the QIAquick gel extraction kit (Qiagen), and the purified DNAs were directly sequenced with the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Tokyo, Japan). Nucleotide sequences were aligned with Clustal W version 1.83 (http://clustalw.ddbj.nig.ac.jp/top-j.html). A phylogenetic tree with 1,000 bootstrap replications was constructed by the neighbor-joining method. The distance of nucleotide substitutions per site was calculated by Kimura's two-parameter method [Kimura, 1980], and was illustrated with NJPlot software (http://pbil.univ-lyon1.fr/software/njplot.html) [Perriere and Gouy, 1996].

Nucleotide Sequence Accession Numbers

The SaV nucleotide sequences corresponding to the partial capsid region determined in this study have been deposited in GenBank/EMBL/DDBJ under the accession numbers AB429079–AB429159.

RESULTS

Detection of Pathogens in Patients With Acute Gastroenteritis

Among 639 stool specimens collected from outpatients with gastrointestinal symptoms, 421 (65.9%) contained at least one of the pathogens (Table I). The monthly distribution of the positive samples showed that the peaks were observed in every winter season (Fig. 1). Among the 421 positive stool samples, NoV was detected in 260 (61.8%), of which 20 were GI (4.8%) and 240 were GII (57.0%). SaV was detected in 81 (19.2%), AstV in 9 (2.1%), KV in 1 (0.2%), group A RoV in 48 (11.4%), group C RoV in 1 (0.2%), AdV in 19 (4.5%), EntV in 13 (3.1%), and bacterial pathogens in 11 (2.6%) (8 *C. jejuni*, 1 *Salmonella enterica* serovar Manhattan, and 2 enteroaggregative *E. coli* [O111:H21,O126:H27]) (Table I). Mixed infections involving two viruses were found in 22 (5.2%) of the positive samples, in which the combinations were NoV GI and GII in 3 (0.7%), group A RoV and NoV GII in 5 (1.2%), SaV and NoV GII in 3 (0.7%), AstV and NoV GII in 2 (0.5%), AdV and NoV GII in 3 (0.7%), KV and NoV GII in 1 (0.2%), EntV and NoV GII in 1 (0.2%), SaV and EntV in 1 (0.2%), SaV and AstV in 1 (0.2%), group A RoV and AstV in 1 (0.2%), and AdV and AstV in 1 (0.2%) (Table II).

NoV was the major pathogen in every year (Table I), and the viral load for 20 NoV GI strains ranged from 4.20×10^4 to 2.34×10^{11} copies/g stool with a geometric mean value of 3.84×10^8 copies/g stool. The viral load for 240 NoV GII strains ranged from 4.20×10^4 to 3.08×10^{11} copies/g stool with a geometric mean value of 8.62×10^8 copies/g stool (data not shown), demonstrating that NoV GII strains were the most prevalent pathogen during the study period. In contrast, the detection of NoV GI, SaV, AstV, KV, RoV(A), RoV (C), Adv, EntV, and bacteria varied, and SaV was found to be

TABLE I. Detection Rate of Pathogen Between 2002 and 2007

Year	No. of tested samples	No. of positive samples	NoVGI	NoV GII	SaV	AstV	KV	RoV(A)	RoV(C)	AdV	EntV	Bacteria
2002	36	23 (63.9)[a]		19 (82.6)	2 (8.7)			2 (8.7)				
2003	115	64 (55.7)	7 (10.9)	28 (43.8)	7 (10.9)	2 (3.1)		20 (31.3)	1 (1.6)	1 (1.6)		
2004	53	40 (75.5)	4 (10.0)	23 (57.5)	2 (5.0)	2 (5.0)		3 (7.5)		4 (10.0)	5 (12.5)	
2005	107	60 (56.1)	6 (10.0)	44 (73.3)	7 (11.7)	1 (1.7)		5 (8.3)				
2006	75	57 (76.0)	1 (1.8)	33 (57.9)	10 (17.5)		1 (1.8)	8 (14.0)		4 (7.0)	2 (3.5)	2 (3.5)
2007	253	177 (70.0)	2 (1.1)	93 (52.5)	53 (29.9)	4 (2.3)		10 (5.6)		10 (5.6)	6 (3.4)	9 (5.1)
Total	639	421 (65.9)	20 (4.8)	240 (57.0)	81 (19.2)	9 (2.1)	1 (0.2)	48 (11.4)	1 (0.2)	19 (4.5)	13 (3.1)	11 (2.6)

[a]The values in parentheses represent the detection rates.

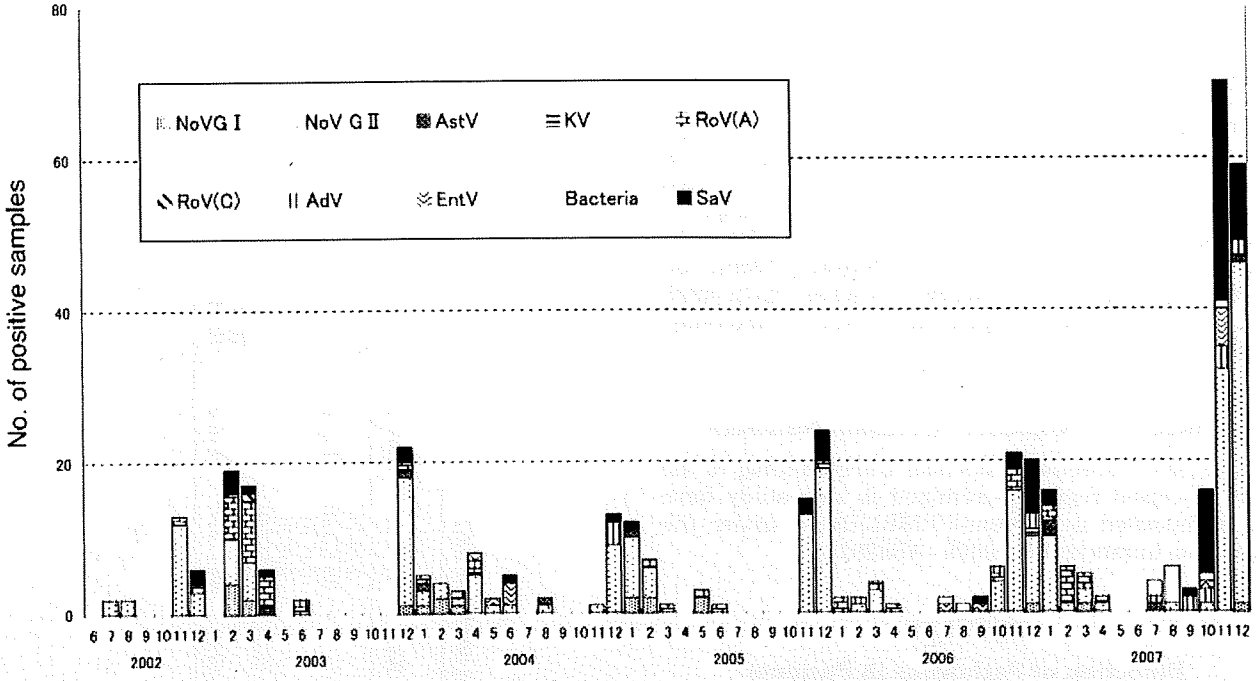

Fig. 1. Seasonal distribution of viral gastroenteritis in pediatric outpatients in Kumamoto Prefecture, Japan, between June 2002 and December 2007.

the secondary prevalent pathogen in 2005 (11.7%), 2006 (17.5%), and 2007 (29.9%) (Fig. 1 and Table I). A significant increase of SaV detection was observed in 2007 in the diarrhea surveillance (Fig. 1 and Table I). Among SaV-positive specimens, multiple viruses were detected in five patients, Kumamoto-18, -41, -67, -69, and -81. SaV and NoV GII were detected in Kumamoto-18, -67, and -69, SaV and EntV were detected in Kumamoto-41, and SaV and AstV were detected in Kumamoto-81 (Table III).

