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Western Blot (Immunoblot) Assay of Small, Round-Structured Virus Associated with an Acute Gastroenteritis Outbreak in Tokyo

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Small, round-structured virus (SRSV) was detected in a stool specimen of a patient during an acute gastroenteritis outbreak in Tokyo and was tentatively named SRSV-9. SRSV-9 was purified by sucrose velocity gradient centrifugation after CsCl density gradient centrifugation. The buoyant density of SRSV-9 appeared to be 1.36 g/ml in CsCl. A Western blot (immunoblot) assay using the biotin-avidin system revealed that SRSV-9 was antigenically related to the Hawaii agent but distinct from the Norwalk agent and contained a single major structural protein with a molecular size of 63.0 ± 0.6 kilodaltons. The prevalence of SRSV-9 infection in Tokyo was surveyed by the Western blot antibody assay by using a crude virus preparation as the antigen. Seroconversion was observed in 56.5% of the patients involved in the outbreaks from which SRSV was detected by electron microscopy.

Small, round-structured virus (SRSV) (2) includes the Norwalk (13), Hawaii (27), Snow Mountain (6), and Otofuke (25) agents, etc. The Norwalk agent, the prototype of SRSV, was first detected by immunoelectron microscopy (IEM) by Kapikian et al. (13) and has been shown to play a significant role in the etiology of acute gastroenteritis in the United States, Australia, and other countries (4, 12, 18, 19). SRSV was also detected from sporadic cases and outbreaks of acute gastroenteritis in Japan (15, 20, 21, 23). Radioimmunoassay (RIA) (7, 10) and enzyme immunoassay (8, 11, 17) techniques were later developed for large-scale epidemiologic studies of these agents. Only two members of SRSV, the Norwalk (9) and Snow Mountain (16) agents, were characterized, and they were shown to contain a single major structural protein, like human calicivirus (26). However, the structural protein of the Hawaii agent, which is antigenically distinct from the Norwalk and Snow Mountain agents, remains unstudied. The Western blot (WB) (immunoblot) assay we present in this report revealed that the SRSV implicated in a nonbacterial acute gastroenteritis outbreak in Tokyo was antigenically related to the Hawaii agent and contained a single major structural protein. SRSV infections in Tokyo were seroepidemiologically surveyed by the WB antibody assay.

MATERIALS AND METHODS

Virus and sera. A single stool specimen and paired serum specimens were obtained from a patient (teacher no. 1 in Table 1) involved in a nonbacterial acute gastroenteritis outbreak (outbreak no. 10 in Table 2) and used in the WB assay. Bacterial pathogens examined were previously described (23). Stool specimens containing rotavirus, enteric adenovirus, *Campylobacter jejuni-C. coli*, or enteropathogenic *Escherichia coli* were obtained from patients with acute gastroenteritis and used as sources of control antigens. Antiserum to the Hawaii agent was kindly provided by R. G. Wyatt and A. Z. Kapikian, National Institutes of Health, Bethesda, Md., and antisera to the Norwalk agent were obtained from G. S. Grohmann, Westmead Hospital, Westmead, New South Wales, Australia, and from M. K. Estes, Department of Virology, Baylor College of Medicine, Houston, Tex. Paired sera were obtained from patients infected with adenovirus type 4, echovirus type 7, coxsackievirus B type 3, rotavirus, *C. jejuni-C. coli*, or *Yersinia pseudotuberculosis* and were used as control sera.

Crude virus preparation. A crude 10% stool homogenate in TN buffer (0.01 M Tris hydrochloride, pH 7.5, 0.15 M NaCl, 0.02% NaN_3) was blended with an equal volume of trichlorotrifluoroethane in a homogenizer (Ultraturrax TP10; Ika-Werk, Federal Republic of Germany) at 0°C for 1 min. The emulsion was centrifuged at 1,500 × *g* for 10 min, and the aqueous layer was further centrifuged at 3,000 × *g* for 30 min and then at 100,000 × *g* (TFT 32.13 rotor; Kontron) for 2.5 h at 4°C. The pellet suspended in 0.1 ml of TN buffer was used as a crude virus preparation for electron microscopy (EM) and WB antibody assay.

