, the genetic analysis on sapovirus in Pakistani children with acute gastroenteritis is not available. The results in this study showed

the majority of Pakistani sequences belonged to two distinct sapovirus genogroups I and II. Of these, the positives were further classified into one sapovirus GI genotype and one sapovirus GII genotype according to the recent sapovirus capsid region classification scheme of Farkas et al. (2004). Moreover, other eight sapoviruses did not cluster with any the published references and presented four novel sapovirus genotypes tentatively called GI/4, GI/5, GI/6, GI/7 and GII/4, respectively. These sapoviruses had a 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. It was noteworthy that sapovirus could be classified into 7 GI and 4 GII genotypes.

Another interesting feature was a high identity at the nucleotide and the amino acid between Karachi/730/1992 and only one representative of a genogroup IV, Hou7-1181/90. Both of them, which revealed unique sequences distinct from known sapoviruses in the DDBJ, were recovered from fecal specimens over 10 years ago. Possibly, these special strains might be emerging in the past. Taken together, our findings clearly indicated that sapovirus strains co-circulating among infants and children with acute gastroenteritis in Karachi city, Pakistan demonstrated the great genetic diversity. Additionally, these data have described the molecular epidemiology as well as the importance of sapovirus causing acute gastroenteritis in Pakistan and increased the evidence for the worldwide distribution of this virus. This is the first indication on molecular epidemiology of sapovirus infection conducted in Karachi city, Pakistan, showing the genetic diversity among them.

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References

1. Chiba S, Sakuma Y, Kogasaka R, Akihara M, Horino K, Nakao T, Fukui S (1979) An outbreak of gastroenteritis associated with calicivirus in an infant home. J Med Virol 4: 249–254
2. Chiba S, Nakata S, Numata-Kinoshita K, Honma S (2000) Sapporo virus: history and recent findings. J Infect Dis 181: 303–308
3. Farkas T, Zhong WM, Jing Y, Huang PW, Espinosa SM, Martinez N, Morrow AL, Ruiz-Palacios GM, Pickering LK, Jiang X (2004) Genetic diversity among sapoviruses. Arch Virol 149: 1309–1323
4. Guo M, Chang KO, Hardy ME, Zhang Q, Parwani AV, Saif LJ (1999) Molecular characterization of a porcine enteric calicivirus genetically related to Sapporo-like human caliciviruses. J Virol 73: 9625–9631
5. Kirkwood CD, Bishop RF (2001) Molecular detection of human calicivirus in young children hospitalized with acute gastroenteritis in Melbourne, Australia, during 1999. J Clin Microbiol 39: 2722–2774

6. Nakata S, Chiba S, Terashima H, Nakao T (1985) Prevalence of antibody to human calicivirus in Japan and Southeast Asia determined by radioimmunoassay. J Clin Microbiol 22: 519–521
7. Nakata S, Estes MK, Chiba S (1988) Detection of human calicivirus antigen and antibody by enzyme-linked immunosorbent assays. J Clin Microbiol 26: 2001–2005
8. Nishio O, Matsui K, Oka T, Ushijima H, Mubina A, Dure-Samin A, Isomura S (2000) Rotavirus infection among infants with diarrhea in Pakistan. Pediatr Int 42: 425–427
9. Okada M, Shinozaki K, Ogawa T, Kaiho I (2002) Molecular epidemiology and phylogenetic analysis of Sapporo-like viruses. Arch Virol 147: 1445–1451
10. Pang XL, Joensuu J, Vesikari T (1999) Human calicivirus associated sporadic gastroenteritis in Finnish children less than two years of age followed prospectively during a rotavirus vaccine trial. Pediatr Infect Dis J 18: 420–426
11. Pang XL, Honma S, Nakata S, Vesikari T (2000) Human caliciviruses in acute gastroenteritis of young children in the community. J Infect Dis 181: 288–294
12. Phan TG, Okame M, Tuan AN, Maneekarn N, Nishio O, Okitsu S, Ushijima H (2004) Human astrovirus, norovirus (GI, GII), and sapovirus infections in Pakistani children with diarrhea. J Med Virol 73: 256–261
13. Sakai Y, Nakata S, Honma S, Tatsumi M, Numata-Kinoshita K, Chiba S (2001) Clinical severity of Norwalk virus and Sapporo virus gastroenteritis in children in Hokkaido, Japan. Pediatr Infect Dis J 20: 849–853
14. Sakuma Y, Chiba S, Kogasaka R, Terashima H, Nakamura S, Horino K, Nakao T (1981) Prevalence of antibody to human calicivirus in general population of northern Japan. J Med Virol 7: 221–225
15. Schuffenecker I, Ando T, Thouvenot D, Lina B, Aymard M (2001) Genetic classification of "Sapporo-like viruses". Arch Virol 146: 2115–2132
16. Suzuki H, Konno T, Kutsuzawa T, Imai A, Tazawa F, Ishida N, Katsushima N, Sakamoto M (1979) The occurrence of calicivirus in infants with acute gastroenteritis. J Med Virol 4: 321–326
17. Vinje J, Deijl H, van der Heide R, Lewis D, Hedlund KO, Svensson L, Koopmans MP (2000) Molecular detection and epidemiology of Sapporo-like viruses. J Clin Microbiol 38: 530–536
18. Wolfaardt M, Taylor MB, Booysen HF, Engelbrecht L, Grabow WO, Jiang X (1997) Incidence of human calicivirus and rotavirus infection in patients with gastroenteritis in South Africa. J Med Virol 51: 290–296
19. Yan H, Yagyu F, Okitsu S, Nishio O, Ushijima H (2003) Detection of norovirus (GI, GII), sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR. J Virol Methods 14: 37–44

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Proteolytic Processing of Sapovirus ORF1 Polyprotein

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The genome of *Sapovirus* (SaV), a causative agent of gastroenteritis in humans and swine, contains either two or three open reading frames (ORFs). Functional motifs characteristic to the 2C-like NTPase (NTPase), VPg, 3C-like protease (Pro), 3D-like RNA-dependent RNA polymerase (Pol), and capsid protein (VP1) are encoded in the ORF1 polyprotein, which is afterwards cleaved into the nonstructural and structural proteins. We recently determined the complete genome sequence of a novel human SaV strain, Mc10, which has two ORFs. To investigate the proteolytic cleavage of SaV ORF1 and the function of protease on the cleavage, both full-length and truncated forms of the ORF1 polyprotein either with or without mutation in ^{1171}Cys to Ala of the GDCG motif were expressed in an in vitro coupled transcription-translation system. The translation products were analyzed directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis or by immunoprecipitation with region-specific antibodies. The ORF1 polyprotein was processed into at least 10 major proteins: p11, p28, p35, p32, p14, p70, p60, p66, p46, and p120. Seven of these products were arranged in the following order: NH_2-p11–p28–p35(NTPase)–p32–p14(VPg)–p70(Pro-Pol)–p60(VP1)-COOH. p66, p46 and p120 were precursors of p28-p35 (NTPase), p32-p14 (VPg), and p32-p14 (VPg)-p70 (Pro-Pol), respectively. Mutagenesis in the 3C-like protease motif fully abolished the proteolytic activity. The cleavage map of SaV ORF1 is similar to those of other heretofore known members of the family *Caliciviridae*, especially to rabbit hemorrhagic disease virus, a member of the genus *Lagovirus*.

