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Antigenic Characterization of Small, Round-Structured Viruses by Immune Electron Microscopy

SHOJIRO OKADA,[1]* SEIJI SEKINE,[1] TAMIE ANDO,[1] YUKINAO HAYASHI,[1] MIYOKO MURAO,[2] KIYOSHI YABUUCHI,[1] TAKASHI MIKI,[1] AND MAKOTO OHASHI[1]

Department of Microbiology, Tokyo Metropolitan Research Laboratory of Public Health, 3-24-1 Hyakunincho, Shinjuku-ku, Tokyo 169,[1] and Saitama Institute of Public Health, 956-1 Kamiokubo, Urawa, Saitama 338,[2] Japan

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Small, round-structured viruses (SRSVs) detected from nonbacterial gastroenteritis outbreaks in Tokyo and Saitama Prefecture, Japan, during the period from 1977 to 1988 were tentatively classified into nine antigenic patterns from SRSV-1 (S-1) to SRSV-9 (S-9) by cross-immune electron microscopy (IEM). S-1 and S-2 appeared pattern specific, while S-3 to S-9, distinguishable from each other in their reactivity, appeared somewhat antigenically related. Their antigenic relatedness to the Norwalk, Hawaii, and Otofuke agents was also examined by IEM by using antisera to these agents. S-3 appeared most closely related to the Norwalk agent. S-4 and S-5 were related to the Norwalk agent and, presumably, were distantly related to the Hawaii and Otofuke agents. S-6 and S-7 were related to the Hawaii and Otofuke agents. S-8 and S-9 were related to the Otofuke agent and, presumably, were distantly related to the Hawaii agent. The prevalence of each antigenic pattern in 38 outbreaks was examined: S-8 was implicated in 24% of the outbreaks, S-5 in 16%, S-4 in 13%, S-9 in 13%, S-6 in 11%, and others in 5%.

A large number of nonculturable small, round viruses (3) have been detected from nonbacterial gastroenteritis outbreaks from several countries since the first detection of the Norwalk agent by immune electron microscopy (IEM) in 1972 (11). From these small, round viruses, a group of viruses similar in size, morphology, and buoyant density to the Norwalk agent was classified into small, round-structured virus (SRSV) (2). The members of SRSV have been generally named after the locations where the outbreak occurred, such as the Norwalk, Hawaii, Snow Mountain, and Otofuke agents, etc.

As for SRSVs detected in the United States, the Norwalk, Hawaii, and Snow Mountain agents were distinguishable from each other by IEM (5, 21), and the Montgomery County agent was shown to be related to the Norwalk agent by cross-challenge studies (22) and IEM (21). A number of SRSVs, including the Otofuke agent (19), Taunton agent (17), etc., have also been detected in the United Kingdom (1, 14), Australia (8, 15), and Japan (20, 23). However, their antigenic relationships have not been extensively studied. The difficulty encountered in these studies is due, in part, to the limited number of fecal specimens available.

We have surveyed the prevalence of SRSV infections among children with epidemic vomiting and diarrhea and patients with acute nonbacterial gastroenteritis in Saitama Prefecture (Pref.) and Tokyo, Japan (16, 18). Through these surveys, we obtained a number of fecal specimens as well as SRSV-infected patients' paired sera for the cross-IEM test. Here, we report antigenic classification of SRSVs by the cross-IEM test. SRSVs from Tokyo and Saitama Pref. were tentatively classified into nine antigenic patterns. Their prevalence in the outbreaks and their antigenic relatedness to the Norwalk, Hawaii, and Otofuke agents were also examined by IEM.

* Corresponding author.

MATERIALS AND METHODS

Virus stocks. Stool specimens containing a number of SRSV particles, as determined by direct electron microscopy (EM), were obtained from gastroenteritis outbreaks in Tokyo and Saitama Pref., and from sporadic cases in young children in Saitama Pref. Specimens were stored undiluted at −20°C.

Serum specimens. Serum specimens used for antigenic characterization of SRSVs were obtained from patients in gastroenteritis outbreaks which occurred in Tokyo and Saitama Pref. Sera of volunteers preinfected and postinfected with the Norwalk and Hawaii agents were kindly provided from M. K. Estes, Department of Virology, Baylor College of Medicine, Houston, Tex., and R. G. Wyatt, National Institutes of Health, Bethesda, Md., respectively. Paired sera of patients infected with the Otofuke and Hawaii agents were kindly provided by S. Urasawa, Department of Hygiene, Sapporo Medical College, Sapporo, Hokkaido, Japan, and M. Oseto, Ehime Prefectural Institute of Public Health, Matuyama, Ehime, Japan, respectively.