Virus purification. The crude SRSV-9 preparation prepared from 20 g of stool specimen was centrifuged at 150,000 × *g* (TST 55.5 rotor; Kontron) for 22 h in a CsCl solution with an initial density of 1.36 g/ml (isopycnic CsCl density gradient centrifugation). Five 0.1-ml fractions were collected and dialyzed against TN buffer containing 1.0 mM $CaCl_2$ and 0.05 mM $MgCl_2$, and the presence of virus particles in each fraction was monitored by EM. The virion-containing fractions were pooled, diluted fivefold with TN buffer, and pelleted by centrifuging at 100,000 × *g* for 3 h. The pellet was suspended in 0.2 ml of TN buffer, layered on a continuous 10 to 30% (wt/vol) sucrose gradient with a 1-ml, 1.6-g/ml CsCl cushion, and centrifuged at 100,000 × *g* (TST 41.14 rotor; Kontron) for 90 min (sucrose velocity gradient centrifugation). Five 0.1-ml fractions were collected, and samples (10 μl per strip) of the virion-containing fractions were used to prepare purified WB strips. The presence of the virus particles was also examined by EM.

WB. The virus preparation was heated at 100°C for 3 min in the sample buffer (24) with sodium dodecyl sulfate and 2-mercaptoethanol and then electrophoresed on a 4 to 20% linear gradient slab gel (Daiichi Pure Chemicals Co., Ltd.,

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TABLE 1. Comparison of SRSV-9 antibody assay by WB with that by IEM

Patient no.[a]	EM[b]	IEM-determined antibody levels in[c]:		WB-determined antibody levels in[d]:	
		Acute-phase serum	Convalescent-phase serum	Acute-phase serum	Convalescent-phase serum (fold rise)
Teachers					
1	+	1+	3+	1,600	51,200 (32)
2	−	1+	1+	1,600	1,600 (1)
3	+	0	2+	3,200	25,600 (8)
4	−	0	0	200	200 (1)
5	+	0	2+	100	12,800 (128)
Pupils					
6	−	0	4+	<100	25,600 (>256)
7	−	0	3+	100	51,200 (512)
8	+	0	1+	<100	1,600 (>16)
9	+	0	2+	1,600	25,600 (16)

[a] Patients in the primary school where the outbreak occurred (outbreak no. 10 in Table 2).
[b] The presence (+) or absence (−) of SRSV particles in the fecal specimens was determined by EM.
[c] Antibody levels were rated in five classes from 0 to 4+ according to the reactivity with SRSV-9 virions by IEM.
[d] The endpoint titer of the WB assay is expressed by the reciprocal number of the highest dilution of serum able to detect the 63-kDa protein.

Tokyo, Japan) with a sodium dodecyl sulfate-discontinuous buffer system. The gel was electroblotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, Calif.) at 25 V/cm for 3 h under ice cooling. The blotted membrane was immersed in 40% fetal calf serum in phosphate-buffered saline, pH 7.4, at 4°C overnight, washed three times with 20 mM Tris hydrochloride–10 mM NaCl, pH 7.5 (Tris-buffered saline), containing 0.3% Tween 20 and then once with phosphate-buffered saline, and stored at 4°C after being cut into 1-mm-wide strips. From 1 g of stool specimen, 240 crude WB strips were obtained. Paired sera of the patient were diluted 1:100 in Tris-buffered saline containing 1% normal goat serum and 5% dry milk and allowed to react with the blotted strips at room temperature overnight. After being washed three times with Tris-buffered saline containing 0.3% Tween 20, immunoglobulin G-antigen immune complex was detected by using a Vectastain ABC-AP kit and an alkaline phosphatase substrate kit II (Vector Laboratories).

EM. For EM, a drop of the crude virus preparation was placed on a 400-mesh Formvar-carbon-coated grid for negative staining with 3% phosphotungustic acid, pH 7.0. The grid was dried and examined in a Hitachi H-7000 electron microscope. IEM was performed by the procedure of Kapikian et al. (14) with a slight modification. A 5-μl sample of fivefold-diluted serum was allowed to react with a 15-μl sample of the fecal extract for 2 h at room temperature. After further overnight incubation in the cold room, the reactivity of the particles with antibody was examined by EM, and antibody levels were rated in five classes from 0 to 4+.

