Genetic analysis of calicivirus genomes detected in intestinal contents of piglets in Japan

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Summary. Enteric caliciviruses, noroviruses, and sapoviruses are emerging pathogens responsible for diarrhea or gastroenteritis in their respective hosts. In this study, swine enteric caliciviruses were detected in ten samples of intestinal contents from 24 piglets in Japan by reverse transcription-polymerase chain reaction using a broadly reactive primer pair (P290/289) that targeted the highly conserved RNA polymerase regions of the enteric caliciviruses. From the positive samples, the entire viral genome of strain K7/JP and 3′-end parts of the genomes of strains K5/JP and K10/JP were cloned and sequenced. K7/JP had an RNA genome of 7144 bases, excluding its 3′ poly (A) tail. The K7/JP genome possessed two open reading frames and characteristics common to sapoviruses. In phylogenetic analysis using amino acid sequences of VP1, K5/JP was demonstrated to be close to the noroviruses previously detected in pigs, and K7/JP and K10/JP were considered to be classified as a new genogroup of sapoviruses.

Introduction

Caliciviruses (family *Caliciviridae*) are small, nonenveloped viruses that are 27 to 35 nm in diameter. The family *Caliciviridae* is divided into four genera, *Norovirus* (NV), *Sapovirus* (SV), *Lagovirus* (LV), and *Vesivirus* (VV) [9]. They possess a single-stranded, plus-sense genomic RNA of approximately 7.3 to 8.3 kb [10]. The VVs and NVs have three separate open reading frames (ORFs) in their genomes, whereas the LVs and SVs have genomic organizations composed of two ORFs. The three ORFs of NVs encodes the non-structural proteins (ORF1), the capsid protein (VP1) (ORF2), and a minor structural protein (VP2) (ORF3), respectively. In contrast, the SVs capsid gene is fused to the non-structural gene, while its ORF2 encodes a small protein, considered to be similar to VP2 of NVs [17].

NVs and SVs, previously known as Norwalk-like viruses and Sapporo-like viruses, respectively, cause acute gastroenteritis in humans. NVs are the leading cause of epidemic, nonbacterial gastroenteritis in humans of all ages [10]. SVs mainly infect infants and young children [22], although SV gastroenteritis outbreaks in adults have been reported [21]. In spite of the inability to grow human NVs and SVs in cell cultures or in animal models, research progress with both viruses has been achieved by electron microscopy and molecular biological analyses, including reverse transcription-polymerase chain reaction (RT-PCR) and nucleotide sequencing [8].

A porcine enteric calicivirus (PEC) (Sw/SV/Cowden/80/US) was first reported in association with diarrhea in piglets in 1980 [24]. PEC resembled other caliciviruses in the composition of its structural and non-structural proteins as well as its morphology in electron microscopic analysis [23]. Molecular biological characterization showed that PEC was genetically similar to SVs, and it was classified into a distinct SV genogroup, GIII [11]. NVs were also detected in mice, pigs, and cattle [3, 15, 18, 28, 30]. Although bovine NVs have been assigned to genogroup GIII within the genus NV [1] and show wide distribution in bovine populations [4, 27, 31], little is known about the distribution and genetic diversity of swine NVs and SVs in the field.

In this paper, we describe the detection of calicivirus sequences in intestinal contents from piglets by RT-PCR. The entire viral genome of the strain K7/JP and 3′-end parts of the genomes of strains K5/JP and K10/JP were cloned and sequenced in order to define the genetic characteristics of enteric caliciviruses of swine origin.

Materials and methods

Intestinal contents of piglets

Intestinal contents from 24 piglets, whose ages were less than 5 months, were collected at a veterinary diagnostic laboratory in a prefecture of Japan between October 2002 and May 2003. Sixteen of the piglets showed diarrheic symptoms. The samples were prepared as 10% suspensions in phosphate-buffered saline (PBS). After centrifugation at $10,000 \times g$ for 10 min at 4 °C, supernatants were collected and used for RNA extraction.

Extraction of viral RNA

Viral RNA was extracted from the supernatants using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). Briefly, 0.5 μl yeast RNA (10 mg/ml) (Ambion, Austin, TX) was dissolved in 560 μl viral lysis buffer and incubated for 5 min at 80 °C, and then 140 μl of each intestinal content supernatant was added to the viral lysis buffer containing the yeast RNA. After extraction of the viral RNA according to the mini kit protocol, the RNA solution was stored at −80 °C until use.