Characterization of SaV

Eighty-one samples were found positive for SaV by either multiplex RT-PCR or RT-PCR or by both, and were further analyzed. The detection rates of SaV with the five methods, multiplex RT-PCR, RT-PCR, universal nested RT-PCR, genogroup-specific RT-PCR, and quantitative real-time RT-PCR, were 27.2%, 100%, 95.1%, 100%, and 97.5%, respectively (Table III). Among the 81 samples, 79 (97.5%) were found positive for SaV by quantitative real-time RT-PCR. The viral loads of the 79 positive samples ranged from 1.32×10^5 to 1.07×10^{11} copies/gram stool (geometric mean value of 8.39×10^8 copies/g stool). SaV strains were detected from September to April except the Kumamoto-10 strain which was detected in June in 2004 (Fig. 1, Table III). The season of SaV was similar to that of NoV (Fig. 1). The ages of 81 patients, 41 males and 40 females, were as follows: 4 individuals were 0 years old (0 (4)), 1 (14), 2 (14), 3 (8), 4 (11), 5 (3), 6 (8), 7 (7), 8 (7), 9 (3), 13 (1), and >15 (1) (Table III). The clinical symptoms of these patients were diarrhea in 60 (74.1%), vomiting in 45 (55.6%), abdominal pain in 28 (34.6%), and fever in 12 (14.8%), as shown in Table III. Phylogenetic analysis based on 270 nt in the capsid showed that SaVs were grouped as follows: 17 GI (21.0%), 10 GII (12.3%), 51 GIV (63.0%), and 3 GV (3.7%) (Table III and Fig. 2). The yearly distribution of SaV was 2 GI in 2002; 1 GI and 6 GII in 2003; 2 GV in 2004; 7 GI in 2005, 6 GI, 4 GII, and 1 GV in 2006; and 1 GI and 51 GIV in 2007 (Table III). The detection rate of SaV showed a sudden increase in 2007, accounting for 29.9% of the pathogen-positive specimens, and 51 of 52 strains were GIV (Kumamoto-31 to -81) (Table III and Fig. 2). These 51 SaV strains were further divided into 8 different groups (Fig. 2), although all of the nucleotide changes were synonymous substitutions (data not shown).

DISCUSSION

The pathogen surveillance of outpatients with gastrointestinal symptoms from June 2002 to December 2007 in Kumamoto Prefecture, Japan, was performed and identified the virus and/or bacteria pathogen in 65.9% of specimens. The season distribution of viral gastroenteritis showed a winter peak (Fig. 1), an observation consistent with other reports [Bon et al., 1999; Hedlund et al., 2000; Chikhi-Brachet et al., 2002; Kirkwood et al., 2005; Blanton et al., 2006; Chen et al.,

TABLE II. Mixed Infections Involving Two Diarrhea Viruses

Year	No. of positive samples	No. of positive samples with co-infection	NoV GI NoV GII	RoV(A) NoV GII	SaV NoV GII	AstV NoV GII	AdV NoV GII	KV NoV GII	EntV NoV GII	SaV EntV	SaV AstV	RoV(A) AstV	AdV AstV
2002	23	0											
2003	64	2		1		1							
2004	40	3	2			1							
2005	60	3	1	1	1		1	1					
2006	57	3		2			2		1	1	1	1	1
2007	177	10		1	2								
Total	421	22 (5.2)[a]	3 (0.7)	5 (1.2)	3 (0.7)	2 (0.5)	3 (0.7)	1 (0.2)	1 (0.2)	1 (0.2)	1 (0.2)	1 (0.2)	1 (0.2)

[a]The values in parentheses represent the detection rates.

2007; Phan et al., 2007b; Sdiri-Loulizi et al., 2008]. Although this study demonstrated that NoV was the major pathogen as recently reported in Japan [Phan et al., 2007b], some reports have indicated that rotavirus is the major pathogen [Bon et al., 1999; Colomba et al., 2006; Chen et al., 2007; Sdiri-Loulizi et al., 2008]. The difference of the pathogen detection rate may also be partly explained by differences in the target country, patients (outpatients vs. hospitalized), settings (sporadic vs. outbreak), and detection methods (ELISA, and multiplex or uniplex PCR with different primer sets).

The novel finding in this study was that SaV is not rare in outpatients with acute gastroenteritis who consult pediatric clinics in Japan. The detection rate of SaV among acute gastroenteritis ranged from 5.0% to 29.9% from 2002 to 2007, and the detection rate of SaV was increased in 2007 in Kumamoto Prefecture. This increase was not due to an improvement in the detection method, because the same SaV screening system was used for all of the samples. It is of note that the SaV detection rate targeting the polymerase region was low compared to that targeting polymerase-capsid junction or capsid. This is mainly due to the inability to detect GIV and GV SaV (Table III) by using the primers SR80 and/or JV 33, and the SaV detection may be underestimated in the previous studies [Buesa et al., 2002; Olesen et al., 2005; Ike et al., 2008; Sdiri-Loulizi et al., 2008].

In this study, dynamic change of the SaV genogroups was confirmed for the first time in the restricted area over 5 years, and found that SaV strains belonging to GIV suddenly appeared in 2007. A possible explanation for the emergence of these strains may be the lack of host immunity against these viruses, because SaV strains belonging to GIV were not found in this area from 2002 to 2006 in the surveillance (Table III). Similar strains (~99% nucleotide identity) were reported from another Prefecture, Osaka, in Japan in 2007 and 2008 (AB327281 and AB433785, Fig. 2), suggesting that GIV SaV may have spread widely throughout Japan as a new dominant strain; nationwide surveillance is necessary to determine whether this is the case.

The transmission route of human SaV remains to be elucidated. However, human-to-human infection is one possible route, because Kumamoto-62, and -67 were found in children attending the same kindergarten in November 2007. Furthermore, Kumamoto-55 and -61 and Kumamoto-74 and -78 were detected from the same elementary school children in November 2007 and December 2007, respectively (data not shown). No food was suspected in these cases. However, food- borne SaV infection may not be ruled out [Noel et al., 1997], because SaV was detected in clams in brackish water [Hansman et al., 2007c] and in environmental samples including river specimens, untreated wastewater, and treated wastewater [Hansman et al., 2007e]. SaV viral loads in these fecal specimens were as high as those of recent outbreak cases [Hansman et al., 2007a, 2007d; Wu et al., 2008], and could be the source of the infection. Further

TABLE III. Description of 81 SaV Strains, PCR Results, Genogroup, Viral Load, Patients Sex, Age, and Clinical Symptoms