RESULTS

Detection of SRSV. A nonbacterial acute gastroenteritis outbreak including 575 patients occurred at a primary school in Tokyo on 15 March 1986. Lunch served at the school was epidemiologically presumed to be implicated in the outbreak, although no particular food item has been identified as a common vehicle. Spherical particles with characteristics of SRSV were detected by EM, and seroconversion was observed in 8 of 28 serum specimens examined by IEM (Fig. 1). The SRSV thus detected was tentatively named SRSV-9.

WB assay of SRSV-9 protein. The fecal extract was banded by CsCl density gradient centrifugation. Virus particles, when examined by EM, appeared as a single peak at a density of 1.36 g/ml (Fig. 2A) but were still contaminated with fecal material. The proteins in each fraction were examined by the WB assay. Treatment of the blotted strips with paired serum samples of the patient from whose stool specimen the fecal extract was prepared revealed the presence of the plural number of bands. However, only one band, with a molecular size of 63 ± 0.6 kilodaltons (kDa), was reactive with convalescent-phase serum but was unreactive or weakly reactive with acute-phase serum. This differentially reactive band was observed only in the strips blotted with material in fractions containing virus particles (fractions 5 to 7) (Fig. 2B). The others, including two dense bands of 28 and 69 kDa, were detected in the strips blotted with material with densities of 1.36 g/ml or less. However, they were presumed to be nonspecific bands since they did

TABLE 2. Detection of SRSV-9 seroconversion by the WB assay

Outbreak no.	Date	Place	No. at risk	No. ill	WB determination of seroconversion rate to SRSV-9[a]	Detection rate of SRSVs by EM[b]
1	January 1984	Restaurant	2	2	2/2	2/2
2	December 1984	Home	6	6	0/1	1/2
3	January 1985	Home	2	2	1/1	1/1
4	February 1985	Restaurant	26	8	0/1	2/2
5	March 1985	Home	3	3	1/1	1/2
6	December 1985	Restaurant	18	16	3/3	3/3
7	December 1985	Restaurant	4	2	1/2	2/2
8	January 1986	Hotel	118	45	3/5	8/13
9	January 1986	Picnic	8	6	1/1	3/7
10	March 1986	Primary school	1,317	575	6/14	8/28
11	December 1987	Restaurant	38	17	0/9	2/12
12	December 1987	Restaurant	8	6	2/3	2/6
13	December 1987	Kindergarten	118	40	10/14	7/19
14	January 1988	Inn[c]	50	20	1/1	7/16
15	February 1988	Restaurant	12	6	4/4	3/5

[a] Patients with more than a fourfold antibody rise in the WB assay were regarded as positive.
[b] Fecal specimens were examined for the presence of SRSV by EM.
[c] Japanese-style hotel.

