not shown). The 58 kDa protein was in agreement with the molecular mass calculated from 530 aa of the SeV ORF2. The supernatant of the infected cells 6 days p.i. was clarified at 10 000 x g for 30 min and centrifuged at 100 000 x g for 2 h in a Beckman TLA-45 rotor. The pellet was suspended in a few drops of water and examined by electron microscopy (EM). Uniform, round-shaped empty VLPs with 38 nm in diameter were observed at 200 particles per EM field (data not shown). A typical yield of the VLPs was 0.1-0.2 mg per 2 x 10^7 Tn5 cells after CsCl equilibrium gradient centrifugation followed by sucrose density gradient centrifugation (9).

Hyperimmune antisera to the purified recombinant SeV (rSeV) were prepared in rabbits (four doses of 250 μg protein/dose with Freund's complete adjuvant). The specificity of rabbit hyperimmune antisera to rSeV or rCV (14, 15) was tested in parallel with indirect ELISAs using rSeV and rCV as antigens. The ELISA method employed was identical to the ELISA for rNV (2,11). ELISA titers were expressed as the reciprocal of the highest dilution of antisera giving an optical density (OD) at 492 nm > 0.15. The titer of anti-rSeV hyperimmune sera to homologous antigen was 4-fold higher than that to heterologous rCV (1:4,096,000 vs. 1:1,024,000). The titer of anti-rCV hyperimmune sera to homologous antigen was 32-fold higher than that to heterologous rSeV (1:8,192,000 vs. 1:256,000). Relatively high cross-reactivity was not unexpected because broad reactivity, especially between strains included in the same genogroup, has been exhibited (7, 18).

An antigen-detection ELISA was developed using the rabbit hyperimmune antisera to rSeV. Microplates were coated with the rabbit preimmune or hyperimmune sera (1:5,000 dilution) to capture the antigen in the stool specimens, and peroxidase-conjugated antisera to rSeV was used as the detector antibody. The sample was considered positive when P-N was >0.15 and P/N was >2, where P and N are the OD value with hyperimmune and preimmune sera (15). In control experiments, hyperimmune antisera to rSeV and rCV efficiently captured 0.2 ng of the homologous antigen, but not the heterologous antigen (Table 1). Preimmune sera captured neither the homologous nor heterologous VLPs at

any concentrations. A panel of 15 stool specimens collected from patients in two SeV-associated outbreaks or a CV-associated outbreak that had been characterized by RT-PCR and Southern hybridization was tested in parallel with SeV and CV antigen-detection ELISAs . In SeV-associated outbreaks, 6 specimens except 125/89 were found to be positive by RT-PCR and Southern hybridization using SeV-specific biotinylated probes, which were prepared by PCR using pCR[SeV] as a template as previously described (21). The probe was specific for the SeV-like strains, but not for other GI or GII NLVs (data not shown). In the CV-associated outbreak, CV-like strains from eight stool specimens were confirmed by Southern hybridization with a CV-specific probe. The antigen-detection ELISA for SeV recognized the viral antigens in stool specimens in the SeV outbreaks, but these samples were negative for the CV assay. In contrast, the CV assay detected viral antigens in a CV-associated outbreak but not in the SeV-associated outbreaks. SeV and CV could be differentiated by antigen-detection ELISAs, although the two viruses belong to the same genogroup.

SeV showed 97% nt identity (97% aa identity) in the capsid region with KY89 and 87% nt identity (98% aa identity) with NV, indicating that SeV is nearly identical to KY89. SeV and KY89 were isolated in 1989 in Japan, while NV was isolated in 1968 in the United States. Although NV and KY89 have not been tested by our antigen ELISA, we predict that these three viruses might be antigenically related.

The hyperimmune antisera to rSeV and rCV revealed minor cross-reactivities to each other when VLPs from cell culture were used to coat the plate to capture antibodies. In contrast, the antigen-detection ELISAs using the antibodies to coat the plate to capture antigens were highly specific. Similar high specificity has also been shown in the ELISAs for the detection of NV, Mexico virus and Grimsby virus (2, 6, 8). The expression of more VLPs representing different antigenic types of NLVs as well as the subsequent development of ELISAs based on the expressed VLPs are necessary for the diagnosis and antigenic classifications of NLVs.

Nucleotide sequence accession number. The nucleotide sequence data of SeV has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB031013.

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FIGURE LEGENDS

Fig. 1 Dendrogram of the ORF2 gene of SeV and known NLVs. The ORF2 genes from four GI NLVs and four GII NLVs were analyzed using a SINCA package (FUJITSU, Ltd., Tokyo), in which tree topology was inferred by the UPGMA cluster analysis with the bootstrap option. The numbers at the branching points are the 50% threshold majority consensus value for 100 bootstrap replicates. The known NLVs sequences are as follows: NV (M87661), KY89 (L23828), OTH25 (L23830), SV (L07418), DSV (U04469), CV (AB022679), LV (X86557), BV (X76716]), CWV (U46500), TV (U02030), MV (U22498), SMV (U70059), MSV (X81879), AV (U46039) and HV (U07611).