Year	Strain name	Accession no.	Multiplex RT-PCR[a]	RT-PCR[b]	Nested RT-PCR[c]	Genogrouping RT-PCR[d]	Genogroup[e]	Real-time RT-PCR copies/g stool	Sex	Year	Month	Diarrhea[f]	Vomiting	Abdominal pain	Fever[g]	Other pathogen
2002	Kumamoto1/ Dec2002/JPN	AB429079	+	+	+	+	GI	4.30×10^{9}	F	1	4	+				
	Kumamoto2/ Dec2002/JPN	AB429080	+	+	+	+	GI	7.84×10^{9}	F	1	9	+	+			
	Kumamoto3/ Feb2003/JPN	AB429081	+	+	+	+	GI	9.12×10^{8}	M	0	10	+	+			
	Kumamoto4/ Feb2003/JPN	AB429082	+	+	+	+	GII	$7.6S \times 10^{6}$	M	1	9	+	+			
	Kumamoto5/ Feb2003/JPN	AB429083	−	+	+	+	GII	5.57×10^{6}	F	0	5	+	+			
2003	Kumamoto6/ Mar2003/JPN	AB429084	+	+	+	+	GII	5.98×10^{8}	F	1	0	+	+			
	Kumamoto7/ Apr2003/JPN	AB429085	+	+	−	+	GII	1.98×10^{7}	M	1	5	+b				
	Kumamoto8/ Dec2003/JPN	AB429086	−	+	−	+	GII	1.01×10^{9}	M	4	11		+	+		
	Kumamoto9/ Dec2003/JPN	AB429087	−	+	+	+	GII	$1.38 \times J01^{9}$	F	2	0	+			+	
2004	Kumamoto10/ Jun2004/JPN	AB429088	−	+	+	+	GV	1.78×10^{9}	M	8	2		+		+	
	Kumamoto11/ Dec2004/JPN	AB429089	−	+	+	+	GV	1.97×10^{9}	F	4	6		+	+		
	Kumamoto12/ Jan2005/JPN	AB429090	+	+	+	+	GI	5.41×10^{8}	F	3	3	+	+			
	Kumamoto13/ Nov2005/JPN	AB429091	+	+	+	+	GI	5.05×10^{5}	F	7	6	+	+	+		
	Kumamoto14/ Nov2005/JPN	AB429092	+	+	+	+	GI	1.73×10^{10}	M	1	8	+	+			
2005	Kumamoto15/ Dec2005/JPN	AB429093	+	+	+	+	GI	2.84×10^{8}	M	7	6		+			
	Kumamoto16/ Dec2005/JPN	AB429094	+	+	+	+	GI	7.58×10^{7}	M	4	7		+	+		
	Kumamoto17/ Dec2005/JPN	AB429095	+	+	+	+	GI	2.40×10^{6}	F	1	6	+	+			
	Kumamoto18/ Dec2005/JPN	AB429096	+	+	+	+	GI	4.57×10^{9}	M	9	7			+		NoV GII
	Kumamoto19/ Sep2006/JPN	AB429097	+	+	+	+	GI	4.54×10^{9}	F	2	1	+	+			
	Kumamoto20/ Nov2006/JPN	AB429098	+	+	+	+	GI	3.06×10^{9}	M	4	9	+	+			
	Kumamoto21/ Nov2006/JPN	AB429099	−	+	+	+	GV	8.35×10^{8}	F	3	7	+b			+	
	Kumamoto22/ Dec2006/JPN	AB429100	+	+	+	+	GI	1.86×10^{5}	F	3	9	+b			+	
	Kumamoto23/ Dec2006/JPN	AB429101	+	+	+	+	GII	7.63×10^{9}	M	3	4		+	+		
2006	Kumamoto24/ Dec2006/JPN	AB429102	+	+	−	+	GI	—	F	4	5	+				
	Kumamoto25/ Dec2006/JPN	AB429103	+	+	+	+	GI	1.19×10^{8}	M	2	7	+				

	Kumamoto26/Dec2006/JPN	AB429104	+	+	−	+	GII	—	F	0	9	+				
	Kumamoto27/Dec2006/JPN	AB429105	−	+	+	+	GII	9.64×10^{9}	F	2	8	+				
	Kumamoto28/Dec2006/JPN	AB429106	−	+	+	+	GII	4.22×10^{10}	M	3	1	+	+	+		
	Kumamoto29/Dec2006/JPN	AB429107	+	+	+	+	GI	1.50×10^{9}	F	6	9	+	+	+		
	Kumamoto30/Jan2007/JPN	AB429108	+	+	+	+	GI	6.08×10^{9}	M	4	5	+	+			
	Kumamoto31/Sep2007/JPN	AB429109	−	+	+	+	GIV	6.60×10^{8}	M	0	10	+		+		
	Kumamoto32/Oct2007/JPN	AB429110	−	+	+	+	GIV	4.87×10^{9}	M	4	11	+b				
	Kumamoto33/Oct2007/JPN	AB429111	−	+	+	+	GIV	3.23×10^{9}	M	9	6		+	+		
	Kumamoto34/Oct2007/JPN	AB429112	−	+	+	+	GIV	3.79×10^{7}	M	8	2	+	+	+		
	Kumamoto35/Oct2007/JPN	AB429113	−	+	+	+	GIV	4.12×10^{9}	F	6	1	+	+	+		
	Kumamoto36/Oct2007/JPN	AB429114	−	+	+	+	GIV	4.75×10^{10}	M	8	2	+	+			
	Kumamoto37/Oct2007/JPN	AB429115	−	+	+	+	GIV	7.42×10^{8}	M	1	11	+	+			
	Kumamoto38/Oct2007/JPN	AB429116	−	+	+	+	GIV	8.45×10^{9}	M	6	11		+	+	+	
	Kumamoto39/Oct2007/JPN	AB429117	−	+	+	+	GIV	1.01×10^{10}	F	4	6		+	+		
	Kumamoto40/Oct2007/JPN	AB429118	−	+	+	+	GIV	1.19×10^{9}	M	2	8	+		+		
	Kumamoto41/Oct2007/JPN	AB429119	−	+	+	+	GIV	4.09×10^{9}	F	1	3	+				EntV
	Kumamoto42/Oct2007/JPN	AB429120	−	+	+	+	GIV	2.86×10^{10}	M	2	0	+				
	Kumamoto43/Nov2007/JPN	AB429121	−	+	+	+	GIV	3.36×10^{8}	F	2	1	+				
	Kumamoto44/Nov2007/JPN	AB429122	−	+	+	+	GIV	1.75×10^{10}	M	5	2		+	+		
	Kumamoto45/Nov2007/JPN	AB429123	−	+	+	+	GIV	4.37×10^{9}	M	6	4	+	+			
	Kumamoto46/Nov2007/JPN	AB429124	−	+	+	+	GIV	7.47×10^{9}	F	1	1	+			+	
	Kumamoto47/Nov2007/JPN	AB429125	−	+	+	+	GIV	1.93×10^{9}	F	4	7	+		+		
	Kumamoto48/Nov2007/JPN	AB429126	−	+	+	+	GIV	5.62×10^{9}	M	6	1	+	+	+	+	
	Kumamoto49/Nov2007/JPN	AB429127	−	+	+	+	GIV	4.84×10^{5}	F	2	11			+		
	Kumamoto50/Nov2007/JPN	AB429128	−	+	+	+	GIV	9.49×10^{9}	F	1	6	+				
	Kumamoto51/Nov2007/JPN	AB429129	−	+	+	+	GIV	1.65×10^{9}	F	44	5	+	+			
	Kumamoto52/Nov2007/JPN	AB429130	−	+	+	+	GIV	2.89×10^{10}	F	1	3	+	+			
	Kumamoto53/Nov2007/JPN	AB429131	−	+	+	+	GIV	1.03×10^{10}	M	1	3	+	+			
	Kumamoto54/Nov2007/JPN	AB429132	−	+	+	+	GIV	1.93×10^{9}	F	1	1	+				
2007	Kumamoto55/Nov2007/JPN	AB429133	−	+	+	+	GIV	2.06×10^{9}	M	8	2	+b			+	
	Kumamoto56/Nov2007/JPN	AB429134	−	+	+	+	GIV	8.51×10^{8}	F	7	10	+				

(*Continued*)

TABLE III. (*Continued*)