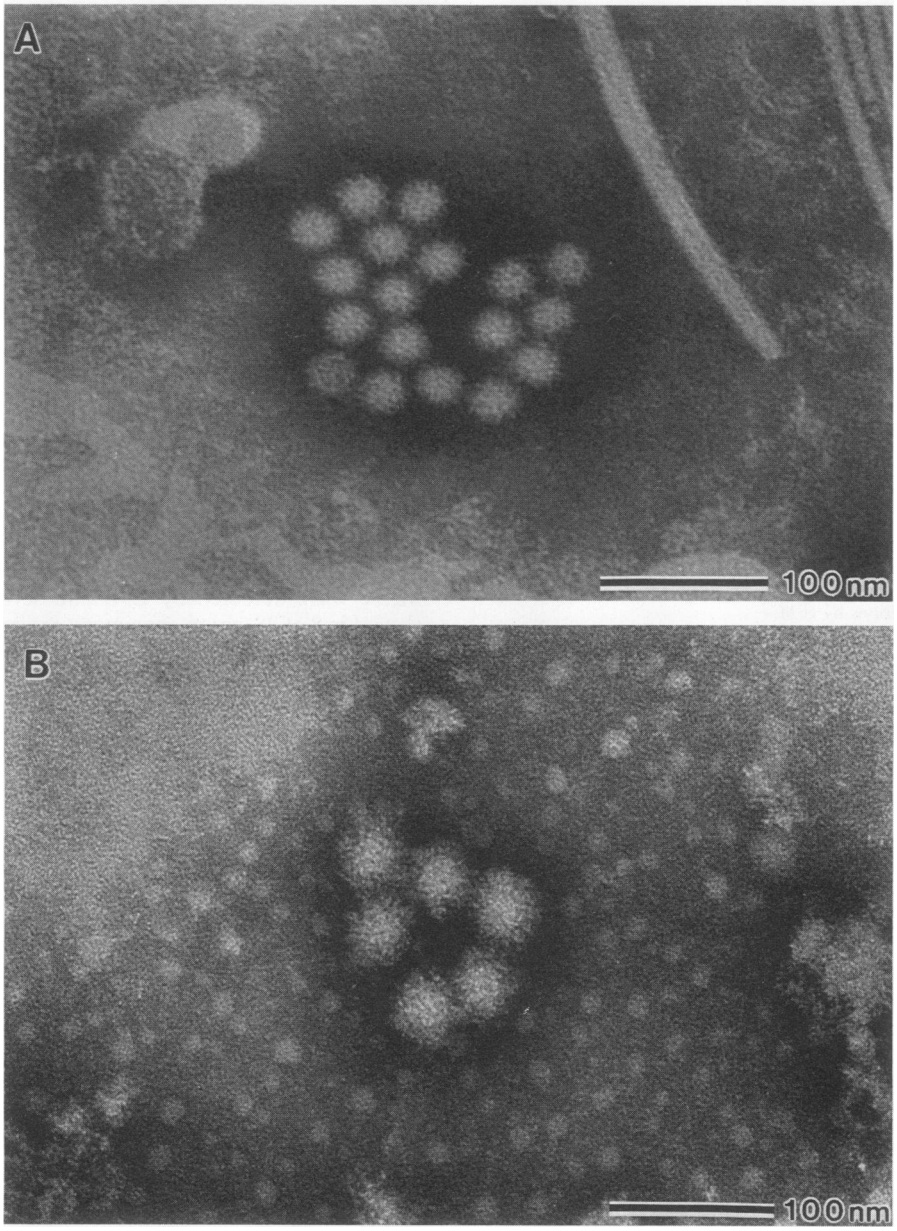

FIG. 1. (A) Electron micrograph of SRSV-9 particles in stool specimen. (B) Immunoelectron micrograph rated as 3+.

not differentially react with paired serum samples of the patient.

The virion band (fractions 5 to 7) in CsCl density gradient centrifugation was pooled, concentrated by centrifugation, and subjected to sucrose velocity gradient centrifugation. Virus particles appeared again as a single peak (Fig. 3A). The 63-kDa protein band which was differentially reactive with the paired serum samples was again detected in the strips blotted with material in virion-containing fractions (fractions 15 and 16), and nonspecific bands were no longer observed (Fig. 3B). The 63-kDa protein band was not detected by treatment with control paired serum samples obtained from patients infected with some enteric viruses and enteropathogenic bacteria (data not shown). From these results we concluded that the 63-kDa protein is a major structural protein of SRSV-9.

WB antibody assay. Since the amount of SRSV antigens available for seroepidemiologic surveys is limited, we tried to detect SRSV-9 antibody by using a crude virus preparation as antigen. A number of nonspecific bands appeared in a wide range of the blotted strip, but a protein band differentially reactive with the paired serum samples was still detectable in the 63-kDa position (Fig. 4). This band appeared to be specific for SRSV-9. Such a band was not observed when the strip was treated with control paired serum samples from patients infected with some enteric viruses and enteropathogenic bacteria or when strips which were blotted with control antigens from stool specimens containing some enteric viruses and enteropathogenic bacteria were treated with paired serum samples of the patient infected with SRSV-9 (Fig. 4A).