The family *Caliciviridae* contains four genera, *Lagovirus*, *Vesivirus*, *Norovirus* (NoV; formerly known as "Norwalk-like virus"), and *Sapovirus* (SaV, formerly known as "Sapporo-like virus"), in which rabbit hemorrhagic disease virus (RHDV), feline calicivirus (FCV), Norwalk virus, and Sapporo virus are assigned as the prototype strains (8, 19). SaV is associated with gastroenteritis in humans and swine (5, 9). Human SaV is predominantly isolated from infants and young children, though it is occasionally associated with outbreaks of gastroenteritis (10, 21, 22, 33). Phylogenetic analysis using SaV capsid protein VP1 revealed five genetic groups, genogroup I (GI) to GV (7). The human SaV are classified into GI, GII, GIV, and GV, whereas porcine SaV belongs to GIII (7, 24, 26). Although porcine SaV can grow in cultured cells (4, 9), neither cell culture nor animal models can support the replication of human SaV.

The SaV genome is a positive-sense, single-strand RNA molecule of approximately 7.5 kb that is polyadenylated at its 3′ terminus. The SaV GI, GIV, and GV genomes are predicted to contain three main open reading frames (ORFs), whereas SaV GII and GIII have two ORFs (9, 16, 22, 23, 25). The SaV ORF1 encodes nonstructural proteins and the capsid protein VP1, while ORF2 and ORF3 encode proteins of yet unknown function. To date, seven complete genome sequences (accession numbers in parentheses) of SaV, GI Manchester (X86560), GI Dresden (AY694184), GII Bristol (AJ249939), and GII Mc10 (AY237420), GII Sakai C12 (AY603425), GIII PEC/Cowden (AF182760), and PEC/LL14 (AY425671) have been published.

The SaV ORF1 polyprotein contains amino acid motifs characteristic of caliciviruses (8, 36), including 2C-like NTPase (NTPase) (GXXGXGKS/T), VPg [KGK(N/T)K and (D/E)EY (D/E)E], 3C-like protease (Pro)(GDCG), 3D-like RNA-dependent RNA polymerase (Pol)(GLPSG and YGDD), and VP1 (PPG) (9, 28). The proteolytic processing of ORF1 by the virus-encoded protease has been reported in RHDV, FCV, and NoV, and detailed cleavage maps of ORF1 have been reported in these viruses (1, 20, 31, 36). A recent study with a full-length RNA transcript derived from the SaV GI Manchester strain indicated six major cleavage products in vitro (6), suggesting that SaV ORF1 is also cleaved into nonstructural and structural proteins by the virus-encoded protease. However, neither the cleavage map of the viral proteins nor the function of the virus-encoded protease involved in this cleavage has been elucidated yet.

In this study, the proteolytic processing of the ORF1 polyprotein of SaV Mc10, a novel human SaV GII strain, was analyzed by using an in vitro coupled transcription-translation system. We also evaluate the function of the virus-coded 3C-like protease on this proteolytic processing.

MATERIALS AND METHODS

Specimen. The Mc10 strain (Hu/SaV/Mc10/2000/Thailand) was isolated in an epidemiological screening of acute gastroenteritis patients in Thailand in June 2000 (10). The RNA extraction, reverse transcription-PCR, and complete nucleotide sequence analysis of the virus genome were performed as previously described (12).

Construction of plasmids containing the full-length genome. A plasmid harboring the entire Mc10 genome was constructed with two overlapping cDNA fragments utilizing a unique HindIII site at nucleotides (nt) 3599 to 3604. The 5′ fragment corresponding to nt 1 to 3735 was amplified with a sense primer

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(5′-GCGGGATCCTAATACGACTCACTATAGGtgattggttagtatggcttccaagccattc tacccaatag-3′) including a BamHI site (underlined) and a T7 RNA polymerase promoter sequence (bold) and an antisense primer (5′-TTGGGCCATGCAGG TGAGCG-3′). The 3′ fragment corresponding to nt 2188 to 7458 was amplified with the sense primer (5′-TCCACCTCCCACATACAGTG-3′) and antisense primer (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGAGCTC AGATCTT$_{20}$ccaagaaagcacggctgc-3′) including a poly(A) tail, a BglII site (underlined), and a SacI site (double underlined). The 5′ fragments were digested with BamHI and HindIII, and the 3′ fragments were digested with HindIII and SacI (New England Biolabs, Beverly, MA). pUC19 vector (Toyobo, Tokyo) was digested with BamHI and SacI (New England Biolabs). These three fragments were purified and ligated to create a plasmid containing a full-length Mc10 genome sequence with the T7 promoter, which was designated as pUC19/SaV Mc10 full-length. The 3′ end of this clone contained a poly(A) tract flanking a unique BglII site which was used to linearize the plasmid for runoff transcription. The complete nucleotide sequence of the insert was determined to confirm the original sequence.

Preparation of region-specific antibodies. A panel of antibodies specific to the ORF1 polyprotein was prepared. DNA fragments corresponding to regions A to J (Fig. 1A) were amplified with pUC19/SaV Mc10 full-length as a template by using the primers listed in Table 1. These regions were first cloned into Gateway pDONR201 vector (Invitrogen, Carlsbad, CA) and then transferred to Gateway pDEST 17 vector (Invitrogen) according to the manufacturer's protocol. All plasmids were verified by sequence analysis. *Escherichia coli* BL21-AI (Invitrogen) was transformed with plasmids A to J and incubated at 37°C in Luria broth (Invitrogen) containing 50 μg/ml of ampicillin until the optical density at 600 nm reached 0.6 to 0.8. The expression was induced in a final concentration of 0.2% (wt/vol) arabinose followed by incubation at 37°C for 3 h. The cultured *E. coli* cells were centrifuged at 10,000 × *g* for 10 min at 4°C and resuspended in equilibration/wash buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7; BD Clontech, Palo Alto, CA) supplemented with 8 M urea. The cell suspension was frozen at −80°C, thawed once, rotated at room temperature for 2 h, and centrifuged at 10,000 × *g* for 10 min at room temperature. The His_6-tagged recombinant protein in the supernatant was absorbed with TALON resin (BD Clontech) and eluted with the elution buffer (50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole, pH 7; BD Clontech) supplemented with 8 M urea. The purified recombinant proteins were dialyzed against phosphate-buffered saline (PBS) containing 4 M urea (pH 7.4). The protein concentration was measured with a protein assay kit (Bio-Rad, Hercules, CA). Five hundred micrograms of each recombinant protein was used to subcutaneously immunize New Zealand White rabbits as described previously (13). The immunoglobulin G (IgG) fraction was purified from serum with rProtein A Sepharose (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions and dialyzed against PBS (pH 7.4). The specificity of the purified IgG fraction was examined by Western blot analysis using the respective recombinant protein.