Year	Strain name	Accession no.	Multiplex RT-PCR[a]	RT-PCR[b]	Nested RT-PCR[c]	Genogrouping RT-PCR[d]	Genogroup[e]	Real-time RT-PCR copies/g stool	Sex	Year	Month	Diarrhea[f]	Vomiting	Abdominal pain	Fever[g]	Other pathogen
	Kumamoto57/Nov2007/JPN	AB429135	–	+	+	+	GIV	1.43×10^{10}	M	2	2	+				
	Kumamoto58/Nov2007/JPN	AB429136	–	+	+	+	GIV	8.30×10^{5}	F	3	6		+			
	Kumamoto59/Nov2007/JPN	AB429137	–	+	+	+	GIV	5.41×10^{10}	F	6	4		+			
	Kumamoto60/Nov2007/JPN	AB429138	–	+	+	+	GIV	1.07×10^{11}	F	7	7	+	+	+		
	Kumamoto61/Nov2007/JPN	AB429139	–	+	+	+	GIV	2.19×10^{9}	F	8	0	+				
	Kumamoto62/Nov2007/JPN	AB429140	–	+	+	+	GIV	4.27×10^{9}	M	2	2	+				
	Kumamoto63/Nov2007/JPN	AB429141	–	+	+	+	GIV	4.26×10^{9}	F	2	4	+				
	Kumamoto64/Nov2007/JPN	AB429142	–	+	+	+	GIV	1.01×10^{10}	M	2	6	+b				
	Kumamoto65/Nov2007/JPN	AB429143	–	+	+	+	GIV	5.11×10^{9}	M	13	5		+	+		
	Kumamoto66/Nov2007/JPN	AB429144	–	+	+	+	GIV	9.18×10^{9}	M	3	8	+	+		+	
	Kumamoto67/Nov2007/JPN	AB429145	–	+	+	+	GIV	3.81×10^{9}	F	2	2	+				NoV GII
	Kumamoto68/Nov2007/JPN	AB429146	–	+	+	+	GIV	3.11×10^{9}	F	4	11		+		+	
	Kumamoto69/Nov2007/JPN	AB429147	–	+	+	+	GIV	3.29×10^{9}	M	5	1		+			NoV GII
	Kumamoto70/Nov2007/JPN	AB429148	–	+	+	+	GIV	4.54×10^{9}	M	7	10		+	+		
	Kumamoto71/Nov2007/JPN	AB429149	–	+	+	+	GIV	5.77×10^{9}	M	5	10		+		+	
	Kumamoto72/Dec2007/JPN	AB429150	–	+	+	+	GIV	6.96×10^{9}	F	2	0	+	+			
	Kumamoto73/Dec2007/JPN	AB429151	–	+	+	+	GIV	2.55×10^{10}	F	7	7		+	+		
	Kumamoto74/Dec2007/JPN	AB429152	–	+	+	+	GIV	3.25×10^{9}	F	8	5	+		+		
	Kumamoto75/Dec2007/JPN	AB429153	–	+	+	+	GIV	2.93×10^{9}	M	7	6	+		+		
	Kumamoto76/Dec2007/JPN	AB429154	–	+	+	+	GIV	1.32×10^{5}	M	6	1		+	+		
	Kumamoto77/Dec2007/JPN	AB429155	–	+	+	+	GIV	4.43×10^{9}	M	4	1	+		+		
	Kumamoto78/Dec2007/JPN	AB429156	–	+	+	+	GIV	2.50×10^{9}	F	8	3	+		+		
	Kumamoto79/Dec2007/JPN	AB429157	–	+	+	+	GIV	5.52×10^{9}	F	6	2	+		+		
	Kumamoto80/Dec2007/JPN	AB429158	–	+	+	+	GIV	9.33×10^{8}	M	9	11	+				
	Kumamoto81/Dec2007/JPN	AB429159	–	+	+	+	GIV	8.04×10^{9}	M	3	10	+			+	AstV

[a]Primers with SR80 and JV33, polymerase region [Vinje et al., 2000].
[b]Primers with SaV124F, SaV1F, SaV5F, and SaV1245R, polymerase-capsid junction [Oka et al., 2006].
[c]Primers F13,F14, R13 JU4 as a first PCR, and the F22 and R2 as second PCR, capsid region [Okada et al., 2006].
[d]Primers F13,F14, R13,R14 as a first PCR, and the F22 and G1R, G2R, G4R, and G5R as second [Okada et al., 2006].
[e]Genogrouping based on the partial capsid sequences [Farkas et al., 2004].
[f]+b is bloody diarrhea.
[g]Higher than 37.0°.

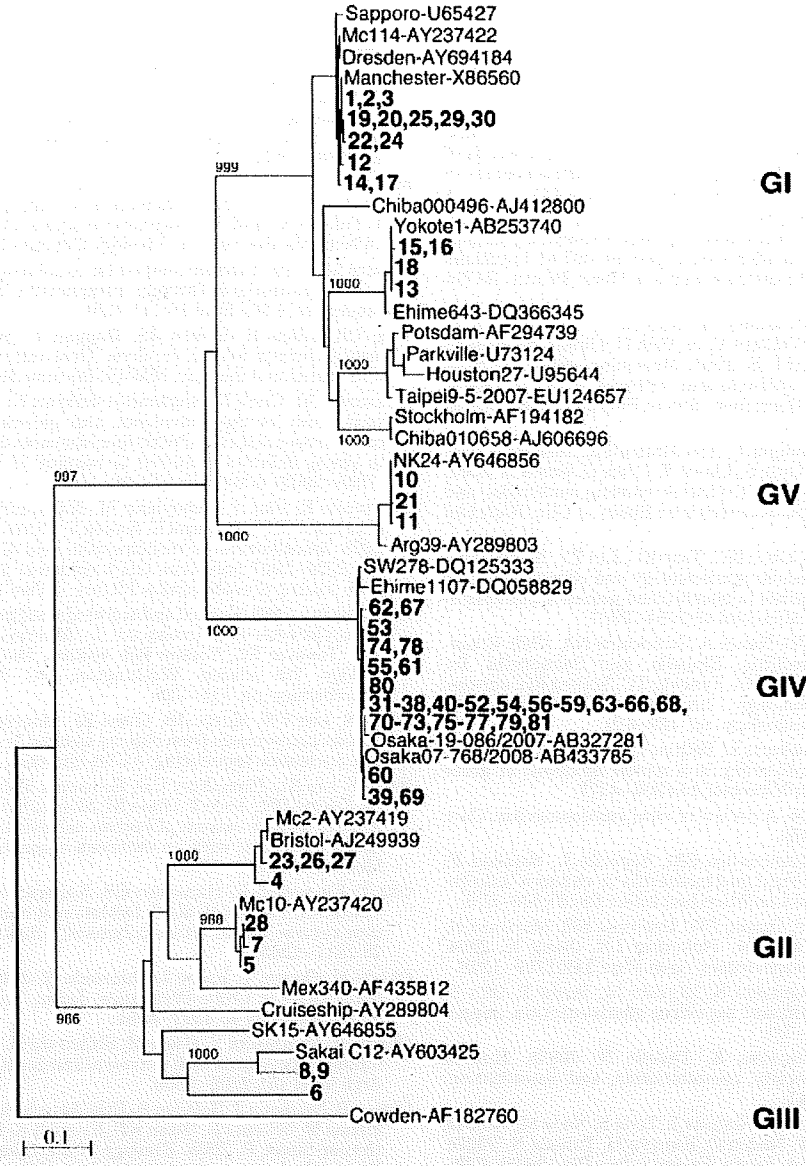

Fig. 2. Phylogenetic tree of SaV based on partial capsid nucleotide sequences. Strain numbers (e.g., 1 corresponds to Kumamoto-1 listed in Table III) of 81 SaV strains are shown in the tree. The numbers on each branch indicate the bootstrap values for the genotype, where the values of 950 or higher were considered statistically significant for the grouping. The scale represents the nucleotide substitutions per site.

clinical and environmental investigations are clearly needed to control SaV infection.

The season and clinical symptoms of NoV and SaV infections are similar (Fig. 1), making it difficult to distinguish between them without a laboratory diagnosis. SaV is not as widely screened for as NoV in Japan. Hence, SaV-associated gastroenteritis cases may be underestimated. Re-examination for the presence of SaV in stool specimens collected in various surveys may be worthwhile to determine the solid causative agent of gastroenteritis in humans.

In conclusion, NoV was the major pathogen in the surveillance, although the detection rate of SaV was not low, and genetically diverse SaV strains were circulated in Kumamoto Prefecture, Japan between 2002 and 2007. Systematic surveillance for gastroenteritis pathogens is important to preserve public health. In addition to NoV, continuous surveillance for other viruses including SaV using novel methods is indispensable to elucidate the genetic diversity of the viruses, to discover and identify novel strains, and eventually to control gastroenteritis.

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BRIEF REPORT

Quantitative analysis of fecal sapovirus shedding: identification of nucleotide substitutions in the capsid protein during prolonged excretion

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Abstract Sapovirus (SaV) is an important pathogen causing gastroenteritis in humans. Quantitative analysis of the viral loads in feces collected from two SaV outbreaks was performed. Our results showed that SaV excretion generally decreased to an undetectable level in 2 weeks; however, some individuals excrete SaV in feces at high concentrations for 2–4 weeks after the onset of illness. In addition, we identified for the first time nucleotide changes in the capsid region during prolonged excretion.