Virus preparation. Stool specimens were prepared as 10% suspensions in TN buffer (0.01 M Tris hydrochloride [pH 7.5], 0.15 M NaCl, 0.02% NaN_3). The suspension was blended with an equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane (Tokyo Kasei Chemical Co., Ltd., Tokyo, Japan) in a homogenizer (Ultraturrax TP10N; Ika-Werk, Staufen Breisgau, Federal Republic of Germany) at 0°C for 1 min. The emulsion was centrifuged at 1,500 × *g* for 10 min. The aqueous layer was removed and immediately centrifuged at 3,000 × *g* for 30 min at 4°C. The supernatant was centrifuged at 100,000 × *g* (model TFT 32.13 rotor; Kontron, Zurich, Switzerland) for 2.5 h at 4°C. The pellet was suspended in 0.1 ml of TN buffer and used as a virus preparation for the direct EM and IEM after storage overnight at 4°C.

EM. For EM, a drop of the virus preparation was placed on a 400-mesh Formvar carbon-coated grid for negative

TABLE 1. Antigenic classification of SRSVs by IEM

Serum for typing			Rating[a] of antibody in acute- and convalescent-phase sera to the following antigens								
Antigenic pattern of SRSV	Patient	Phase[b]	S-1 (AC-13/83)[c]	S-2 (Y.A6-86)	S-3 (Sa-1283)	S-4 (88-217)	S-5 (85-28)	S-6 (84-2120)	S-7 (UN-85)	S-8 (85-2193)	S-9 (86-510)
S-1	83-SA1	C	$3–4^+$	0	0	0	0	0	0	0	0
	83-SA2	C	$3–4^+$	0	0	0	0	0	0	0	0
S-2	85-SH2	C	0	3^+	0	0	0	0	0	0	0
S-3	77-SK5	A	0	$0–1^+$	0	2^+	0	$0–1^+$	0	2^+	0
		C	0	$0–1^+$	3^+	2^+	0	1^+	$0–1^+$	2^+	0
	77-SK2	A	0	1^+	0	0	0	0	0	0	0
		C	0	1^+	2^+	2^+	2^+	0	0	0	0
S-4	83-SW4	A	0	0	0	0	0	0	0	0	$0–1^+$
		C	0	0	1^+	$2–3^+$	$1–2^+$	0	1^+	$2–3^+$	$1–2^+$
	83-SW3	A	$1–2^+$	2^+	$0–1^+$	0	$2–3^+$	0	0	$0–1^+$	$0–1^+$
		C	2^+	$2–3^+$	$0–1^+$	$3–4^+$	$2–3^+$	$1–2^+$	1^+	$0–1^+$	$0–1^+$
S-5	88-124	A	0	$0–1^+$	0	2^+	$0–1^+$	$0–1^+$	0	0	0
		C	0	$0–1^+$	0	2^+	3^+	2^+	$1–2^+$	$0–1^+$	0
	85-433	A	ND[d]	0	0	3^+	0	$1–2^+$	2^+	$2–3^+$	$0–1^+$
		C	ND	0	0	3^+	$2–3^+$	$1–2^+$	2^+	3^+	$0–1^+$
S-6	84-SU1	C	0	0	0	0	0	4^+	0	0	0
	84-SK8	A	0	$0–1^+$	0	$0–1^+$	2^+	0	$1–2^+$	$0–1^+$	0
		C	0	$0–1^+$	0	$0–1^+$	2^+	3^+	2^+	$2–3^+$	0
S-7	85-565	A	0	$0–1^+$	0	2^+	0	1^+	0	0	0
		C	0	2^+	0	3^+	0	3^+	$2–3^+$	$1–2^+$	0
	88-SB4	A	ND	0	0	0	0	0	0	0	0
		C	ND	$2–3^+$	0	0	0	$3–4^+$	4^+	$0–1^+$	0
S-8	85-2193	A	0	1^+	0	0	0	0	0	0	0
		C	0	2^+	0	0	2^+	0	$2–3^+$	$3–4^+$	$2–3^+$
	86-137	A	$0–1^+$	2^+	0	$1–2^+$	0	$1–2^+$	2^+	0	0
		C	$0–1^+$	3^+	0	$1–2^+$	$2–3^+$	$3–4^+$	$2–3^+$	$3–4^+$	0
S-9	86-510	A	$2–3^+$	$0–1^+$	0	0	0	$2–3^+$	0	0	0
		C	3^+	$0–1^+$	0	0	2^+	4^+	2^+	$3–4^+$	$2–3^+$
	86-545	A	0	$1–2^+$	0	1^+	0	0	0	0	0
		C	0	$2–3^+$	0	1^+	$2–3^+$	$3–4^+$	3^+	4^+	$3–4^+$