JCM-Table 1

TABLE1. Detection of NLVs in stools by ELISAs using hyperimmune antisera to rSEV and rCV

	Antigen-detection ELISA for :		
Samples	SEV	CV	RT-PCR/hybridization
rSEV[a]	**1.05**[b]	0.00	N.T.[c]
rCV[a]	0.00	**0.93**	N.T.
121/89	**1.31**	0.01	+
122/89	**1.61**	0.00	+
124/89[d]	**0.53**	0.00	+
125/89	**0.23**	0.00	−
131/89	**1.48**	0.01	+
1381/93	**0.35**	0.04	+
1382/93	**1.55**	0.04	+
663/99	0.03	**0.62**	+
665/99	0.04	**1.32**	+
666/99	0.07	**2.01**	+
669/99	0.04	**0.51**	+
675/99	0.01	**0.28**	+
676/99	0.07	**1.72**	+
680/99	0.05	**1.62**	+
687/99	0.06	**1.04**	+

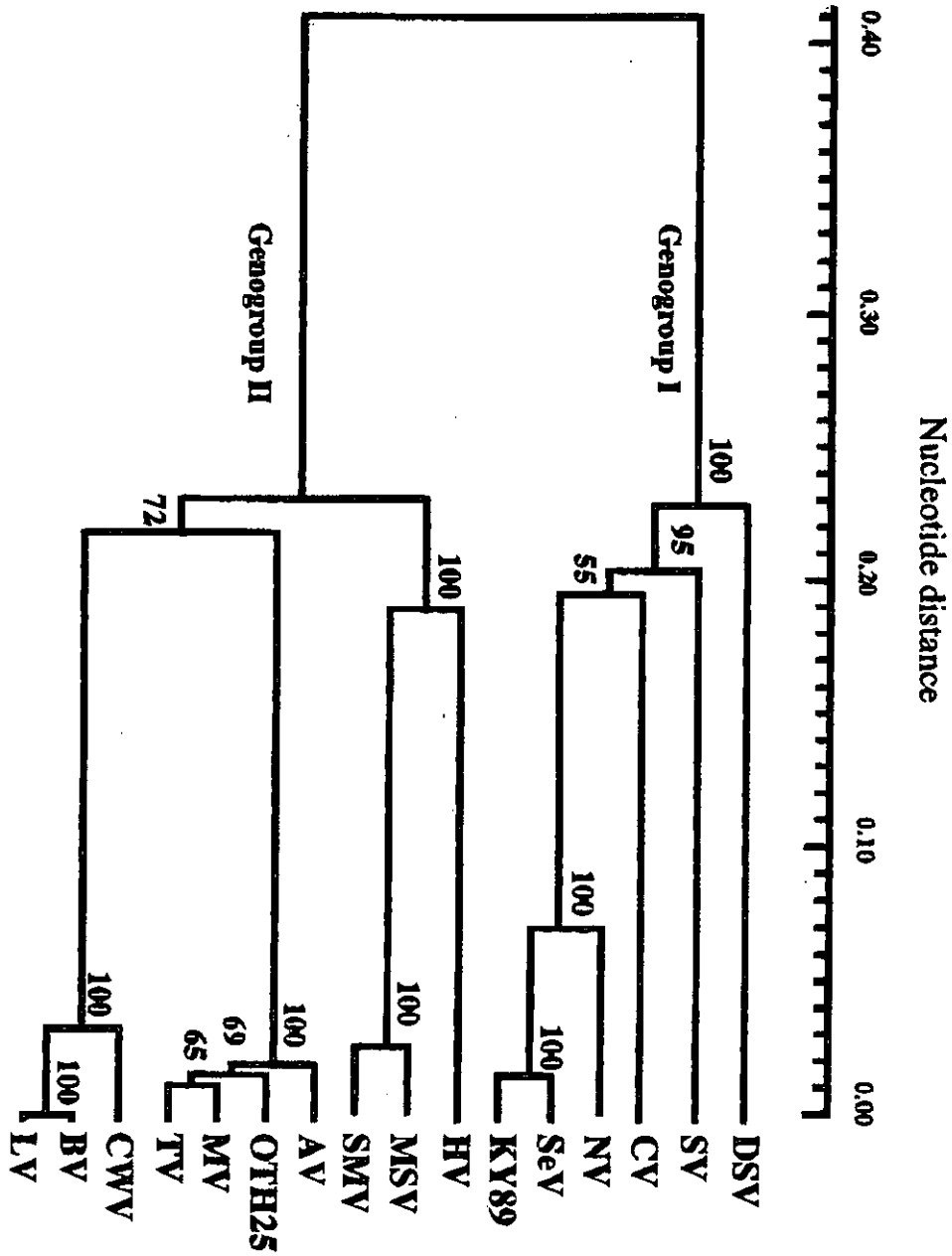
[a] Purified VLPs (2 ng/ml) were used as antigens

[b] OD values at 492 nm. Homologous titers were shown by boldface.

[c] Not tested

[d] Stool was used for the expression of rSEV VLPs.

JCM - Fig. 1


Genogroup II
Genogroup I
100
72
95
55
100
100
100
100
100
100
100
69
65
100
100
LV
BV
CWV
TV
MV
OTH25
AV
SMV
MSV
HV
KY89
SeV
NV
CV
SV
DSV
0.40
0.30
0.20
0.10
0.00
Nucleotide distance