Sapovirus (SaV) is an important pathogen causing gastroenteritis that is associated with sporadic cases as well as outbreak cases [4, 5, 7, 12, 17, 20]. The SaV genome is a polyadenylated, single-stranded positive-sense RNA molecule of approximately 7.5 kb with two or three open reading frames (ORFs). ORF1 encodes nonstructural proteins (i.e., NTPase, VPg, 3C-like protease, and RNA-dependent RNA polymerase) and capsid protein (VP1), while ORF2 and ORF3 encode proteins with unknown functions. SaV strains are divided into five genogroups, GI–GV, based on the VP1 gene sequences, of which the GI, GII, GIV, and GV strains infect humans, while the GIII strains infect swine [3]. Human SaV strains are noncultivable, and electron microscopy (EM) and single-round or nested reverse transcription-polymerase chain reaction (RT-PCR) are used to detect SaV [2, 6, 8, 14, 16, 19, 21]. Recently, we developed a real-time RT-PCR assay that detects a broad range of genetically diverse human SaV strains and can be used for quantitative analysis [13].

There are two reports on SaV viral shedding after the onset of illness, one in which EM was used [9] and another in which single-round RT-PCR was used [18]. In these reports, it was shown that SaV shedding continues for approximately 2 weeks, i.e., 12 days [9] and 15 days [18], after the onset of illness. However, little is known about the amount of virus shed into feces after the onset of illness. In addition, there are no data regarding nucleotide changes that may occur during the prolonged excretion period.

The purposes of this study were, first, to analyze consecutive fecal specimens collected from two SaV outbreaks using a recently developed real-time RT-PCR [13] and nested RT-PCR [16], and, second, to determine the nucleotide sequence of the 2.3-kb 3′ end of the genome.

Outbreaks occurred at a care center for disabled people from 11 to 23 May 2005, in Nobeoka (outbreak 1), and at an elementary school from 11 May to 13 June 2005, in Nichinan (outbreak 2) in Miyazaki Prefecture, Japan. Twenty (45.4%) of 44 people and 86 (16.2%) of 531 people reported gastroenteritis symptoms in outbreak 1 and outbreak 2, respectively. Fecal specimens were collected from 18 patients in outbreak 1 and from 41 patients in outbreak 2 and initially used for screening of noroviruses by using a real-time RT-PCR [10]; however, all specimens

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were negative (data not shown). The fecal specimens were then examined for SaV using RT-PCR with SV-F11 and SV-R1 primers [14] and were positive for SaV in 13 (72%) of 18 patients in outbreak 1, and 7 (17%) of 41 patients in outbreak 2. Among them, 11 patients (designated as 1-1 to 1-11) in outbreak 1, and 6 patients (designated as 2-1 to 2-6) in outbreak 2 were monitored for the SaV viral load because two or more sequential fecal specimens were available from these patients (Table 1). Viral RNA was extracted from 10% fecal specimens using a QIAamp Viral RNA Mini Kit according to the manufacturer's instructions. The quantitative real-time RT-PCR was carried out as described previously [13]. The viral loads of these patients were found to be between 1.91×10^5 and 2.23×10^9 (outbreak 1, $n = 11$) and between 1.32×10^5 and 8.25×10^9 (outbreak 2, $n = 6$) cDNA copies per gram of feces at the first collection point (day 1–day 15 after onset) (Table 1). The level decreased below the detection limit, 1.29×10^5 cDNA copies per gram of feces [13], at the second collection point(s), except in 3 of 11 patients, patients 1-1, 1-2, and 1-3, in outbreak 1, and in 1 of 6 patients in outbreak 2, patient 2-1, as shown in Table 1. Patient 1-1 (age 14, male) had 2.23×10^9 and 2.32×10^5 copies per gram of feces at days 3 and 11, respectively. Patient 1-2 (age 12, male) had 2.24×10^7 and 7.94×10^5 copies per gram of feces at days 15 and 25, respectively, and the copy number decreased below the detection limit at day 30. Patient 1-3 (age 15, male) had 2.87×10^8, 2.66×10^6 and 2.38×10^5 copies per gram of feces at days 6, 14 and 28, respectively, and the copy number decreased below the detection limit at day 31. Patient 2-1 (age 10, male) had 8.25×10^9 and 4.43×10^5 copies per gram of feces at days 3 and 12, respectively, and the copy number decreased below the detection limit at day 26 and day 33. To confirm the real-time RT-PCR results, a recently developed RT-PCR with primers SV-F13, SV-F14, SV-R13, and SV-R14 for the first PCR [16] and SV-F22 and SV-R2 for the second PCR [16] was performed. The results were consistent with those of real-time RT-PCR, except in the specimen collected from patient 1-4 at day 7 after onset, the specimen from patient 1-11 at day 3 after onset, the specimen from patient 2-2 at day 19 after onset, and the specimen from patient 2-3 at day 4 after onset of illness (Table 1). Sequence analysis of the nested RT-PCR products (approx. 350 nt) demonstrated that each outbreak was caused by a single SaV strain belonging to a different GI cluster (data not shown).

To further characterize the SaV strains, sequential specimens collected from four patients (1-1, 1-2, 1-3, and 2-1) were subjected to long sequence analysis. For this purpose, cDNA was re-synthesized as follows: 10 μl of the extracted viral RNA solution with 1 μl TX30SXN primer [7] (20 pmol/μl) was incubated at 92°C for 2 min, annealed at 37°C, and then mixed with 4 μl of RT buffer (Invitrogen), 2 μl 100 mM DTT (Invitrogen), 1 μl 10 mM dNTP (Toyobo), 1 μl SuperScript III reverse transcriptase (200 U/μl) (Invitrogen), and 1 μl RNaseOUT ribonuclease inhibitor (40 U/μl) (Invitrogen). This mixture was then incubated first at 50°C for 2 h and then at 95°C for 5 min. The 2.3-kb 3′ end of the genome corresponding to the entire capsid region, the ORF2 gene, and the 3′ untranslated region was amplified by semi-nested RT-PCR; two forward primers, SV-F13 [16] and SV-F14 [16], and a reverse primer, TX30SXN, were used for the first PCR, and SV F11 [14] and TX30SXN primers were used for the second PCR with high-fidelity PCR enzyme. A final volume of 100 μl of the reaction mixture containing 2 μl of the cDNA or the first PCR reaction mixture, 10 μl of 10 × KOD-Plus DNA polymerase buffer, 10 μl of 2.5 mM dNTPs, 4 μl of 25 mM $MgSO_4$, 2.5 μl of dimethylsulfoxide (Sigma), 2 μl of forward primers (20 pmol/μl each), 2 μl of reverse primers (20 pmol/μl), and 2 μl of KOD-Plus DNA polymerase (1 U/μl) (Toyobo), was subjected to PCR at 94°C for 10 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 5 min, and a final extension at 72°C for 15 min, and was then held at 4°C. The PCR products were separated by 1% agarose gel electrophoresis, purified using a QIAquick PCR Purification Kit (Qiagen), and directly sequenced with the Big Dye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems). Phylogenetic analysis, nucleotide sequences analysis, and amino acid sequences analysis were performed as described previously [11]. The nucleotide sequences, 2,307 nt from patients 1-1, 1-2, and 1-3, and 2,277 nt from patient 2-1, were determined and deposited in DDBJ under the accession numbers AB455796–AB455804.

Sequence analysis of the 3′ terminal 2,307 nt of the viral genome showed a single nucleotide difference in an ORF2 protein-encoding region (nt 1,713–2,204) in patients 1-1 (AB455796 and AB455797), 1-2 (AB455798 and AB455799), and 1-3 (AB455800, AB455801, and AB455802) in outbreak 1; i.e., T^{1972} (the number indicates the nucleotide residue from the putative VP1 start codon) in patients 1-1 and 1-2 was C^{1972} in patient 1-3, although this change was synonymous (data not shown). In patient 1-1, no difference was found in the VP1-encoding region at day 3 and day 11 (AB455796 and AB455797). In patient 1-2, two nucleotide changes were found at day 25 (AB455799) when compared with those at day 15 (AB455798), i.e., microheterogeneity G/A which was recognized by the superimposed signals in the sequencing reaction at nucleotide position 613 (GTC of Val^{205} [the number indicates the amino acid residue from the putative starting Met of the VP1] at day 15, GTC of Val^{205} and ATC of Ile 205 at day 25, respectively) and G–C at nucleotide position 699 (GAG of Glu^{233} at day 15, GAC of

