Epidemiological Study of Norwalk Virus Infections in Japan and Southeast Asia by Enzyme-Linked Immunosorbent Assays with Norwalk Virus Capsid Protein Produced by the Baculovirus Expression System

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In this study, we investigated Norwalk virus (NV) antigen and antibody to recombinant NV (rNV) in human populations in Japan and Southeast Asia by enzyme-linked immunosorbent assays (ELISAs). Baculovirus-expressed recombinant NV (rNV) capsid protein was used for preparing antisera to rNV or used as an antigen for detecting antibody to rNV. The ELISAs were specific for NV and had sensitivities equivalent to or higher than those of the previously developed radioimmunoassays. In 159 stool samples obtained from children, mainly younger than 10 years old, with acute gastroenteritis due to small round structured viruses in Japan, only 1 was positive for NV antigen. The pattern of acquisition of antibody to rNV was quite different from those of antibodies to group A rotavirus and human calicivirus Sapporo (HuCV-Sa) strain. The prevalence of antibody to rNV remained at a low level throughout childhood and then showed a steep rise during school age and early adulthood in Japan. A high prevalence of antibody was observed in samples collected from healthy adults in Japan and Southeast Asia. These results suggested that NV infection is common in adults in Japan and Southeast Asia but may be rare in infants in Japan. The HuCV-Sa strain was negative by the ELISA, and no serological relationship between NV and the HuCV-Sa strain was found. NV may be quite different from the HuCV-Sa strain, although both viruses are classified in the family *Caliciviridae*.

Norwalk virus (NV) is one of the small round structured viruses (SRSVs) and the most important causative virus of food-borne or waterborne outbreaks of acute nonbacterial gastroenteritis in humans, especially in adults (8, 14). Until recently, because NV has not been cultivated in tissue culture systems, it was unclassified and the research work on NV infection was limited to several institutes in the United States, where immunologic methods, such as radioimmunoassays (RIAs) or enzyme-linked immunosorbent assays (ELISAs), using reagents from volunteer studies were available (5, 7, 9, 10). The recent success of the NV gene cloning (11) and the production of the recombinant NV (rNV) capsid protein by a baculovirus expression system (13) has resulted in the availability of an unlimited amount of rNV antigen and high titer of immune sera to rNV to enable large-scale epidemiological studies.

In previous seroepidemiological studies (7, 15), NV infection was prevalent throughout the world, especially in developing countries. The prevalence of antibody to NV seemed to be low in infants and younger children and then was elevated during adulthood in developed countries, whereas NV infection seemed to start in early childhood in developing countries (7, 15). Because an antibody ELISA system using rNV protein has been developed and validated (4, 6), we conducted an epidemiological study to clarify the prevalence of NV infection in infants and adults mainly in Japan, where NV gastroenteritis has rarely been reported so far. The age-related prevalence of antibody to rNV in one district in Japan and the prevalence of antibody to NV in

adult sera obtained in Japan and Southeast Asia were examined.

NV is now proposed to be classified as a member of the family *Caliciviridae* on the basis of its genomic organization and biochemical properties (2, 11, 13, 15). Previous antigenic and serologic comparisons between NV and human calicivirus suggested that NV was related to some strains of human caliciviruses but was not related to the human calicivirus Sapporo (HuCV-Sa) strain (2). Because these data were obtained by immunoassays using human acute- and convalescent-phase sera, it was our interest to examine the relationship between the HuCV-Sa strain and NV by a newly established antigen ELISA (4).

MATERIALS AND METHODS

Stool samples. Two hundred three stool samples were tested by the antigen ELISA for NV. Two hundred stool samples from patients, mainly younger than 10 years old, with acute gastroenteritis in Sapporo or in Ehime prefecture, Japan, were used as negative controls or as test samples in the ELISA for NV. These specimens had been examined for virus by electron microscopy (EM) or by conventional ELISA for group A rotaviruses or by ELISA for human calicivirus (18) or by both methods. In these specimens, 20 samples obtained in Sapporo were positive for group A rotaviruses by conventional ELISA and genome analysis, 10 samples collected in Ehime prefecture were positive for enteric adenoviruses by EM and ELISA using monoclonal antibodies to either type 40 or type 41 (21), 11 samples obtained from infants during the outbreak of acute gastroenteritis due to human calicivirus in an orphanage in Sapporo were positive for the HuCV-Sa strain by ELISA (17), 109 samples collected in Sapporo from 1987 to 1990 were posi-