Next, the result of such a WB assay was compared with that obtained by IEM (Table 1). All the patients with more than an eightfold antibody rise in the WB assay showed

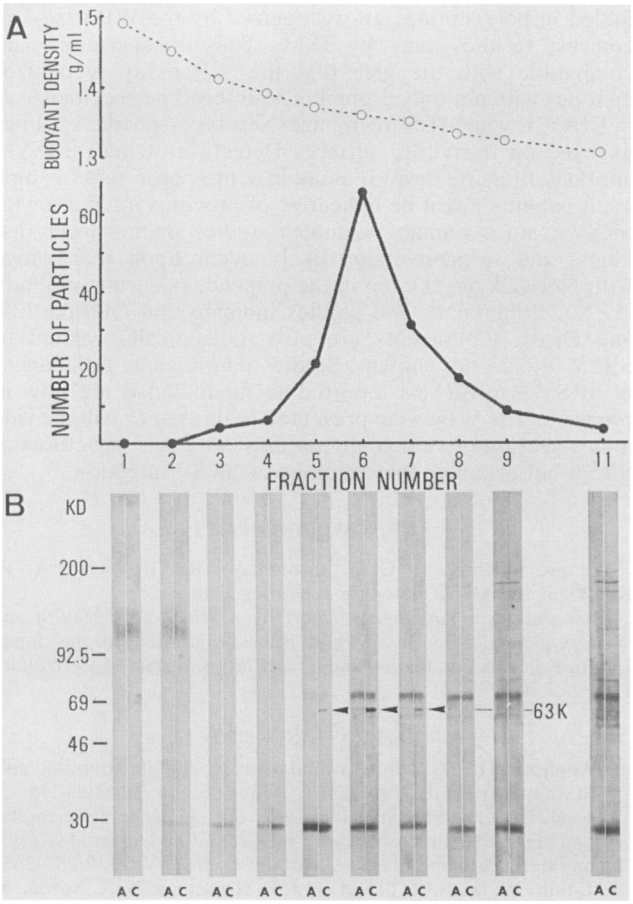

FIG. 2. (A) Isopycnic CsCl density gradient centrifugation of an SRSV-9 crude preparation. The fecal extract was centrifuged in CsCl (initial density, 1.36 g/ml) at 150,000 × *g* for 22 h, and each fraction was examined for the presence of the particles in an electron microscope. (B) WB assay. Material in each fraction was pelleted by high-speed centrifugation, and proteins separated by electrophoresis on a 4 to 20% linear gradient acrylamide slab gel in sodium dodecyl sulfate were electroblotted onto a nitrocellulose membrane. The blotted strips were treated with 100-fold-diluted acute-phase (lanes A) and convalescent-phase (lanes C) sera of the patient. The immunoglobulin G-antigen immune complex was detected by using the biotin-avidin system. Myosin (200 kDa), phosphorylase *b* (92.5 kDa), albumin (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa) were run as internal markers.