Site-directed mutagenesis of the full-length cDNA clone. Site-directed mutagenesis was performed with pUC19/SaV Mc10 full-length as a template, using the GeneTailor site-directed mutagenesis system (Invitrogen) according to the manufacturer's instructions. The sense primer, 5′-CCAACAAAGCGTGGGGA CGCGGGCACACCC-3′, contains nucleotide changes (underlined) converting TGT (^{1171}Cys) to GCG (^{1171}Ala) in the GDCG motif. As the antisense primer, 5′-GTCCCCACGCTTTGTTGGGTATCCATTTATGATGCG-3′ was prepared. The nucleotide sequence of the full-length mutant clone was confirmed and designated as pUC19/SaV Mc10 full-C1171A/ORF1.

In vitro transcription-translation assay. In vitro protein synthesis was performed using TNT T7 Quick for PCR DNA (Promega, Madison, WI). The primers used to generate the templates for the T7 polymerase coupled transcription-translation assay in rabbit reticulocytes are represented in Table 2, and the translation products are depicted in Fig. 1B. PCR was performed with 500 ng of pUC19/SaV Mc10 full-length, or pUC19/SaV Mc10 full-C1171A/ORF1 in 100 μl of the reaction mixture containing 40 pmol of each primer, KOD polymerase buffer, 0.2 mM each deoxynucleoside triphosphate (dNTP), 1 mM $MgSO_4$, and 2 units of KOD-Plus-DNA polymerase (Toyobo). After initial denaturation at 94°C for 5 min, 25 cycles consisting of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and primer extension at 72°C for the appropriate period were performed, followed by a final extension at 72°C for 15 min. Five microliters of the PCR mixture was added to 50 μl of the TNT reaction mixture containing 40 μl of TNT master mixture (Promega) and 40 μCi of Redivue Pro-mix L-^{35}S in vitro cell labeling mix (Amersham Biosciences). The TNT reaction was performed at 30°C for 1.5 h, and 2 μl of the solution was mixed with 20 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris-HCl [pH 6.8], 5% [wt/vol] sucrose, 2% [wt/vol] SDS, 0.002% [wt/vol] bromophenol blue with 5% [vol/vol] 2-mercaptoethanol), heated at 95°C for 5 min, and loaded onto 5 to 20% Tris-Gly polyacrylamide gel (D.R.C., Tokyo). The proteins in the gel were blotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) using a semidry electroblotting apparatus. The radiolabeled proteins were detected by a Bioimage Analyzer BAS 2500 (Fuji Film, Tokyo).

Immunoprecipitation. For radioimmunoprecipitation, 10 μl of the TNT translation reaction mixture was diluted with 80 μl of radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP40, 1 mM EDTA; Upstate Biotechnology Inc., Lake Placid, NY) and incubated with 5 μg of region-specific antibodies. After incubation for 1 h on ice, 25 μl of a suspension of protein A magnetic beads (New England Biolabs) and 900 μl of RIPA buffer were added. The mixture was gently rotated at 4°C for 1 h and then washed three times with 1 ml of RIPA lysis buffer. The immunoprecipitated proteins were resuspended in 20 μl of SDS-PAGE sample buffer and heated at 95°C for 5 min prior to analysis with 5 to 20% Tris-Gly polyacrylamide gel. The proteins were blotted onto an Immobilon-P polyvinylidene difluoride membrane. Immunoprecipitated radioactive proteins were detected with a Bioimage Analyzer BAS 2500 (Fuji Film).

Nucleotide sequence analysis. Nucleotide sequence analysis was performed with the Big Dye Terminator version 3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems, Tokyo) and an automated sequencer, the 3100 Avanti genetic analyzer (Applied Biosystems). Nucleotide sequences were assembled with Sequencher version 4.1 (Gene Codes Corporation, Ann Arbor, MI). Nucleotide and amino acid sequences were analyzed with GENETYX Mac version 11.2.2 (Genetyx Corporation, Tokyo).

Nucleotide sequence accession number. The GenBank accession number of the SaV Mc10 genome sequence is AY237420.

RESULTS AND DISCUSSION

Functional motifs in Mc10 ORF1. The complete nucleotide sequence analysis demonstrated that the Mc10 genome consists of 7,458 nt, excluding the 3′ poly(A) tail, and has two ORFs. ORF1 consists of 6,837 nucleotides (nt 14 to 6850) and encodes a protein of 2,278 amino acids (aa) with an estimated molecular mass of 250 kDa. ORF2 consists of 501 nucleotides (nt 6850 to 7350) and encodes a protein of 167 aa that overlaps the 3′ end of ORF1 by 1 nt. An amino acid homology search revealed that the Mc10 ORF1 polyprotein contains conserved sequence motifs characteristic of calicivirus, which include NTPase (^{481}GPPGIGKT^{488}), VPg (^{942}KGKTK^{946} and ^{964}DEYDE^{968}), Pro (^{1169}GDCG^{1172}), Pol (^{1504}GLPSG^{1508} and ^{1552}YGDD^{1555}), and VP1 (^{1856}PPG^{1858}) (Fig. 1A). These motifs are also found in other SaV strains and allowed us to predict several cleavage sites of putative mature nonstructural and structural proteins, which in turn served as templates to generate region-specific antibodies.