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tive for SRSV by EM, and 50 samples obtained in Ehime prefecture from 1984 to 1988 were positive for SRSV by EM. Three of these stool samples that served as positive controls were obtained from volunteers experimentally infected with NV in Houston, Tex., and were shown to contain NV by RIA and dot blot hybridization (11). Stool samples were prepared as a 10% (wt/vol) suspension in 10 mM phosphate-buffered saline (PBS; pH 7.4) and clarified by centrifugation at $3,000 \times g$ for 20 min. The supernatant was extracted with an equal volume of Difulon sorbent (trichlorotrifluoroethane; Daikin Kogyo Ltd., Tokyo, Japan) and clarified by centrifugation at $7,000 \times g$ for 20 min. The aqueous phase was stored at 4°C until tested.

Baculovirus-expressed NV capsid protein. NV capsids (rNV) produced by the baculovirus expression system were obtained as described previously (13). The rNV was diluted to a concentration of 1 µg/ml and used for the following experiments.

Preimmune and hyperimmune antisera. Guinea pig and rabbit hyperimmune antisera to rNV were prepared by inoculating guinea pigs and rabbits with rNV in Houston as described previously (4, 13). Preimmune sera were collected from the animals before inoculating them with rNV. Guinea pig, rabbit, and two mouse samples of hyperimmune antiserum to the HuCV-Sa strain, group A rotavirus (KU strain), group B rotavirus (adult diarrhea rotavirus), and group C rotavirus (Cowden strain) were prepared as described previously (16, 18, 19).

Serum samples. Thirty-two paired serum samples obtained from patients with acute gastroenteritis and one paired serum sample from a chimpanzee experimentally infected with NV, which were kindly provided by A. Z. Kapikian, and 908 serum samples collected from healthy adults or from ill children without gastroenteritis were tested by ELISA for antibody to rNV. Four paired serum samples obtained from volunteers before and after the oral administration of NV served as positive controls (11). Paired serum samples from 8 patients with group A rotavirus, 6 patients with enteric adenovirus (1), and 14 patients with HuCV-Sa (17) gastroenteritis were used to determine the specificity of the ELISA. Three hundred forty-four serum samples were collected from children (1 month to 19 years of age) without gastroenteritis who were outpatients or inpatients in Sapporo Medical University Hospital from 1986 to 1991. Thirty-four cord bloods were obtained in Sapporo Medical College in 1991. One hundred ninety serum samples were collected from healthy adults (over 20 years of age) in 1992 in Hokkaido, Japan, and kindly supplied by S. Sekiguchi (Japan Red Cross Society, Hokkaido, Japan). The other 190 serum samples from healthy adults aged 20 to 49 years were collected in four prefectures in Japan in 1978; 40 were from Miyagi-ken, 50 were from Saitama-ken, 50 were from Kyoto-fu, and 50 were from Fukuoka-ken. These samples were kindly provided by S. Yamazaki (National Institute of Health, Tokyo, Japan). One hundred fifty serum samples were obtained from healthy adults in Singapore, Indonesia, or Papua New Guinea and were kindly supplied by S. Yamazaki; 50 were collected from adults aged 20 to 37 years in Singapore in 1975, as part of a seroepidemiological survey to assess the immune status against vaccine-preventable disease in Singapore; 50 were collected from adults aged 20 to 29 years in Indonesia in 1975 and 1976, as part of a multiple serological survey in Indonesia; and 50 were collected from adults aged 20 to 44 years old in Papua New Guinea in 1979, as part of an epidemiological survey to assess the efficacy of immunization in Papua New Guinea.

All serum samples were stored at -20°C until used and were tested without knowledge of the age and sex of subjects.