[a] A 1.5^+ or more change in antibody rating between acute- and convalescent-phase serum was considered significant.
[b] A, Acute-phase sera; C, convalescent-phase sera.
[c] Antigenic pattern of SRSV, followed by the patient identification in parentheses.
[d] ND, Not done.

staining with 3% phosphotungustic acid, pH 7.0. The grid was dried and examined in an electron microscope (model H-7000; Hitachi, Ltd., Tokyo, Japan). IEM was performed by the procedure of Kapikian et al. (12), with a slight modification. A 5-μl portion of acute- or convalescent-phase serum diluted fivefold was allowed to react with a 15-μl portion of the virus preparation for 2 h at room temperature. After further overnight incubation in the cold room, the preparation was processed as described for direct EM and the reactivity of the particles with antibody was examined by EM. Antibody levels were rated in five classes from 0 to 4^+ according to the following scale: 4^+, the particles were heavily coated with antibody, thereby showing somewhat obscured appearance; 3^+, the particles were heavily coated with antibody but less than those rated 4^+; 2^+ and 1^+, the particles were coated with still lesser amounts of antibody; and 0, the particles were not coated with antibody.

RESULTS

Antigenic classification of SRSVs by IEM. During the period from October 1977 to January 1985, SRSVs similar in morphology to the Norwalk agent were detected by EM in 297 (30%) of 982 sporadic cases of acute gastroenteritis in children and in 162 (60%) of 272 cases from 21 outbreaks examined in Saitama Pref. In Tokyo, SRSVs have been detected in 239 (45.8%) of 522 cases from 80 outbreaks examined since 1984. A number of patients' paired sera and fecal specimens containing SRSV were obtained through these surveys and submitted to the cross-IEM test, which allowed antigenic classification of SRSVs.

In the cross-IEM, SRSV from a given outbreak or sporadic case appeared to react differently, in some cases, with paired sera originated from different sporadic cases or outbreaks. The results of repeated cross-IEM tests are summarized and presented in Table 1. SRSVs were classified into nine antigenic patterns from SRSV-1 (S-1) to SRSV-9 (S-9). S-1 was reactive only with anti-S-1, and S-2 was reactive with anti-S-2 and anti-S-7. S-3 was reactive only with anti-S-3, and S-4 was reactive with anti-S-4 and was weakly reactive with anti-S-3. S-5 reacted with antisera to S-5, S-8 and S-9, and was weakly reactive with anti-S-3 and anti-S-4. S-6 reacted with antisera to S-6, S-7, and S-9, and reacted

TABLE 2. Reactivity of SRSV antigenic patterns with antisera to Norwalk, Hawaii, and Otofuke agents

Strain	Serum: Volunteer or patient	Serum: Phase[a]	Rating of antibody in sera to the following antigens: S-1 (AC-13/83)[b]	S-2 (Y.A6-86)	S-3 (Sa-1283)	S-4 (88-217)	S-5 (85-28)	S-6 (84-2120)	S-7 (UN-85)	S-8 (85-2193)	S-9 (86-510)
Norwalk	519B	Pr	ND[c]	0–1+	0	0–1+	0	1+	ND	0	0
		Po	ND	0–1+	4+	1+	2+	1+	ND	0–1+	0
	523	Pr	ND	0–1+	0	0–1+	0	0	ND	0	0
		Po	ND	0–1+	3+	3+	2–3+	0	ND	0	0
	528	Pr	ND	0	0	0–1+	0	0–1+	ND	0–1+	0
		Po	ND	0	3+	2–3+	1–2+	0–1+	ND	1–2+	0
Hawaii	S10	Pr	ND	0	0	0	0	0	0	0	0
		Po	ND	0	0	0	0	2+	2–3+	0–1+	0–1+
	82-21[d]	A	ND	ND	ND	ND	ND	0–1+	0	ND	ND
		C	ND	ND	ND	ND	ND	2–3+	2–3+	ND	ND
	82-27[d]	A	ND	ND	ND	ND	ND	1–2+	0	ND	ND
		C	ND	ND	ND	ND	ND	2+	2–3+	ND	ND
Otofuke	OT	A	ND	1–2+	0	0	1+	0	2+	0	0
		C	ND	1–2+	0	0	1+	2–3+	2+	2–3+	0
	KN	A	ND	0	0	0–1+	0	2+	2+	1+	0
		C	ND	0–1+	0	0–1+	0	3+	3–4+	3+	2+