Table 1 Fecal specimens collected from two outbreaks

Specimens	Sex	Age	Symptoms at onset of illness					Specimen collected days after onset of illness	Real-time RT-PCR (copies/g stool)	Nested RT-PCR		Accession no.	Nucleotide differences in capsid
			Diarrhea	Vomiting	Nausea	Abdominal pain	Fever[a]			1st	2nd		
Outbreak 1													
1-1	M	14	+	+				3	2.23×10^9	+	+	AB455796	
								11	2.32×10^5	−	+	AB455797	
1-2	M	12				+	+	15	2.24×10^7	+	+	AB455798	
								25	7.94×10^5	−	+	AB455799	613 G/A, 699 G–C
								30	−	−	−	−	
1-3	M	15	+	+			+	6	2.87×10^8	+	+	AB455800	
								14	2.66×10^6	−	+	AB455801	
								28	2.38×10^5	−	+	AB455802	894 T–A
								31	−	−	−	−	
1-4	M	15	+					7	3.95×10^5	−	−	−	
								22	−	−	−	−	
1-5	M	18	+		+	+		4	2.88×10^6	−	+	−	
								21	−	−	−	−	
1-6	M	16	+					2	5.62×10^7	+	+	−	
								14	−	−	−	−	
1-7	F	19	+			+		1	9.49×10^7	+	+	−	
								16	−	−	−	−	
1-8	M	16	+	+				5	5.77×10^8	+	+	−	
								26	−	−	−	−	
1-9	F	20	+			+		5	1.18×10^8	+	+	−	
								28	−	−	−	−	
1-10	M	13	+					3	1.02×10^8	+	+	−	
								25	−	−	−	−	
1-11	F	16	+					3	1.91×10^5	−	−	−	
								31	−	−	−	−	
Outbreak 2													
2-1	M	10	+	+				3	8.25×10^9	+	+	AB455803	
								12	4.43×10^6	−	+	AB455804	
								26	−	−	−	−	
								33	−	−	−	−	
2-2	M	10		+				2	1.32×10^9	+	+	−	
								19	−	−	+	−	
								26	−	−	−	−	
2-3	M	34	+					4	1.32×10^5	−	−	−	
								9	−	−	−	−	
2-4	F	26	+					5	3.84×10^7	−	+	−	
								10	−	−	−	−	
2-5	M	11				+		10	5.31×10^7	+	+	−	
								21	−	−	−	−	
								28	−	−	−	−	
2-6	M	12	+					14	9.38×10^5	−	+	−	
								28	−	−	−	−	

[a] Higher than 37.0 degrees

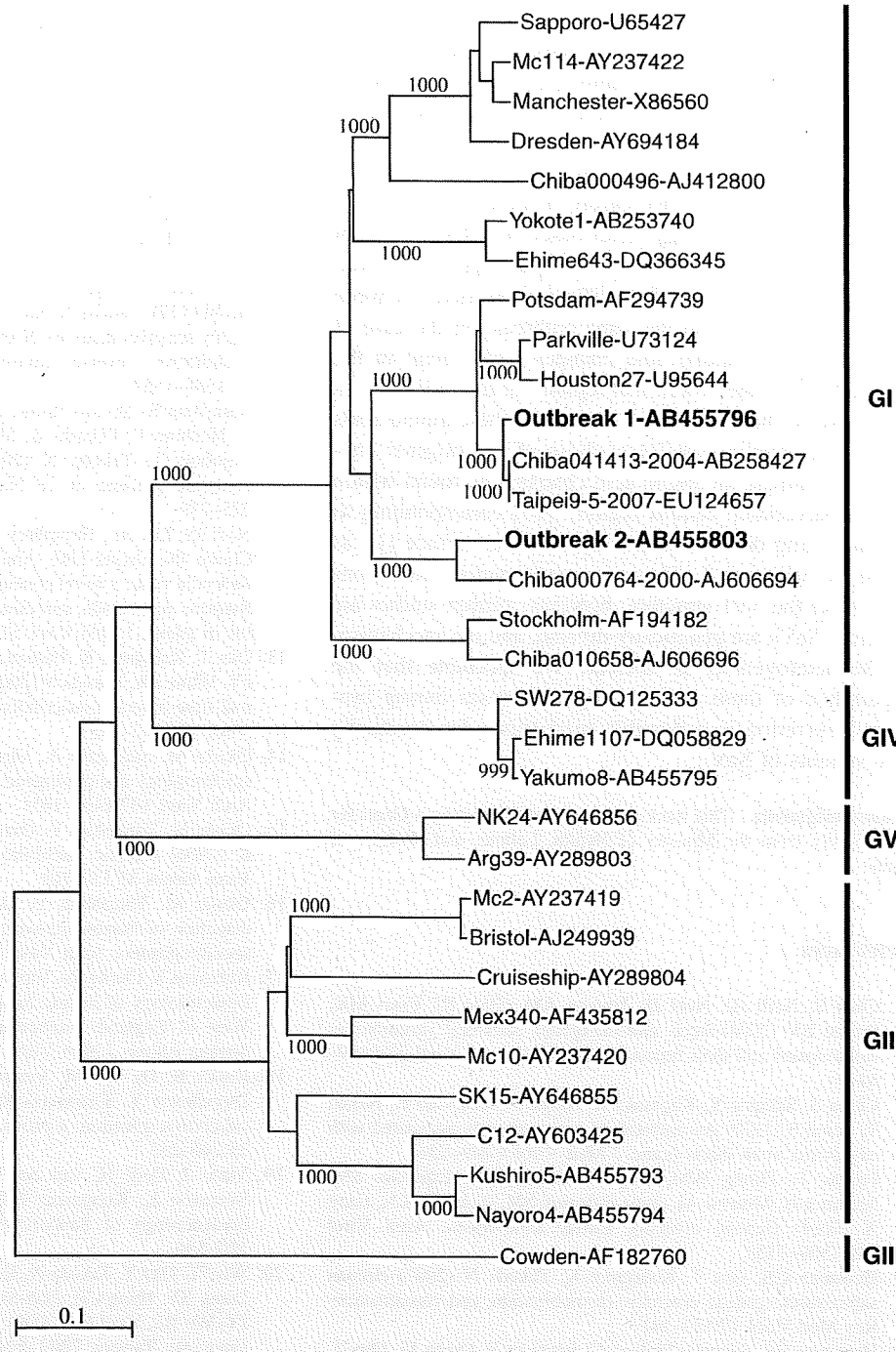

Fig. 1 Phylogenetic tree of SaV based on complete nucleotide sequences of the capsid gene. Two representative SaV strains at the first collection point from patients in outbreak 1 (1-1, AB455796) and outbreak 2 (2-1, AB455803) are shown as *bold letters* in the *tree*. The *number on each branch* indicates the bootstrap value, where a value of 950 or higher is considered statistically significant for the grouping. The *scale* represents nucleotide substitutions per site

Asp[233] at day 25, respectively) (Table 1). In patient 1-3, a single nucleotide change was found in VP1-encoding region at day 28 (AB455802) compared to those at day 6 and day 14 (AB455800 and AB455801), i.e., T–A at nucleotide position 894 (AAT of Asn[298] at day 6 and day 14, AAA of Lys[298] at day 28) (Table 1). Sequence analysis of 2,277 nt of the 3′ terminus of the genome from patient 2-1 in outbreak 2 showed no nucleotide substitution at day 3 and day 12 (AB455803 and AB455804). Phylogenetic analysis with the entire VP1 sequence revealed that SaV strains from outbreak 1 and outbreak 2 belong to GI with different clusters (Fig. 1).

Our results showed that SaV excretion decreased with time and generally decreased to an undetectable level in 2 weeks; however, some individuals excrete SaV in feces at high concentrations for 2–4 weeks after the onset of

illness. The presence of individuals with high viral loads could be a possible source of SaV infection via the human-to-human route through a contaminated lavatory; however, the transmission route of human SaV remains to be elucidated.