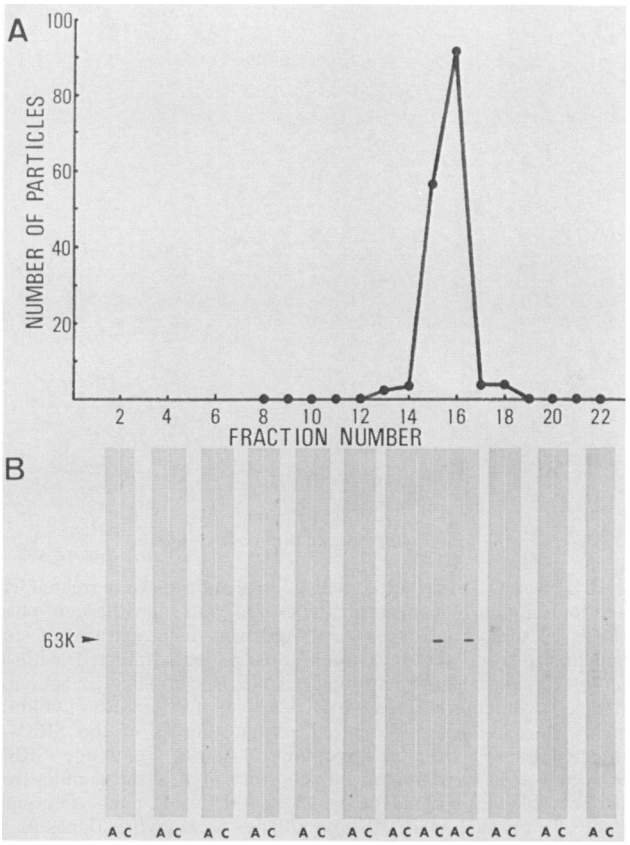

FIG. 3. (A) Sucrose velocity gradient centrifugation of an SRSV-9 preparation. The virion-containing fractions in CsCl density gradient centrifugation were pooled and pelleted by high-speed centrifugation. The pellet was centrifuged at 100,000 × *g* for 90 min at 4°C in a 10 to 30% (wt/vol) sucrose gradient. (B) WB assay. Material in each fraction was electroblotted onto a nitrocellulose membrane after electrophoresis; the blotted membrane was treated with acute-phase (lanes A) and convalescent-phase (lanes C) sera. The immunoglobulin G-antigen immune complex was detected by using the biotin-avidin system. The molecular size of the reactive protein was determined by using standard protein markers.

seroconversion in IEM and vice versa. Antibody titers were high in acute-phase sera of some adult teachers when examined by the WB assay (Table 1). This may indicate previous SRSV infection, since serum antibody appeared to be ineffective in preventing SRSV reinfection (5, 22). The WB antibody assay was very sensitive: an antibody rise of as much as 512-fold was detectable in some cases.

Relatedness of SRSV-9 to the Hawaii agent. Whether SRSV-9 was antigenically related to the Norwalk and Hawaii agents was examined by the WB assay. SRSV-9 reacted with antiserum to the Hawaii agent but not with antiserum to the Norwalk agent (Fig. 4B). However, when examined by IEM, SRSV-9 did not react with either anti-Norwalk agent or anti-Hawaii agent serum (data not shown). This favors the ideas that more epitopes may be recognized by the WB assay than by IEM and that SRSV-9 is a virus sharing some epitopes common to the Hawaii agent.

Prevalence of SRSV-9 infection. Paired serum samples from 62 patients involved in 15 nonbacterial acute gastroenteritis outbreaks (13 oyster-associated and 2 oyster-unassociated outbreaks) which occurred in Tokyo during the 4-year period from January 1984 to February 1988 were examined for SRSV-9 antibody by the WB assay, using a crude SRSV-9 preparation as antigen (Table 2). Seroconversion was observed in 35 (56.5%) of 62 tested patients involved in 12 (80.0%; 10 oyster-associated and 2 oyster-unassociated outbreaks) of 15 outbreaks examined, suggesting that SRSVs with epitopes common to SRSV-9 are prevalently implicated in acute gastroenteritis outbreaks in Tokyo.

DISCUSSION

SRSV-9, which was implicated in a nonbacterial acute gastroenteritis outbreak in Tokyo, was found to be antigenically related to the Hawaii agent but distinct from the Norwalk agent by the WB antibody assay. SRSV-9 appeared to share properties common to other members of SRSV so far studied. The density of SRSV-9 virions in CsCl was 1.36 g/ml, consistent with the values reported for the other members of SRSV, such as Norwalk, Otofuke, and Snow Mountain agents, etc. (1). SRSV-9 contained a single major