[a] Pr, Serum from a preinfected volunteer; Po, serum from a postinfected volunteer; A, acute-phase serum of the patient; C, convalescent-phase serum.
[b] Patient identification indicated in parentheses.
[c] ND, Not done.
[d] Amounts of provided antisera were limited, so IEM tests were run only against S-6 and S-7.

weakly with antisera to S-4, S-5, and S-8. S-7 reacted with anti-S-7 and anti-S-9 and reacted weakly with anti-S-5 and anti-S-8. S-8 reacted with anti-S-8 and anti-S-9 and reacted weakly with antisera to S-4, S-6, and S-7. S-9 reacted with anti-S-9 and was weakly reactive with anti-S-8. The reaction patterns observed were reproducible by using different strains belonging to a given antigenic pattern. Thus, S-1 and S-2 appeared pattern specific, while S-3 to S-9 appeared to be somewhat antigenically related, although distinguishable from each other by their reactivity, which differed from pattern to pattern.

Relatedness of SRSV antigenic patterns to Norwalk, Hawaii, and Otofuke agents. Serological reactivity of SRSV antigenic patterns with antisera to the Norwalk, Hawaii, and Otofuke agents was examined by IEM, although in a one-way serological reaction. S-2 to S-6, S-8, and S-9 were tested with three paired serum specimens of volunteers pre- and postinfected with the Norwalk agent. All paired sera demonstrated seroconversion to S-3 and S-5, and two of them demonstrated seroconversion to S-4. The rating of antibody in sera to S-3 appeared higher than that to S-5 (Table 2). These results are consistent with the observation that anti-S-3 sera were reactive not only with S-3 but also with S-4 and S-5 (Table 1) and strongly suggest that the Norwalk agent is related to S-4 and S-5 and most closely to S-3.

Their relatedness to the Hawaii agent was also examined by IEM. S-2 to S-9 were also tested with one serum pair of a volunteer pre- and postinfected with the Hawaii agent. The serum pair demonstrated seroconversion to S-6 and S-7 but to none of the others (Table 2). To know which of S-6 and S-7 is more closely related to the Hawaii agent, they were tested with two patients' paired serum specimens from the outbreak in which the Hawaii agent was implicated (23). Seroconversion to S-7 was observed in two paired serum specimens, but that to S-6 was observed in one serum pair (Table 2), suggesting that S-7 is more closely related to the Hawaii agent.

To know their relatedness to the Otofuke agent, S-2 to S-9 were tested with two patients' paired serum specimens from the outbreak caused by the Otofuke agent (19). Seroconversion to S-8 was demonstrated in two paired serum specimens and that to S-6, S-7, and S-9 was demonstrated in one serum pair, suggesting that the Otofuke agent is related to S-6, S-7, and S-9 and most closely to S-8.

These observations, consistent with the antigenic relationships among the Norwalk, Hawaii, and Otofuke agents so far obtained (19, 21), were combined with the data from Table 1 and summarized in Table 3. S-1 and S-2 were pattern specific. S-3 was most closely related to the Norwalk agent. S-6 and S-7 were related to the Hawaii agent as well as to the Otofuke agent. S-8 and S-9 were related to the Otofuke

TABLE 3. Antigenic relationships among Norwalk, Hawaii, Otofuke agents and SRSV antigenic patterns

Strain/SRSV antigenic pattern	SRSV antigenic pattern: S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8	S-9
S-1	+[a]	−[b]	−	−	−	−	−	−	−
S-2	−	+	−	−	−	−	−	−	−
S-3	−	−	+	±[c]	±	−	−	−	−
Norwalk	ND[d]	−	+	±	+	−	ND	−	−
S-4	−	−	−	+	±	±	−	±	−
S-5	−	−	−	−	+	±	±	−	−
S-6	−	−	−	−	−	+	−	±	−
S-7	−	+	−	−	−	+	+	±	−
Hawaii	ND	−	−	−	−	+	+	−	−
S-8	−	−	−	−	+	±	±	+	±
S-9	−	−	−	−	+	+	+	+	+
Otofuke	ND	−	−	−	−	±	±	+	±

[a] +, All sera examined were seroconverted to a given SRSV.
[b] −, No seroconversion.
[c] ±, Seroconversion to a given SRSV was demonstrated not in all but at least in 50% in serum pairs.
[d] ND, Not done.

agent. They were also presumed to be distantly related to the Hawaii agent, since their antisera were reactive with S-6 and S-7, which were related to the Hawaii and Otofuke agents. S-4 and S-5 were related to the Norwalk agent and presumably were distantly related to the Hawaii and Otofuke agents, judged from the reactivity of their antisera with S-6 and S-7.