In addition, we identified for the first time the nucleotide change(s) in the SaV VP1 during a prolonged excretion period (i.e., 25 and 28 days after onset of illness in patient 1-2 and patient 1-3 in outbreak 1, respectively). However, the mutations were found in different positions between patients 1-2 and 1-3 in the same outbreak. In the case of patient 1-2, two amino acid changes were found in the "second half of the N-terminal region" of the SaV VP1, as described by Okada et al. [15]; however, these amino acids have characteristics similar to those of the original residues. In contrast, an amino acid change was found within the "hypervariable central region" [15] corresponding to the protruding domain located on the viral surface [1], as found in patient 1-3. These non-synonymous mutations may affect the VP1 structure; however, further studies are required. SaVs are genetically diverse, and the mechanism of the heterogeneity is unclear. We speculate that the appearance of these mutant progeny viruses during prolonged excretion is a possible mechanism for producing new variants of SaV.

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ノロウイルス抗原キット“クイックEx-ノロウイルス®”の行政検査における有用性の検討

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Summary

Noroviruses (NVs) are the representative pathogen virus of acute gastroenteritis which is prevalent in winter. Every year, NVs also cause much gastroenteritis outbreaks in closed colonies as a result of their high infectivity. In this study, we evaluated practicality of NV antigen detection kit "Qick Ex Norovirus®" (Ex-NV) associated with immunochromatography which was put on the market in November 2007 at the administration inspection of outbreaks in comparison with real-time PCR that is common method at present. As compared with real-time PCR, the efficient sensitivity and specificity were 94.0% (47/50), 88.5% (23/26) and 100% (24/24) respectively using sufficient amount of 50 stool samples. There were 3 stool samples that belonged to genogroup Ⅰ that did not correlate with real-time PCR. Whereas, the sensitivity using 32 rectum swabs as samples was 22.2% (4/18). Although the specificity and efficiency were 100% (14/14) and 56.3% (18/32), respectively. We found that the limited number of NV detected by Ex-NV was 10^6 copies per gram of stool. Using Ex-NV, we could complete brief examination protocol in about 30 minutes after receiving samples, without expensive apparatus such as thermal cycler. It this study, we conclude that Ex-NV is useful for examination in gastroenteritis outbreak cases. On the other hand, it is necessary to use more sensitive method such as real-time PCR, in the case of all negative samples or examination of no symptomatic persons, probably less amount of NV in their stool samples.

キーワード

ノロウイルス　迅速診断　リアルタイムPCR　イムノクロマト法

ノロウイルス(NV)は毎年冬期に流行する感染性胃腸炎の代表的な原因ウイルスであり，近年になってNVが原因とされた患者報告数と施設等における集団感染事例報告数が増大している[1,2)]．NVは少ないウイルス量で感染し，患者の糞便や吐物中に多量に排出されることから，ひとたび流行すると容易に拡大する危険性を孕んでいる．さらに，NVの感染様式は経口感染であるが，体内に取り込まれる経路としては食品，水，汚染された手指，空気中を漂う塵埃[3)]など多様であることも一因であろう．

このような背景から，地域の衛生管理を担う保健所は感染症情報を的確かつ迅速に把握し，感染拡大防止策をとらなくてはならない．行政機関でNV検査を行う場合，現状では，保健所が採取した検体は各都道府県および政令市ごとに設置された地方衛生研究所に搬送され，リアルタイムPCR法等による遺伝子検査を行い，成績を保健所へ報告する体制をとっている自治体がほとんどである．しかしながら，流行期においては限られた人員で短時間に作業を実施することは困難であり，感染防止対策も遅れがちになる危険がある．そのため，検体運搬の必要のない，各保健所で簡単に実施できる検査法の導入が望まれる．

現在NVの検査法として原理の異なる5種類

1) 秋田県健康環境センター保健衛生部微生物班（〒010-0874 秋田県秋田市千秋久保田町6-6）
(平成20年4月4日受付・平成21年2月12日受理)

(ELISA 法，RT-PCR 法，NASBA 法，RT-LAMP 法，TRC 法)のキットが市販されているが，保健所の検査室の規模によっては常備されていない機器が必要である．そこで，イムノクロマト法を原理とした NV 抗原検査キット“クイック Ex-ノロウイルス®”(Ex-NV)の迅速性と特殊な機器を必要としない簡便な操作性に着目し，行政検査における有用性を検証すべく，胃腸炎集団感染事例の検体を用い，現行のリアルタイム PCR 法と比較検討した．

Ⅰ　対象および方法

1. 対　象

2007 年 12 月～2008 年 5 月の間に，秋田県内の保健所から当センターに NV 検査依頼のあった 494 検体 69 事例のうち，82 検体(14 事例)を無作為に抽出し対象とした．

2. 材　料

検査材料は，糞便 50 検体，直腸スワブ 32 検体を用いた．糞便は自然排便されたものを用い，ウイルス検査用滅菌容器に採取した．直腸スワブは各患者に 1 本滅菌綿棒を配布し，綿球を隠れる程度まで患者肛門へ挿入し，検体を採取した．検体は患者本人または施設従業員が家庭や施設において採取するという特殊な事情があるため，挿入時の痛みを軽減したい場合は水道水で湿らせるように指導している．検体は冷蔵保存して当センターに搬送され，採取から 1 日以内に検査を行った．

3. 試薬と方法

1) Ex-NV 法

Ex-NV 法は添付の文書に準拠して以下の通り実施した．

前処理用チューブに糞便検体(固形便は小豆大で約 0.1 g，液状便は約 0.1 m*l*)と検体浮遊液を，1：9 の割合になるように入れ，混和し試料とする．その試料を 6,000×g で 5 分間遠心した後，上清を 300μl 採取し，検体浮遊チューブに移す．チューブに試料濾過フィルターを装着し，試料全量を反応容器に滴下する．テストストリップを反応容器に挿入し，反応容器キャップを装着する．15～30℃ で 15 分静置後，目視判定する．判定部にコントロールライン(青色)とテストライン(青色)が現れた場合を陽性とし，コントロールラインのみが現れた場合は陰性，コントロールラインが現れない場合は試験無効とし，再測定を行う．

2) リアルタイム PCR 法

糞便は約 0.01 g，直腸スワブは綿球部分を蒸留水 1 m*l* に攪拌混合し，乳剤とした後，UltraClean 15 を用いたグラスミルク法[4]により RNA を抽出した．抽出した RNA は，総量 50μl となるように蒸留水で溶解した．NV の検出とコピー数の測定は，Kageyama ら[5]のリアルタイム PCR 法に準じて行った．使用試薬は LightCycler RNA Amplification Kit Hybridization Probes(ロシュ・ダイアグノスティクス)，機器は LightCycler 320 S(ロシュ・ダイアグノスティクス)で反応容量は 20μl である．

4. 検討方法

1) リアルタイム PCR 法と Ex-NV 法の相関

糞便と直腸スワブ検体について，リアルタイム PCR 法と Ex-NV 法を同時に実施した．結果の基準はリアルタイム PCR 法とし，これに対する Ex-NV 法の一致率，感度，特異度を求めた．

2) 検出感度

Ex-NV 法の検出感度の検討には，本法で陽性と判定された genogroup Ⅱ(GⅡ)/4 を含む糞便 3 検体と genogroup Ⅰ(GⅠ)/6 を含む 1 検体を用いた(No. 1，2，3，4)．検体はそれぞれ別事例由来である．前述の操作により作製した試料を原液とし，検体浮遊液で 10 倍段階希釈を行い，10^4 までの各希釈液について Ex-NV 法による検査を実施した．

Ⅱ　結　果

1. リアルタイム PCR 法と Ex-NV 法の相関

1) 糞便検体について

糞便検体における PCR 法と Ex-NV 法の一致状況を表 **1** に示した．

50 検体中リアルタイム PCR 法陽性は 26 検体であり，genogroup の内訳 GⅠ：GⅡ は 4：22 であった．このうち，Ex-NV 法で陽性と判定されたのは 23 検体であり，NV 量は糞便 1 g 当たり 5.80×10^6～7.25×10^9 コピー(copies/g)であった．なお，Ex-NV 法で陰性となった不一致例 3 検体はいずれも GⅠ に属するものであり，NV 量は 3.69×10^8～2.23×10^9copies/g であっ

表1　糞便検体を用いたリアルタイムPCR法とEx-NV法の相関

		リアルタイムPCR			
		陽性		陰性	合計
		GⅠ	GⅡ		
Ex-NV	陽性	1	22	0	23
	陰性	3	0	24	27
	合計	26		24	50