Generation of region-specific antibodies. To generate a panel of antibodies specific to the ORF1 polyprotein, 10 protein fragments (Fig. 1A, proteins A to J) were expressed in *E. coli* BL21-AI cells as N-terminal His_6-tagged fusion proteins, as described in the Materials and Methods. We selected these regions based on the characteristic amino acid motifs and multiple alignments of the available SaV ORF1 aa. Because the residues Q (or E)/G, A, S, T, D, and N have been identified as the cleavage sites in other caliciviruses (1, 20, 31), the boundaries of some of these regions were selected between the highly conserved dipeptides among SaV strains (data not shown). SDS-PAGE analysis of the arabinose-induced proteins in *E. coli* demonstrated that all of these proteins were efficiently expressed with the expected sizes, except for region I. Inefficient expression of a corresponding region in *E. coli* was reported in RHDV and FCV (31, 36), and a common highly hydrophobic domain observed in these viruses may be the reason. Most of the proteins were expressed as insoluble forms,

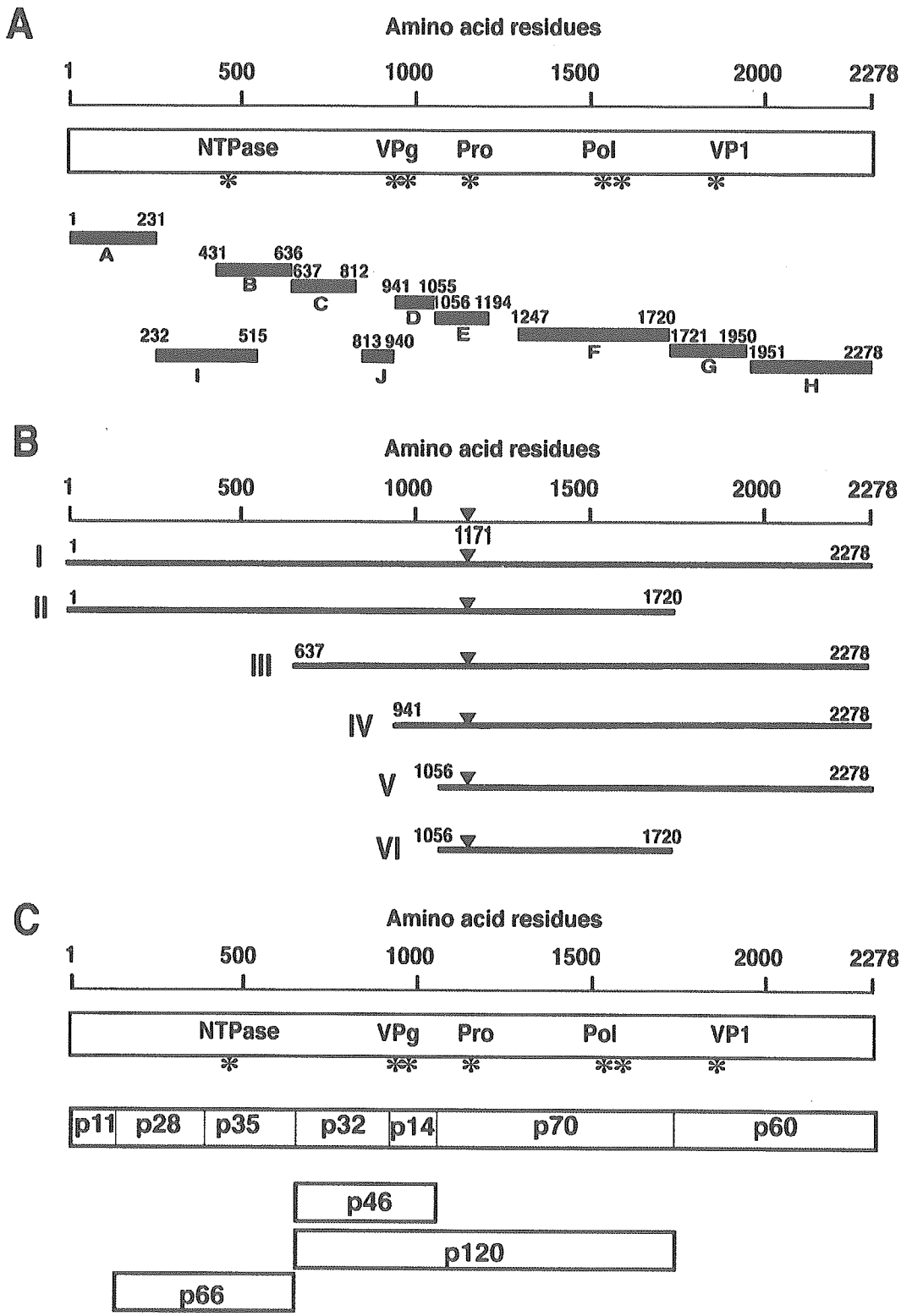

FIG. 1. (A) Genome organization and expression of Mc10 ORF1 proteins. The motifs of 2C-like NTPase (^{481}GPPGIGKT^{488}), VPg (^{942}KGKTK^{946} and ^{964}DEYDE^{968}), Pro (^{1169}GDCG^{1172}), Pol (^{1504}GLPSG^{1508} and ^{1552}YGDD^{1555}), and VP1 (^{1856}PPG^{1858}) are indicated by asterisks below the ORF1 polyprotein. The regions A to J were expressed in *E. coli*. (B) Mc10 ORF1 proteins expressed in vitro with a coupled transcription-translation system. The template DNAs were generated by PCR using primers listed in Table 2, and proteins I to VI were synthesized in vitro. Triangles indicate the position in the putative 3C-like protease where a ^{1171}Cys-to-^{1171}Ala change was introduced. Both wild-type (Pro^{w}) and mutant (Pro^{mut}) forms of the constructs were used for the expression. (C) A proposed genetic map of Mc10 ORF1. The motifs of NTPase, VPg, Pro, Pol, and VP1 are indicated by asterisks below the ORF1 polyprotein. The identified products were p11, p28, p35, p32, p14, p70, p60, p66, p46, and p120.

TABLE 1. PCR primers to construct bacterial expression plasmids

Region	Sequence (5′→3′)[a]	Nucleotide positions	Amino acid positions
A	ATGGCTTCCAAGCCATTCTACCCAATAG TTCACAAAAGTGGACCAGAACCTGTCTAAC	14–706	1–231
B	GGTACTGAGTTGATACAGGAATTTGGTACATC CTCGGGGTTGTACCCCAAGAACGGTCTAACAG	1304–1921	431–636
C	GGGGACACCTTGGATGGTGTCCGAGTGAAACCTG ATGGTGCCTGAGGCCTCGGATGAAGTCAAATGC	1922–2449	637–812
D	GCCAAAGGAAAGACCAAGCATGGCCGTG TTCTTCATCATAACCTTGATTACGGG	2834–3178	941–1055
E	GCTCCCACACCAATTGTTACATTCACATCTG TTCTCCATTTGTTGATGTGGCTGCGTGCAGTCCG	3179–3595	1056–1194
F	ACACACGCCCCTGCGCCGTTTGGTTCCGGTGATG TTCAAACACTAATTTGGTGGTCTCTTCACTG	3752–5173	1247–1720
G	ATGGAGGGCCTAGGCCAACCACAGCCACAG CTGCTGCTCTGGGGCCTTCAACAGACAAAAG	5174–5863	1721–1950
H	ATGGACAATGGAATTTCGCCCGCCTCTTTGTTG TTATTCTAAGAACCTAACGGCCCGGCCACTG	5864–6850	1951–2278
I	GGGATGAGGTCGCAGTTTTCCACCAATGC GGTGTAAGAGTCGTGGTGGTCCAACGTGACG	707–1558	232–515
J	CTTGGTCTGTGTTCAGTCAAAGGCATGTGGAGAG CTCCTCCTCCCGACCAGACCGGGTGGCGAGGTATG	2450–2833	813–940