ELISA for antigen. ELISAs were performed with 96-well polyvinyl chloride flat-bottom microtiter plates (Dynatech Laboratories, Inc., Chantilly, Va.). PBS (pH 7.4) containing 10% fetal calf serum (FCS) and 1% bovine serum albumin (BSA) was used as the diluent for stool samples, blocking serum, and detector antibody. The conjugate, a peroxidase-conjugated goat antibody to rabbit immunoglobulin G, was diluted in PBS containing 5% normal guinea pig serum and 1% BSA. Duplicate wells of the microtiter plates were each coated with 100 µl of a 1:10,000 dilution of either hyperimmune guinea pig serum to rNV or preimmune guinea pig serum diluted in PBS, and the plates were incubated at 4°C overnight. The plates then were washed five times with PBS containing 0.1% Tween 20 (PBS-T) and blocked with in PBS containing 1% BSA for 60 min at 37°C. After the blocking fluid was removed, 50 µl of each test sample (10% stool suspension) was added, and the plates were incubated for 2 h at 37°C or overnight at 4°C. After five washings in PBS-T, 50 µl of a 1:5,000 dilution of hyperimmune rabbit antiserum to rNV was added, and the plates were incubated for 60 min at 37°C. After five washings in PBS-T, 50 µl of a 1:1,000 dilution of peroxidase-conjugated goat immunoglobulin G antibody to rabbit immunoglobulin G (Immuno-Biological Laboratories) in PBS containing 5% normal guinea pig serum and 1% BSA was added. The plates were incubated for 60 min at 37°C and washed five times in PBS-T. A 100-µl portion of 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (Sigma Chemical Co., St. Louis, Mo.) in phosphate citrate buffer (pH 4.0) containing 1.5 µl of 30% H₂O₂ per ml was added to each well, and the plates were incubated for 30 min at room temperature. The A_{405} was measured with an ELISA reader (Easy Reader EAR400; SLT-Labinstruments).

For each sample, the results were expressed as the ratio of the A_{405} in wells coated with hyperimmune serum (positive) to the A_{405} in wells coated with preimmune serum (negative) (P/N ratio). The cutoff value of this system was obtained by testing 20 stool suspensions from the patients with the group A rotavirus gastroenteritis. The mean P/N ratio for these samples was 1.06, with a standard deviation of 0.25, and the cutoff value was defined as the mean plus 3 standard deviations (i.e., a P/N ratio of >1.8). A P/N ratio of >1.8 and an A_{405} of >0.1 were considered to indicate a positive reaction. All tests were performed in duplicate, and the results were averaged. The optimal dilutions of reagents were determined by checkerboard titrations with rNV capsid protein and 10% stool suspensions containing group A rotavirus.

ELISA-BL. An ELISA-blocking test (ELISA-BL) previously used in this laboratory to measure antibody to a variety of enteric viruses (18) was modified to measure antibody to rNV. Wells of the microtiter plates were coated with guinea pig hyperimmune serum to NV (1:10,000 dilution), and the plates were incubated at 4°C overnight. After five washings in PBS-T, the plates were blocked with 1% BSA in PBS for 60 min at 37°C. In a separate tube, 50 µl of fourfold dilutions (in 10% FCS and 1% BSA in PBS) of the test serum (beginning with a 1:50 dilution) was mixed with 25 µl of rNV capsid protein (20 ng/ml) or diluent and incubated for 2 h at 37°C. The mixture then was added to each of the wells of the blocked plate and incubated for 90 min at 37 or 4°C overnight. After five washings, the wells received rabbit hyperimmune serum to rNV (1:5,000 dilution) and were tested as described above. In each microtiter plate, an

TABLE 1. Specificity of ELISA for distinguishing NV from other viruses

Virus in stool specimens	No. of specimens	No. of results of ELISA for NV	
		Positive	Negative
NV	3	3	0
Rotavirus ^a	20	0	20
Adenovirus	10	0	10
Human calicivirus	11	0	11

^a Includes specimens used to determine the range of negative results.

optimal dilution of convalescent-phase serum from a volunteer infected with NV, which produced about 80% reduction in the A_{405} , served as a positive control; diluent alone and an acute-phase serum sample served as negative controls. A result of >50% inhibition of the A_{405} produced by the serum samples compared with that of the buffer control was considered to be positive for antibody to rNV. All tests were performed in duplicate, and the results were averaged.

RESULTS

Specificity and sensitivity of ELISA to detect NV. The specificity of the ELISA for detection of NV antigen was initially evaluated with 44 stool specimens containing different viruses (Table 1). All three stool samples containing NV by EM and RIA (unpublished data) were positive in the ELISA. None of the stool specimens collected from patients with group A rotavirus (20 specimens), enteric adenovirus (10 specimens), or the HuCV-Sa strain (11 specimens) was positive. The data from testing 20 group A rotavirus-positive samples were used to determine the cutoff criteria for positive results.