リアルタイムPCR法との一致率：94.0%
感度：88.5%
特異度：100%

表2　直腸スワブ検体を用いたリアルタイムPCR法とEx-NV法の相関

		リアルタイムPCR			
		陽性		陰性	合計
		GⅠ	GⅡ		
Ex-NV	陽性	0	4	0	4
	陰性	0	14	14	28
	合計	18		14	32

リアルタイムPCR法との一致率：56.3%
感度：22.2%
特異度：100%

た．PCR法で陰性であった24検体はすべてEx-NV法でも陰性と判定され，疑陽性例は認められなかった．以上から，リアルタイムPCR法に対するEx-NV法の一致率は94.0%であり，感度は88.5%，特異度は100%であった．

2）直腸スワブ検体について

直腸スワブ検体におけるPCR法とEx-NV法の一致状況を表2に示した．

32検体中リアルタイムPCR法陽性は18検体であり，genogroupはすべてGⅡであった．このうち，Ex-NV法で陽性と判定されたのは4検体であった．リアルタイムPCR法で陰性であった14検体はすべてEx-NV法でも陰性と判定され，疑陽性例は認められなかった．以上から，リアルタイムPCR法に対するEx-NV法の一致率は56.3%であり，感度は22.2%，特異度は100%であった．

2．Ex-NV法の検出感度

Ex-NVの検出感度に関する検討結果を表3に示した．検体No.1(GⅡ/4：1.15×10^{9}copies/g)およびNo.2(GⅡ/4：4.88×10^{8}copies/g)はいずれも原液から10^{2}倍希釈までEx-NVで陽性を示したが，10^{3}倍以上の希釈では検出できなかった．検体No.3(GⅡ/4：2.39×10^{7}copies/g)およびNo.4(GⅠ/6：5.05×10^{6}copies/g)は原液のみ陽性であり，希釈検体では検出できなかった．

Ⅲ　考　察

わが国におけるNV感染症は冬期に流行し，時に大きな集団発生につながることが知られている．保健福祉施設や病院などでは，抵抗力の低下した患者が脱水や吐物窒息などによって重篤化，時に死に至るケースも報告されている．ひとたびNVアウトブレイクが発生すると，就業制限等による業務の停滞や公的機関の閉鎖，検査や感染制御に多くの資金が投入されるなど，一般社会において混乱と損害を招くことはこれまでの事例から明らかである．NVの拡大を防止するには感染者の早期発見と適切な衛生管理が最も効果的であり，そのためには地域医療の最前線にある保健所において，NVの迅速診断が随時実施できる体制が望ましい．

行政におけるNV検査は，国立感染症研究所の通

表3　Ex-NV法の検出感度

No.	genogroup	糞便1g中のNV量(copies)	希釈系列					
			原液	×10	$\times10^{2}$	$\times10^{3}$	$\times10^{4}$	$\times10^{5}$
1	GⅡ/4	1.15×10^{9}	+	+	+	−	−	−
2	GⅡ/4	4.88×10^{8}	+	+	+	−	−	NT
3	GⅡ/4	2.39×10^{7}	+	−	−	−	NT	NT
4	GⅠ/6	5.05×10^{6}	+	−	−	NT	NT	NT

NT：not tested

知[6]に基づいて遺伝子増幅法，ELISA法，電子顕微鏡によるウイルス粒子の直接検出の3法が実施されている．遺伝子増幅法は検出感度が10 copies/μlと良好であるものの，サーマルサイクラーなどの特殊機器を必要とする．ELISA法および電子顕微鏡法も一般の保健所では常備されていない機器が必要であり，さらに感度が$10^{6\sim7}$copies/g[6,7]と高くない．このような検査法の特徴を理解したうえで，各機関の実情にあった方法が選択されている．

新たに開発されたEx-NV法は，イムノクロマト法を用いたNV抗原の検出キットである．本法は簡便性(特殊機器を用いない)と迅速性(検査時間短縮)に優れ，従来法の問題点を解決した方法といえる．本検討では集団感染事例の検体を用いて，リアルタイムPCR法とEx-NV法を比較し，保健所をはじめとする行政機関での活用が可能かどうかを検証した．

糞便を用いた検討では，Ex-NV法はリアルタイムPCR法と同等の検出率であり，本法の有用性が確認された．これはELISA法で報告されているPCR法との一致率(64.8～83.2%)[7]を上回るものであった．PCR法陽性であったにもかかわらずEx-NV法で検出できなかった検体の抗原型は，それぞれGⅠ/4，GⅠ/8，GⅠ/11(DDBJ Accession No. AB 430878，AB 430338，AB 428374)であった．Ex-NVにはグループで6遺伝子型(GⅠ/1～4，GⅠ/6，GⅠ/11)，GⅡグループで13遺伝子型(GⅡ/1～8，GⅡ/12～15，GⅡ/17)の抗体が混用されている[8]．GⅠ/8の検体が偽陰性であった原因は，Ex-NVの使用抗体が対応していなかったことによると考えられた．また，使用抗体に含まれているにもかかわらずGⅠ/4とGⅠ/11が検出できなかった要因として，Ex-NVの使用抗体はバキュロウイルス発現によって作製されたNV様人工粒子に対するものであるため，作製時に使用したNVと現在流行しているNVとは抗原構造に乖離する部分がある可能性が考えられた．

Ex-NVの検査材料は原則として自然排便であり，直腸擦過物の使用は可としていない．しかしながら，地域や施設内等で保健所職員が限られた時間で一斉に便を回収するには，時間を選ばず簡単に実施できる直腸採便が効率的である．このような背景から，本検討では直腸スワブを用いた場合の検出状況も検討に加えた．その結果，直腸スワブの一致率，感度は糞便検体に比べて著しく低いものであった．スワブで採取した場合，Ex-NV法で必要な0.1 gの糞便が採取されているか否かが肉眼的に判断できない．検出感度が低かった要因は，採取量不足の検体が含まれていたことによると考えられた．さらに直腸スワブは，今現在下痢をしていない場合でも採取できるため，NV感染の急性期を過ぎた患者が検査の対象とされた可能性もある．糞便中のNV量は有症期が最も多く，症状の改善とともに減少する．したがって，自然排便が困難だからとの理由で，安易に直腸スワブを使用することは避けるべきである．

糞便検体を用いた感度に関する検討結果から，Ex-NV法で検出できるウイルス量は10^6copies/g以上と判断された．この値は，ウイルス含有量が既知の糞便検体をキット添付の希釈液で段階的に希釈することにより求めた予測値である．実際には糞便中の反応阻害物質などの影響により，これとは若干の差異があるかもしれない．しかし，NV感染時の糞便中ウイルス量は急性期にピークとなり，感染から数日間は$10^{7\sim8}$copies/g程度が排出される[9]．したがってEx-NVは，発症初期に適切に使用されれば十分検出できる感度であると考えられた．なお，ELISA法で報告[10]されているように，NVの抗原型によっては反応性や検出感度が異なることがある．本検討でも，検出できなかった型がGⅠで認められた．しかし，近年のわが国におけるNVは80%以上がGⅡ型(そのうち2006～2008年は80%以上がGⅡ/4)である[11～13]．Ex-NVは日本の流行株の抗原型は網羅しており，集団発生時における利用価値は高いと思われた．GⅠ型で経験される感度のばらつきについては，今後さらなる改良が望まれる．われわれも抗原型の動向に留意し，継続的に監視することが必要であろう．

以上のことから，Ex-NV法は感染症をより迅速・正確・効率的に検出する義務をもつ保健所で使用される場合に有用なキットであると思われた．集団感染発生時にはEx-NVを使用して初期診断を下し，感染源や感染経路，広がりを追跡調査して適切な感染対策を講じる．そのためには適切な時期に糞便を採取し，直ちに検査に供することが必要である．高感度のPCR法などは，抗原型の精査や無症状者の検査，あるいは検体すべての結果がEx-NV法で陰性であった場合のみに用いることとし，運用法を工夫するこ

とで検査費用や人件費等の削減にも貢献できると考えられた.

Ⅳ 結　語

イムノクロマト法によるEx-NVは，行政検査においてPCR等に併せて利用することで，迅速な原因究明と行政対応に有用であると考えられた.

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