Prevalence of each antigenic pattern in outbreaks in Tokyo and Saitama Pref. SRSVs from 38 outbreaks, from which stool specimens containing relatively plenty of virus particles were available, were examined by IEM. S-8 was detected from 24% of the outbreaks examined, S-5 from 16%, S-4 from 13%, S-9 from 13%, S-6 from 11%, S-7 from 8%, and others from 5%. As for their prevalence in relation to the authentic SRSVs, the Norwalk agent-related patterns (S-3, S-4, and S-5) were implicated in 34% of the outbreaks examined, the Hawaii agent-related patterns (S-6 and S-7) were implicated in 18%, and the Otofuke agent-related patterns were implicated in 37% (S-8 and S-9). If S-6 and S-7 were also counted in the Otofuke agent-related patterns, their prevalence was strikingly high, reaching 55% of the outbreaks. As for their transmission vehicles, 26 outbreaks were foodborne, 2 were waterborne, and 10 were unknown. The most prevalent vehicle in foodborne outbreaks was oysters, occupying 92% of the foodborne outbreaks. Clams and lunch meat were implicated in one outbreak. Outbreaks in which transmission vehicles remained unknown occurred in one kindergarten, seven elementary schools, and one middle school. The existence of a common source transmission was strongly suspected for these outbreaks, but we failed to find out what caused the outbreak. SRSVs implicated in oyster-associated outbreaks appeared not to be restricted to particular antigenic patterns but were widely distributed from S-3 to S-9.

Morphological and physical characteristics of some SRSV antigenic patterns. S-1 to S-9 were morphologically indistinguishable from the Norwalk, Snow Mountain, and Taunton agents, which had capsomerelike structures on the surface. Diameters of virus particles ranged in size from 28 to 36 nm; in S-1 to S-8, particles with a diameter around 32 nm were most prevalent, while in S-9, 28-nm particles were prevalent. Very rarely, depression structures characteristic of calicivirus were observed in S-1, S-4, S-6, and S-7 particles from some outbreaks. Buoyant density examined in CsCl appeared as 1.36 g/ml in S-8 and S-9, 1.38 g/ml in S-4, and 1.40 g/ml in S-7.

DISCUSSION

SRSVs from sporadic cases and outbreaks of acute nonbacterial gastroenteritis in Saitama Pref. and Tokyo were tentatively classified into nine antigenic patterns by the cross-IEM test. S-1 and S-2 appeared pattern specific. On the other hand, S-3 to S-9 appeared to be antigenically related, although distinguishable from each other in their reactivity. They are presumed to be related to each other by sharing a common epitope(s), and the observed antigenic differences might be due to the difference in combination of the common epitope(s) or to the presence of additional specific epitope(s). From their reaction patterns and reactivity with some of authentic SRSVs, they may be grouped into five groups: (i) antigenic patterns which are pattern specific (S-1 and S-2); (ii) one most closely related to the Norwalk agent (S-3); (iii) ones related to the Norwalk and presumably distantly related to the Hawaii and Otofuke agents (S-4 and S-5); (iv) ones related to the Hawaii and Otofuke agents (S-6 and S-7); and (v) ones related to the Otofuke and presumably distantly related to the Hawaii agent (S-8 and S-9).

Radioimmunoassay (RIA) has been used to identify the causative agents of nonbacterial gastroenteritis outbreaks (6, 7). Kaplan et al. (13) reported that an outbreak was considered to be caused by the Norwalk agent when seroconversion to the Norwalk virus was demonstrated by RIA in at least 50% of paired sera from cases in the outbreak. However, such a criterion seems not to be appropriate for antigenic typing of SRSV, since at least a part of antigen of the Norwalk agent was also recognized by antibody to the Snow Mountain agent or calicivirus by RIA, but none was recognized by IEM (4, 5, 9). It is likely that RIA is not type specific and recognizes epitopes exposed on the virion surface as well as those hidden, in contrast to IEM which recognizes only those on the virion surfaces (4, 10). We also obtained the same sort of the finding in the Western (immuno-) blot (WB) assay: S-9 reacted with a broad range of antisera against S-3 to S-9 in the WB assay (unpublished data), while in IEM, S-9 reacted with anti-S-9 and was weakly reactive with anti-S-8. Accordingly, we must still rely on the troublesome IEM, since it is the only reliable method so far available for antigenic typing of SRSV, while RIA and WB assays seem to be the more effective methods in determining whether or not a given gastroenteritis outbreak was caused by SRSV. Comparative studies on epitopes of SRSV antigenic patterns recognized by IEM and by WB assay are now under way.

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