[a] The top line indicates a forward primer that includes attB1 sequence (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTC-3′) at the 5′ end. The bottom line indicates a reverse primer that includes attB2 sequence (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTC**CTA**-3′) with stop codon (bold) at the 5′ end.

and they were solubilized with 8 M urea. Under this condition, the proteins A to H, but not J, were efficiently extracted and purified by affinity chromatography using His_6 tag.

We expressed eight viral proteins, A to H (Fig. 1A), which correspond to 83% of the ORF1 polyprotein, and region-specific antibodies were prepared.

Proteolytic processing of ORF1 polyprotein in vitro. Proteolytic processing of Mc10 ORF1 was investigated with an in vitro translation using a rabbit reticulocyte lysate. In order to produce a full-length ORF1 fragment, an experiment using runoff RNA products from the pUC19/SaV Mc10 full-length clone was originally performed. However, this was unsuccessful due to the low translation level (data not shown). Therefore, a DNA fragment containing the entire ORF1 was amplified by PCR with a set of primers, which included a T7 promoter sequence in the sense primer (Table 2). The ORF1 polyprotein was then expressed by using an in vitro coupled transcription-translation system in the presence of ^{35}S-labeled methionine and cysteine (Fig. 1B, construct I). SDS-PAGE of the radiolabeled proteins followed by image analysis showed that at least seven proteins, p28, p32, p35, p46, p60, p66, and p120, were generated, demonstrating that ORF1 polyprotein "I-Pro^{w}" was translated and proteolytically cleaved (Fig. 2A, lane I-Pro^{w}). A time course analysis revealed that there was no accumulation of the 250-kDa primary translation product even at the early stage of the incubation, indicating that the processing of ORF1 occurred cotranslationally or rapidly after translation, as observed in RHDV, FCV, and NoV (1, 2, 11, 15, 20, 32).

To determine whether the cleavage is dependent on the virus-encoded 3C-like protease, a DNA fragment containing a C1171A mutation was prepared and used for an in vitro transcription-translation. As shown in the lane I-Pro^{mut} (Fig. 2A), only a single major band of 250 kDa was observed. Thus, the viral protease is responsible for the processing, and the Cys residue in the GDCG motif is critical for the proteolytic activity, as seen in other caliciviruses (1–3, 15, 27, 32).

TABLE 2. PCR primers to prepare templates for in vitro transcription-translation

Fragment[a]	Sequence (5′→3′)[b]	Nucleotide positions	Amino acid positions
I	GCTTCCAAGCCATTCTACCCAATAGAG TTCTAAGAACCTAACGGCCCGG	17–6847	1–2278
II	GCTTCCAAGCCATTCTACCCAATAGAG TTCAAACACTAATTTGGTGGTCTC	17–5173	1–1720
III	GGGGACACCTTGGATGGTGTCCGAGTG TTCTAAGAACCTAACGGCCCGG	1922–6847	637–2278
IV	GCCAAAGGAAAGACCAAGCATGGC TTCTAAGAACCTAACGGCCCGG	2834–6847	941–2278
V	GCTCCCACACCAATTGTTAC TTCTAAGAACCTAACGGCCCGG	3179–6847	1056–2278
VI	GCTCCCACACCAATTGTTAC TTCAAACACTAATTTGGTGGTCTC	3179–5173	1056–1720

[a] The AUG codon is derived from the original sequence, whereas those of fragments II to V were provided by forward primers.

[b] The top line indicates a forward primer that includes sequence (5′-GGATCCTAATACGACTCACTATAGGGAACAGCCACC**ATG**-3′) at the 5′ end, with the T7 promoter (underlined) and an additional start codon (bold). The bottom line indicates a reverse primer (5′-T_{30} **TTA**-3′) that includes poly(A) with a stop codon (bold) at the 5′ end.

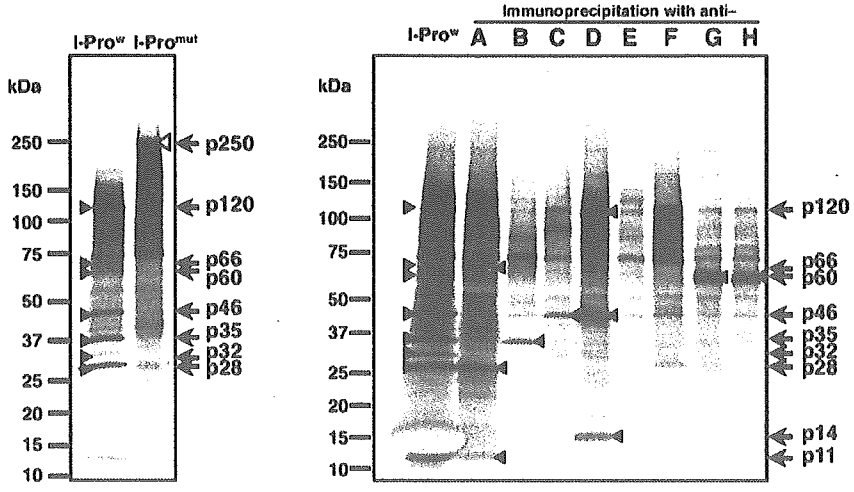

FIG. 2. The processing products of Mc10 ORF1 polyprotein. (A) SDS-PAGE of in vitro ^{35}S-labeled proteins derived from I-Pro^{w} and I-Pro^{mut}. The protein bands specific to either the wild-type or mutant form are indicated by triangles or an open triangle, respectively. The molecular sizes of viral proteins are shown on the right. (B) Immunoprecipitation with region-specific antibodies. In vitro ^{35}S-labeled proteins derived from I-Pro^{w} were immunoprecipitated with anti-A to -H antibodies and analyzed by SDS-PAGE. The proteins of interest are indicated by arrows.