The sensitivity of the ELISA for detection of NV antigen was determined by using rNV capsid particles serially diluted in 10% FCS-1% BSA. The end point dilution of 1:640 obtained in the ELISA contained approximately 1.56 ng of NV capsid protein per ml (Fig. 1). Figure 1 shows the ELISA results of titrations of rNV capsid protein and an NV-positive stool specimen whose end point dilution was 1:160.

Detection of NV in stools of children with SRSV gastroenteritis by the NV ELISA. One hundred nine stool specimens obtained from children younger than 10 years old with acute gastroenteritis in Sapporo and 50 similar samples obtained Ehime prefecture were tested by the ELISA for NV antigen (Table 2). All of those samples contained SRSV by EM. Only 1 of the 109 stool specimens from Sapporo was positive for NV antigen. This positive sample was tested by reverse transcription PCR for NV using the 35-36 primer set for the RNA polymerase regions or the 1-4 primer set (12), dot blot hybridization with a cDNA probe that was from the RNA polymerase regions (11, 13), and the ELISA for human calicivirus (18). The 35-36 pair of primers gave a single major band of the expected size after the amplification, but the 1-4 pair of primers did not give a specific band for NV, and strong positive signal was obtained by dot blot hybridization (data not shown). The sample was negative for HuCV-Sa by ELISA. These results indicated that the ELISA result for NV antigen was correct, and the lack of 1-4 amplification of the sample suggests that this virus is closely related but not identical to the 8FIIa prototype NV strain. None of 50 stool samples obtained from children mainly younger than 10

FIG. 1. Titration of rNV capsid protein (○) and NV antigen (●) in a stool specimen from an experimentally infected volunteer. A P/N ratio of >1.8 and A_{405} (O.D.₄₀₅) of >0.1 were considered to indicate a positive reaction. The antigen titers of positive samples were 1:640 (1.56 ng/ml) for the rNV and 1:160 for the stool sample.

years old in Ehime prefecture was positive in the NV antigen test.

Specificity and sensitivity of ELISA-BL to detect antibody to rNV. The specificity of the ELISA-BL for detecting antibody to NV was evaluated by using 5 hyperimmune, 5 preimmune, and 15 paired serum samples. Guinea pig and rabbit hyperimmune sera to NV were positive, with antibody titers of 1:4,000,000 and 1:1,000,000, respectively. Convalescent-phase serum from a chimpanzee experimentally in-

TABLE 2. Results of NV ELISA to detect NV antigen in stools from patients with SRSV gastroenteritis in Sapporo and in Ehime prefecture

Age (yr)	No. of patients in:			
	Sapporo		Ehime	
	Positive	Negative	Positive	Negative
0-1	0	24	0	3
1	1	45	0	5
2	0	11	0	6
3	0	6	0	8
4	0	6	0	6
>5	0	9	0	22
Unknown	0	7	0	0
Total	1	108	0	50

FIG. 2. Titration of serum antibody to rNV in paired serum samples obtained from experimentally infected volunteers. Serum samples were portions of a fourfold dilution in PBS containing 10% FCS-1% BSA beginning with a 1:100 dilution. Samples were from volunteer 519 (○ and ●), volunteer 523 (△ and ▲), volunteer 528 (◇ and ◆), and volunteer 529 (□ and ■). Before (open symbols) and after (closed symbols) oral administration of NV. A >50% inhibition of optical density produced by the serum samples compared with that produced by buffer control was considered to be positive result for antibody to NV.

ected with NV was positive, with an antibody titer of >1:3,200, and preinoculation serum was negative (<1:50). Three samples of hyperimmune serum to three groups of rotaviruses and one rabbit and four guinea pig preimmune serum samples were negative. Eight paired serum samples collected from patients with group A rotavirus gastroenteritis and six paired serum samples collected from patients with enteric adenovirus gastroenteritis did not reveal a significant rise in antibody titer to rNV. Interestingly, none of 1 guinea pig, 1 rabbit, and 2 mouse hyperimmune serum samples to HuCV-Sa was positive for antibody to rNV, and none of 14 paired serum samples collected from the patients with HuCV-Sa gastroenteritis showed a significant elevation of antibody to rNV.