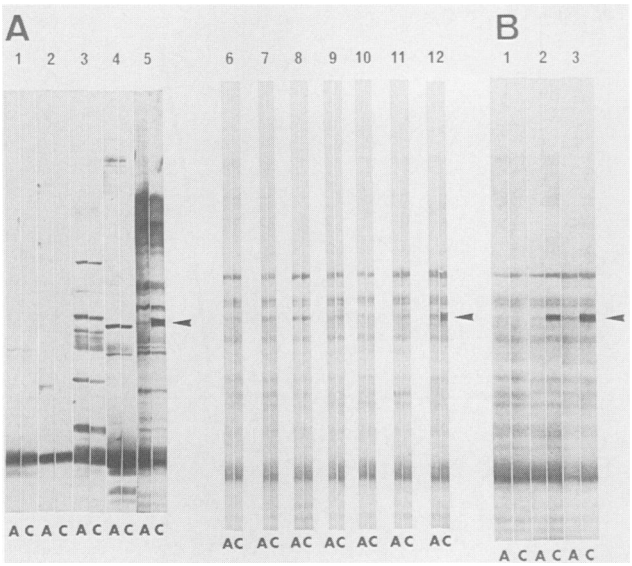

FIG. 4. (A) Strips blotted with control antigens were treated with 100-fold-diluted acute-phase (lanes A) and convalescent-phase (lanes C) sera. Control antigens used were fecal extracts of stool specimens from acute gastroenteritis patients infected with *C. jejuni*- *C. coli* (lanes 1), enteropathogenic *E. coli* (lanes 2), rotavirus (lanes 3), or enteric adenovirus (lanes 4). Crude SRSV-9-blotted strips were treated with paired serum samples of the SRSV-9-infected patient as a positive control (lanes 5). Crude SRSV-9-blotted strips were treated with control paired serum samples from patients infected with adenovirus type 4 (lanes 6), echovirus type 7 (lanes 7), coxsackievirus B type 3 (lanes 8), rotavirus (lanes 9), *C. jejuni*-*C. coli* (lanes 10), or *Y. pseudotuberculosis* (lanes 11). Lanes 12 are a positive control treated with paired serum samples of the SRSV-9-infected patient. (B) Crude SRSV-9-blotted strips were treated with serum samples of volunteers preinfected (lanes A) and postinfected (lanes C) with the Norwalk agent (lanes 1) or the Hawaii agent (lanes 2) and acute-phase (lanes A) and convalescent-phase (lanes C) sera of the SRSV-9-infected patient (lanes 3). Arrowheads indicate the position of the 63-kDa protein.

structural protein, as did the Norwalk and Snow Mountain agents, and its molecular size (63 kDa) was compatible with the 59- and 62-kDa sizes reported for the Norwalk (9) and Snow Mountain (16) agent proteins, respectively. The members of *Caliciviridae*, including human calicivirus (26), are distinct in having one major structural protein of 60 to 71 kDa, in contrast to other animal viruses, which possess a plural number of structural proteins. Accordingly, SRSV-9 may be regarded as a possible candidate for a member of the *Caliciviridae*, as proposed for the Norwalk and Snow Mountain agents (9, 16).

IEM has proved valuable in detecting SRSV, but it is not a tool suitable for epidemiologic survey. The later-developed enzyme immunoassay and RIA are currently used for this purpose, but it takes much time to prepare reagents for these tests. The WB assay widely used in the field of virology has not yet been successfully used in epidemiologic survey of SRSV infections. The WB antibody assay presented in this paper appeared to be specific for SRSV-9 and was as sensitive as enzyme immunoassay and RIA; the extents of the antibody rises and endpoint titers of the sera (Table 1) were almost equivalent to those reported in blocking enzyme immunoassay and RIA (17). Cross-reaction of SRSV-9 with the Hawaii agent was observed when examined by the WB but not by IEM. It is likely that not only epitopes exposed on the virion surface but also those buried in the particles or folded in polypeptides are recognized by the WB assay, in contrast to the assay by IEM. These observations are compatible with the idea that the WB assay recognizes epitopes with not only group but also serotype specificity, as in RIA (3), while IEM recognizes serotype-specific epitopes exposed on the virion surface. Detection of high SRSV-9 antibody titers by the WB assay in acute-phase sera of some adult patients might be indicative of previous infection with SRSVs, since human volunteer studies demonstrate that illness and seroconversion likely occur upon rechallenge with Norwalk agent even in the presence of serum antibody (5, 22). Preliminary WB studies indicate that Otofuke (25) and Osaka (20) agents are also antigenically related to SRSV-9 (data not shown). Studies on antigenic relatedness of SRSV-9 to SRSVs reported so far in Japan are now in progress. The WB assay presented in this paper will provide a powerful tool to elucidate not only antigenic structures of SRSV but also seroepidemiology of SRSV infection.

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