Immunoprecipitation with region-specific antibodies. To identify the cleavage products, an in vitro reaction mixture of I-Pro^{w} protein was subjected to the immunoprecipitation with region-specific antibodies (anti-A to -H antibodies). As shown in Fig. 2B, lane A, three major proteins of 11, 28, and 66 kDa were precipitated with anti-A antibody (Fig. 2B, lane A), whereas p11 was not identified as a specific cleavage product in the direct SDS-PAGE analysis due to an abundance of cellular proteins (Fig. 2A and 2B, lane I-Pro^{w}). Anti-B antibody precipitated 35-kDa proteins (Fig. 2B, lane B). Anti-C antibody precipitated 46-kDa proteins (Fig. 2B, lane C). Three major proteins of 14, 46, and 120 kDa were precipitated with anti-D antibody (Fig. 2B, lane D), whereas p14 was not detectable in the direct SDS-PAGE analysis due to an abundance of cellular proteins (Fig. 2B, lane I-Pro^{w}). Neither anti-E nor -F antibodies precipitated a specific cleavage product (Fig. 2B, lanes E and F), although these antibodies reacted with I-Pro^{mut} and with proteins E and F expressed in *E. coli* (data not shown). Both anti-G and -H antibodies precipitated 60-kDa proteins (Fig. 2B, lanes G and H).

The above results indicated the following: (i) p11, p28, p35, and p66 were products derived from the N-terminal region of the ORF1; (ii) p14 and p46 were products derived from the central region of the ORF1; (iii) p46 was either unprocessed or stable p14-p32, since this protein was precipitated with both anti-C and -D antibodies; and (iv) p60 was a product derived from the C-terminal region of ORF1, since this protein was precipitated with both anti-G and -H antibodies. The cleavage products of ORF1, except p32 and p120, were thus identified by the region-specific antibodies. Furthermore, two additional products, p11 and p14, were found which were not detected by direct SDS-PAGE analysis.

Proteolytic processing of N- and C-terminally truncated translation products. To further analyze the cleavage products, five truncated templates—one C-terminally truncated, three N-terminally truncated, and one both C- and N-terminally truncated—were generated and expressed by an in vitro coupled transcription-translation system (Fig. 1B, II to VI). The expressed regions were selected according to the cleavage products sizes. We used identical boundaries to the regions to prepare the region-specific antibodies. The expression was carried out with both wild-type (designated as Pro^{w}) and C1171A mutant (designated as Pro^{mut}) templates, and the ^{35}S-labeled products were analyzed by direct SDS-PAGE (Fig. 3) and immunoprecipitation with region-specific antibodies (Fig. 4). When mutant proteins I- to VI-Pro^{mut} were expressed, only single major protein bands of p250, p190, p180, p145, p130, or p70, which corresponded to their respective estimate sizes, were observed (Fig. 3, I- to VI-Pro^{mut}). Similarly, VI-Pro^{w} was not further processed (Fig. 3, VI-Pro^{w}). In contrast, I- to V-Pro^{w} were extensively processed and the following cleavage patterns were obtained. (i) II-Pro^{w} was processed into p11, p28, p35, p32, p14, p66, p46, and p120, which were identical to I-Pro^{w}, except for p60, confirming that p60 is located in the C-terminal ORF1 (Fig. 3, II-Pro^{w}; Fig. 4, II Pro^{w}). (ii) III-Pro^{w} was cleaved into p32, p14, p70, p46, p120, and p60 (Fig. 3, III-Pro^{w}; Fig. 4, III-Pro^{w}). (iii) IV-Pro^{w} was cleaved into p14, p70, p84, and p60 (Fig. 3, IV-Pro^{w}; Fig. 4, IV-Pro^{w}); and (iv) V-Pro^{w} was processed into p70 and p60 (Fig. 3, V-Pro^{w}). p66 was identified as an unprocessed or stable p28-p35, since this protein was detected in additional N-terminally truncated Pro^{w} forms II′ (corresponding to aa 70 to 1720) but not in II″ (corresponding to aa 326 to 1720), and since the p66 that appeared in the cleavage products of Pro^{w} forms II′ could be precipitated with anti-A antibody (data not shown).

Since p32 and p120 were found in I- to III-Pro^{w} but not in IV- or V-Pro^{w}, p32 and p120 are derived from the central region of ORF1 (Fig. 3, I- to V-Pro^{w}). Although p70 was difficult to detect in the I- and II-Pro^{w} processed products due to the nonspecific or the inappropriate internal translation protein(s) seen in I- and II-Pro^{mut} (Fig. 3, I- and II-Pro^{mut}), this protein appeared in the cleavage products of III-, IV-, and V- Pro^{w} (Fig. 3, III-, IV-, and V-Pro^{w}), and was identical in size to the VI-Pro^{w} and VI-Pro^{mut} products (Fig. 3, VI-Pro^{w} and

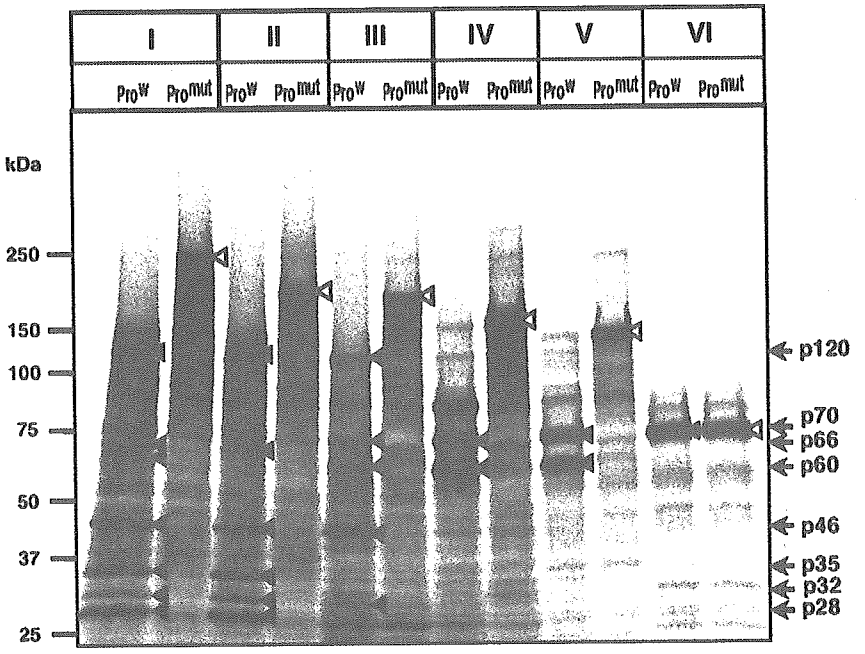

FIG. 3. SDS-PAGE of in vitro ^{35}S-labeled proteins derived from I- to VI-Pro^{w} and I- to VI-Pro^{mut}. The proteins derived from the wild-type form are indicated by triangles, and those from the mutant form are indicated by open triangles.