The sensitivity of the test was evaluated by using four paired serum samples obtained from volunteers before and after oral administration of NV. Figure 2 shows the titration of serum antibody to NV in these samples. The antibody titers were higher in the convalescent-phase sera by the new ELISA than by the RIA developed previously (>1:25,600 to >1:102,400 versus 1:25,600 to >1:25,600) but lower in the preinoculation sera by the new ELISA (<1:100 to 1:400 versus 1:100 to 1:1,600).

Prevalence of antibody to rNV. A total of 908 serum samples were tested by ELISA-BL. Five hundred thirty-four children and adults were tested for antibody to rNV in Hokkaido. They were divided into 10 age groups, and at least 50 samples were tested in each group, except for over-50-year age group, in which 40 samples were tested (57 were in the 0 to 4-month age group, 58 were in the 4-month to 1-year group, 52 were in the 1- to 3-year group, 57 were in the 3- to 7-year group, 60 were in the 7- to 12-year group, 60 were in the 12- to 20-year group, 50 were in the 20- to 30-year

FIG. 3. Age-related prevalence of antibody to rNV in serum samples from children and adults in Hokkaido.

group, 50 were in the 30- to 40-year group, 50 were in the 40- to 50-year group, and 40 were in the over-50-year group). The age-related prevalence of antibody to rNV in these serum samples is shown in Fig. 3. Of 34 cord blood samples, 24 (71%) had antibody to rNV, and the positive rate was similar to that of the 20 (70%)- and 30 (76%)-year-old age groups. The prevalence of antibody decreased thereafter and reached a minimum in the 4-month to 6-year age group, in which the prevalence of antibody was only 6 to 11%. Then the positive rate showed a steep rise during school age and early adulthood, steadily increased thereafter, and reached to 98% in the over 50-year-old age group. Overall, 70% or more of adults had antibody to rNV.

Table 3 shows the prevalence of antibody to rNV by age and sex in 380 serum samples from healthy adults in five prefectures of Japan. More than 75% of serum samples in each of the five districts had antibodies to rNV. There was no significant difference between those areas in antibody prevalence, whereas the positive rate steadily increased by age. The total proportion of seropositivity to rNV in Japan was 81% (308 of 380 samples).

The prevalence of antibody to rNV by age and sex in 150 serum samples from healthy adults in three Southeast Asian countries is shown in Table 4. Interestingly, a low prevalence of antibody to rNV (64%) was obtained in Singapore, one of the moderately developed countries, compared with the high prevalence rate (90 to 100%) obtained in two developing countries.

DISCUSSION

The recent success of cloning of the Norwalk virus genome (11) and the production of rNV capsid protein by the baculovirus expression system (13) permitted the development of new ELISAs for large-scale epidemiological studies. This is the first report of use of these assays in Japan and Southeast Asia, where relatively few studies on NV infection have been done. These assays were specific for NV, and their sensitivities were equivalent to or higher than those of

TABLE 3. Prevalence of antibody to rNV by age and sex in sera of healthy adults from five districts in Japan

District	No. of samples positive for antibody/no. tested (% positive) in adults					
	With an age (yr) of:				Male	Female
	20-29	30-39	40-49	>50		
Hokkaido	35/50 (70)	38/50 (76)	44/50 (88)	39/40 (98)	85/97 (87)	71/93 (76)
Miyagi-ken	21/30 (70)	9/10 (90)	0	0	11/15 (73)	19/25 (76)
Saitama-ken	31/39 (79)	7/10 (70)	1/1 (100)	0	1/1 (100)	38/49 (78)
Kyoyo-fu	32/40 (80)	8/10 (80)	0	0	12/15 (80)	28/35 (80)
Fukuoka-ken	31/38 (82)	10/10 (100)	2/2 (100)	0	1/1 (100)	42/49 (86)
Total	150/197 (76)	72/90 (80)	47/53 (89)	39/40 (98)	110/129 (85)	198/251 (79)

the RIAs previously developed, as determined by measuring titers of NV antigen and antibody to rNV. When rNV capsid protein was used, the sensitivity of ELISA was estimated to be 1.56 ng/ml. In this study, we used an ELISA-BL to detect antibody to rNV. This format has the advantage that antibody in sera from humans and from various species of animals can be measured under the same conditions without a need to change and optimize separate the detector antibodies for each species.