VI-Pro^{mut}). p70 in III- to VI-Pro^{w} and VI-Pro^{mut} was immunoprecipitated with both anti-E and -F antibodies (Fig. 4, III- to VI-Pro^{w}, and VI-Pro^{mut}). Although the identification of p70 in I- and II-Pro^{w} by immunoprecipitation was not clear (Fig. 4, I- and II-Pro^{mut}), we concluded that p70 was the cleavage product derived from the central region of ORF1. In addition, we observed p84 and assigned it to p14-p70 based on its molecular size and immunoreactivity to anti-D, -E, and -F antibodies. We did not further analyze this product in this study, because this protein was detected only in IV-Pro^{w} and is likely to be a construct-dependent unprocessed or stable product.

Cleavage map of SaV ORF1. The ORF1 cleavage map was generated using full-length and/or truncated forms of the ORF1 polyprotein. We concluded that Mc10 ORF1 polyprotein was processed into at least 10 major proteins—p11, p28, p35, p32, p66, p14, p46, p70, p120 and p60—by an in vitro coupled transcription-translation system. Seven of these products were arranged in the order NH_2-p11–p28–p35–p32–p14–p70–p60-COOH, and the amino acid motifs indicated that p35, p14, p70, and p60 correspond to NTPase, VPg, Pro-Pol, and VP1, respectively (Fig. 1C). p66, p46, and p120 were identified as p28-p35 (NTPase), p32-p14 (VPg), and p32-p14 (VPg)-p70 (Pro-Pol).

The cleavage map and product sizes are strikingly similar to those of RHDV, except in the case of Pro-Pol, which is processed into Pro and Pol, and the RHDV polyprotein is ar-

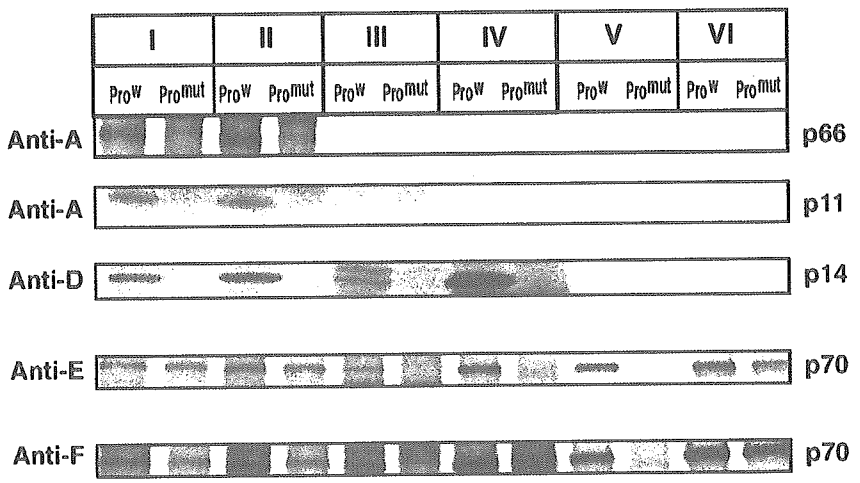

FIG. 4. Identification of p66, p11, p14, and p70. In vitro ^{35}S-labeled proteins expressed with I- to VI-Pro^{w} and I- to VI-Pro^{mut} were immunoprecipitated with anti-A, anti-D, anti-E, or anti-F antibodies.

ranged in the order NH_2-p16–p23–p37 (NTPase)–p29–p13 (VPg)–p15 (Pro)–p58 (Pol)–p60 (VP1)-COOH (36).

Although p70 (Pro-Pol) was not further cleaved under the conditions used in this study, it should be noted that Pro-Pol has also been identified as a stable product in RHDV, FCV, and NoV in in vitro translation systems (1, 18, 32, 36). Extension of the incubation time from 1.5 h to 24 h or the presence of a canine pancreatic microsomal membrane fraction had no effect (data not shown). Further cleavage of Pro-Pol to Pro and Pol has been shown in RHDV and NoV, but not in FCV, when the Pro-Pol-containing region was expressed in mammalian cells (14, 20, 27, 31, 32), and a similar event was observed in RHDV, FCV, and NoV in *E. coli* (17, 29, 30, 32, 34, 35). These observations indicated that the cleavage of Pro-Pol to Pro and Pol is dependent on the expression system.

In this study, SaV ORF1 cleavage was investigated for the first time with full-length and N- and C-terminally truncated constructs of the ORF1 polyprotein either with or without mutation in ^{1171}Cys to Ala of the GDCG motif derived from the GII Mc10 strain. After the in vitro transcription-translation reaction, the cleavage products were analyzed with region-specific antibodies against a panel of viral protein fragments. When both the N- and C-terminally truncated ORF1 constructs were expressed, the cleavage products of the expected size were generated from each truncated form of ORF1, indicating that our prediction of the boundaries of each product was almost accurate except for the N-terminal region. Our preliminary data showed that SaV Pro-containing region expressed in *E. coli* has cleavage activity between Q/G within the rhinovirus 3C-like protease recognition sequence as reported in Chiba virus (29; Oka et al. unpublished observation). This result supports the possibility that SaV protease has a similar dipeptide recognition pattern (cleaves after Q or E) to those of other caliciviral proteases. At the beginning of our study, four SaV full-length genome sequences including Mc10 were available, but now, seven strains of SaV full genome sequences which belong to GI, GII, and GIII, respectively, are available. Multiple alignments of the available SaV ORF1 amino acid sequence revealed the presence of the conserved dipeptide-QG, -QA, -EG, and -EA, which are likely used as cleavage sites. From these data, we speculated that selected sites, E^{940}/A^{941} and E^{1055}/A^{1056} in the central region, are likely the cleavage sites, but in the case of N- and C-terminal regions, several candidate sites exist close to the selected sites described in this study (data not shown); therefore, we did not conclude any real cleavage sites in this study. The identification of each cleavage site by another approach, i.e., N-terminal amino acid sequencing and/or site-directed mutagenesis of the candidate cleavage sites, is now under investigation.

In conclusion, we determined the SaV ORF1 cleavage map and showed that the viral 3C-like protease was shown to be responsible for this proteolytic processing, and the proteolytic activity was fully abolished by replacing ^{1171}Cys within the GDCG motif of the 3C-like protease.

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REFERENCES

1. **Belliot, G., S. V. Sosnovtsev, T. Mitra, C. Hammer, M. Garfield, and K. Y. Green.** 2003. In vitro proteolytic processing of the MD145 norovirus ORF1 nonstructural polyprotein yields stable precursors and products similar to those detected in calicivirus-infected cells. J. Virol. **77:**10957–10974.
2. **Blakeney, S. J., A. Cahill, and P. A. Reilly.** 2003. Processing of Norwalk virus nonstructural proteins by a 3C-like cysteine proteinase. Virology **308:**216–224.
3. **Boniotti, B., C. Wirblich, M. Sibilia, G. Meyers, H.-J. Thiel, and C. Rossi.** 1994. Identification and characterization of a 3C-like protease from rabbit hemorrhagic disease virus, a calicivirus. J. Virol. **68:**6487–6495.
4. **Chang, K. O., S. V. Sosnovtsev, G. Belliot, Y. Kim, L. J. Saif, and K. Y. Green.** 2004. Bile acids are essential for porcine enteric calicivirus replication in association with down-regulation of signal transducer and activator of transcription 1. Proc. Natl. Acad. Sci. USA **101:**8733–8738.
5. **Chiba, S., S. Nakata, K. Numata-Kinoshita, and S. Honma.** 2000. Sapporo virus: history and recent findings. J. Infect. Dis. **181**(Suppl. 2)**:**S303–S308.
6. **Clarke, I. N., and P. R. Lambden.** 2000. Organization and expression of calicivirus genes. J. Infect. Dis. **181**(Suppl. 2)**:**S309–S316.
7. **Farkas, T., W. M. Zhong, Y. Jing, P. W. Huang, S. M. Espinosa, N. Martinez, A. L. Morrow, G. M. Ruiz-Palacios, L. K. Pickering, and X. Jiang.** 2004. Genetic diversity among sapoviruses. Arch. Virol. **149:**1309–1323.
8. **Green, K. Y., T. Ando, M. S. Balayan, T. Berke, I. N. Clarke, M. K. Estes, D. O. Matson, S. Nakata, J. D. Neill, M. J. Studdert, and H. J. Thiel.** 2000. Taxonomy of the caliciviruses. J. Infect. Dis. **181**(Suppl. 2)**:**S322–S330.
9. **Guo, M., K. O. Chang, M. E. Hardy, Q. Zhang, A. V. Parwani, and L. J. Saif.** 1999. Molecular characterization of a porcine enteric calicivirus genetically related to Sapporo-like human caliciviruses. J. Virol. **73:**9625–9631.
10. **Hansman, G. S., K. Katayama, N. Maneekarn, S. Peerakome, P. Khamrin, S. Tonusin, S. Okitsu, O. Nishio, N. Takeda, and H. Ushijima.** 2004. Genetic diversity of norovirus and sapovirus in hospitalized infants with sporadic cases of acute gastroenteritis in Chiang Mai, Thailand. J. Clin. Microbiol. **42:**1305–1307.
11. **Hardy, M. E., T. J. Crone, J. E. Brower, and K. Ettayebi.** 2002. Substrate specificity of the Norwalk virus 3C-like proteinase. Virus Res. **89:**29–39.
12. **Katayama, K., H. Shirato-Horikoshi, S. Kojima, T. Kageyama, T. Oka, F. Hoshino, S. Fukushi, M. Shinohara, K. Uchida, Y. Suzuki, T. Gojobori, and N. Takeda.** 2002. Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. Virology **299:**225–239.
13. **Kobayashi, S., K. Sakae, K. Natori, N. Takeda, T. Miyamura, and Y. Suzuki.** 2000. Serotype-specific antigen ELISA for detection of Chiba virus in stools. J. Med. Virol. **62:**233–238.
14. **König, M., H.-J. Thiel, and G. Meyers.** 1998. Detection of viral proteins after infection of cultured hepatocytes with rabbit hemorrhagic disease virus. J. Virol. **72:**4492–4497.
15. **Liu, B., I. N. Clarke, and P. R. Lambden.** 1996. Polyprotein processing in Southampton virus: identification of 3C-like protease cleavage sites by in vitro mutagenesis. J. Virol. **70:**2605–2610.
16. **Liu, B. L., I. N. Clarke, E. O. Caul, and P. R. Lambden.** 1995. Human enteric caliciviruses have a unique genome structure and are distinct from the Norwalk-like viruses. Arch. Virol. **140:**1345–1356.
17. **Liu, B. L., G. J. Viljoen, I. N. Clarke, and P. R. Lambden.** 1999. Identification of further proteolytic cleavage sites in the Southampton calicivirus polyprotein by expression of the viral protease in E. coli. J. Gen. Virol. **80:**291–296.
18. **Martin Alonso, J. M., R. Casais, J. A. Boga, and F. Parra.** 1996. Processing of rabbit hemorrhagic disease virus polyprotein. J. Virol. **70:**1261–1265.
19. **Mayo, M. A.** 2002. Virus taxonomy—Houston 2002. Arch. Virol. **147:**1071–1076.
20. **Meyers, G., C. Wirblich, H. J. Thiel, and J. O. Thumfart.** 2000. Rabbit hemorrhagic disease virus: genome organization and polyprotein processing of a calicivirus studied after transient expression of cDNA constructs. Virology **276:**349–363.
21. **Nakata, S., S. Honma, K. K. Numata, K. Kogawa, S. Ukae, Y. Morita, N. Adachi, and S. Chiba.** 2000. Members of the family caliciviridae (Norwalk virus and Sapporo virus) are the most prevalent cause of gastroenteritis outbreaks among infants in Japan. J. Infect. Dis. **181:**2029–2032.
22. **Noel, J. S., B. L. Liu, C. D. Humphrey, E. M. Rodriguez, P. R. Lambden, I. N. Clarke, D. M. Dwyer, T. Ando, R. I. Glass, and S. S. Monroe.** 1997. Parkville virus: a novel genetic variant of human calicivirus in the Sapporo virus clade, associated with an outbreak of gastroenteritis in adults. J. Med. Virol. **52:**173–178.
23. **Numata, K., M. E. Hardy, S. Nakata, S. Chiba, and M. K. Estes.** 1997. Molecular characterization of morphologically typical human calicivirus Sapporo. Arch. Virol. **142:**1537–1552.
24. **Okada, M., K. Shinozaki, T. Ogawa, and I. Kaiho.** 2002. Molecular epidemiology and phylogenetic analysis of Sapporo-like viruses. Arch. Virol. **147:**1445–1451.
25. **Robinson, S., I. N. Clarke, I. B. Vipond, E. O. Caul, and P. R. Lambden.** 2002. Epidemiology of human Sapporo-like caliciviruses in the South West