In the survey of 159 stool samples obtained from children, mainly younger than 10 years old, with SRSV gastroenteritis in Japan, we found only 1 positive stool sample. The positive result was confirmed by subsequent experiments with reverse transcription PCR using one primer set for the RNA polymerase region (35-36 primers) and dot blot hybridization with cDNA for the RNA polymerase region, but the reverse transcription PCR using another primer set (1-4 primers) showed a negative result. These results indicated that NVs are present in Japan and may be one of the causative agents of gastroenteritis in infants in Japan, but these strains of NV are not identical to the prototype NV. Because the new ELISA for NV antigen is quite specific for the prototype of NV and it does not detect NV-related viruses (4), detection of NV in stool samples might be very low. Genomic analysis of this positive sample will be required to answer this question. The low proportion of NV detected in stool samples from children younger than 10 years old agreed with the low prevalence of antibody acquisition in children less than 6 years old. These results suggested that NV infection in younger children is rare in Japan. Our data combined with results of previous studies in the United States (7, 15) indicate that a relatively low prevalence of antibody to NV is seen in younger children in developed countries whereas acquisition of antibody to NV starts in early childhood in developing countries. It may be of interest to examine the diarrhea stool samples obtained from infants in developing

countries by this new ELISA to know whether NV gastroenteritis is common in infancy in those areas.

The age-related prevalence of antibody to rNV in Hokkaido was strikingly different from that of those to group A rotavirus and HuCV-Sa. In our study, acquisition of antibody to rNV remained at a low level for children less than 6 years old and then showed a steep rise during school age and early adulthood, whereas the antibody to group A rotavirus or human calicivirus was acquired in early childhood (3, 18, 20). The prevalence of antibody to rNV steadily increased through adulthood and reached 98% in adults over 50 years old; this prevalence was much higher than those determined previously by RIA or immune adherence hemagglutination assay in the United States (7, 15). This might be explained by the difference of the sensitivity between each method or by the difference of the period when the serum samples were collected. When the prevalences of antibody to rNV in healthy adults of five different districts in Japan were compared, there was no significant difference by sex and by area, and NV infection has constantly occurred through Japan from before 1978 to 1991. The high prevalence of antibody detected in cord blood, which was similar to that found in the 20- to 30-year-old group, and in serum samples obtained from infants 0 to 3 months old, which declined to 6 to 11% in infants aged from 4 months to 6 years, suggests that antibody detected in early infancy was probably of maternal origin. Our data suggested that NV infection in Japan is rare in younger children and is increasing from school age to early adulthood and that reinfection might occur during adulthood. The high prevalence of antibody to rNV found in the 20-year-old group in Indonesia and in Papua New Guinea agreed with the previous data (7) and suggested that NV infection may be common in childhood in developing countries.

A previous report (2) of RIAs using human paired sera for NV suggested that NV was related to some calicivirus

TABLE 4. Prevalence of antibody to rNV by age and sex in sera of healthy adults from three Southeast Asian countries

Country	No. of samples positive for antibody/no. tested (% positive) in adults				
	With an age (yr) of:			Male	Female
	20-29	30-39	40-49		
Singapore	19/30 (63)	13/20 (65)	0	32/50 (64)	0
Indonesia	44/49 (90)	1/1 (100)	0	23/25 (92)	22/25 (88)
Papua New Guinea	27/27 (100)	19/19 (100)	4/4 (100)	18/18 (100)	32/32 (100)
Total	90/106 (85)	33/40 (83)	4/4 (100)	73/93 (78)	54/57 (95)

strains but not to the HuCV-Sa strain. In that report, only serological comparison was done by testing paired sera from patients infected with each virus. When stool samples from volunteers infected with NV, which were shown to contain NV by immunoelectron microscopy or RIA, were tested by the ELISA for HuCV-Sa, all samples were negative (18). In the previous study, stool samples containing the HuCV-Sa strain were not positive for rNV by the new ELISA and no serological relationship between the HuCV-Sa strain and NV was demonstrated by the new ELISA-BL for antibody to rNV. These data suggest that NV and HuCV-Sa are serologically distinct from each other, although both viruses are now classified in the family *Caliciviridae*.

Further study is necessary to clarify the importance of NV infection in Japan and Southeast Asia, and these new ELISA methods should be useful for these purposes. Moreover, genome analysis of the HuCV-Sa strain will be important to clarify the differences between NV and HuCV-Sa.

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