Detection of viral RNA by RT-PCR

The primer pair P290/289 (Table 1) has been shown to universally amplify the calicivirus RNA polymerase region by RT-PCR [14]. The RT-PCR was performed in 50 μl reaction mixture containing 1 μl RT/Platinum Taq mix (Invitrogen, Carlsbad, CA), 25 μl 2× reaction

Table 1. Nucleotide primers used in this study

Primer	Polarity	Location*	Primer sequence, 5′ to 3′
AAP	+		GGC CAC GCG TCG ACT AGT ACG CGI IGG CII GGG IIG
AUAP	–		GGC CAC GCG TCG ACT AGT AC
GSP1	–	4312–4330	CTT GTG TTT GAC ATC TCG C
GSP2	–	4285–4304	TCC GTG CTC AAG CAG CGG CC
P290	+	4480–4202	GAT TAC TCC AAG TGG GAC TCC AC
P289	–	4489–4510	TGA CAA TGT AAT CAT CAC CAT A
TX30SXN	–		GAC TAG TTC TAG ATC GCG AGC GGC CGC CC(T)30
TX30SXN/A	–		GAC TAG TTC TAG ATC GCG AGC GGC CGC CC

*Numbers are nucleotide position in the K7/JP virus genome

mix (0.4 mM of each dNTP and 2.4 mM MgSO$_4$), 2.5 μl of each primer (10 μM), 14 μl DEPC-treated water, and 5 μl of the extracted RNA. The thermocycler program was 50 °C for 30 min and 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, and a final 10-min extension at 72 °C. RT-PCR products were analyzed by agarose gel electrophoresis followed by observation under UV light. The amplified cDNA with expected sizes was cloned and sequenced as described below.

cDNA synthesis by 3′ and 5′ rapid amplification of cDNA ends (RACE)

cDNA synthesis was conducted using a 3′-terminal primer TX30SXN (Table 1) with SuperScript^{TM} III reverse transcriptase (Invitrogen) and RNase H (Invitrogen). The PCR for the 3-kbp fragment was performed using the primer pair P290/TX30SXN/A (Table 1) with KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan). For cloning of the genomic 5′-terminal sequence, the specific reverse primer GSP1 (Table 1) was used for first-strand cDNA synthesis. The homopolymeric dC tail was added to the 3′ end of the first-strand cDNA, and the cDNA was purified using the 5′ RACE system, Version 2.0 (Invitrogen). A 4.2-kbp fragment was amplified by PCR with LA Taq polymerase (TaKaRa, Otsu, Japan) using the primer GSP2 (Table 1) in combination with the 5′ RACE abridged anchor primer (AAP) and abridged universal amplification primer (AUAP).

Sequence determination

The RT-PCR products of the RNA polymerase gene and the 3′ and 5′ RACE were cloned into the TOPO TA vector (Invitrogen). The internal sequences were determined with an ABI PRISM 3100-Avant genetic analyzer after performing sequence reactions using primers based on the vector and newly obtained sequences. All sequences were determined by sequencing both strands of at least two clones. If any sequence difference was observed between the two clones, we sequenced additional clones to confirm the sequence.

Phylogenetic analysis

Nucleotide (nt) and amino acid (aa) sequences of the following caliciviruses in Genbank (Table 2) were used in the phylogenic analysis: SVs: Hu/Manchester/93/UK (MV) (X86560), Hu/Sapporo/82/JP (SV) (U65427), Hu/Houston/90/US (Hu90) (U95644), Hu/Hou7-1181/90/US (Hou7) (AF435814), Hu/London/29845/92/UK (Ln92) (U95645), Hu/Argentina39/98/Arg (Arg39) (AY289803), and Sw/Cowden/80/US (PEC) (AF182760); NVs: Hu/Norwalk/68/US (NV) (M87661), Hu/Chiba407/87/JP (Chiba) (AB042808), Hu/Hawaii/71/US (HV)

(U07611), Hu/Oth-25/89/JP (OTH.25) (L23830), Bo/Jena117/80/GE (JV) (AJ011099), and Sw/SW43/97/JP (SW43) (AB074892); VVs: Fe/FCV/F4/68/JP (FCV) (D31836) and Pi/VESV/SMSV1/95/US (SMSV) (AF181081); LVs: Ra/RHDV/GH/88/GE (RHDV) (M67473) and Ra/EBHSV/GD/96/FR (EBHSV) (Z69620); Genus-unknown strain Bo/NB/80/US (NB) (AY082891). The nt and aa sequence analyses and homology comparisons were performed using Genetyx-Win version 6 software (GENETYX, Tokyo, Japan). The aa neighbor-joining phylogenetic trees were constructed using the Clustal W program, accessed through the DNA Data Bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp/).

Results

Detection of viral RNA from intestinal contents of piglets by RT-PCR

Since the cloning of the NV genome in 1990 [13], a number of primer pairs for RT-PCR have been designed by different laboratories for the detection of NVs and SVs [2]. The P290/289 primer pair is useful for broad detection of NVs and SVs in clinical and epidemiologic studies as well as for environmental monitoring [5, 7, 12, 14]. In our study, RNA samples from the intestinal contents of 24 piglets were tested by using the P290/289 primer pair, and the expected products were detected in ten samples. Although six (K5/JP, K7/JP, K8/JP, K10/JP, K11/JP, and K13/JP) of the ten samples were obtained from piglets that had diarrhea, it was
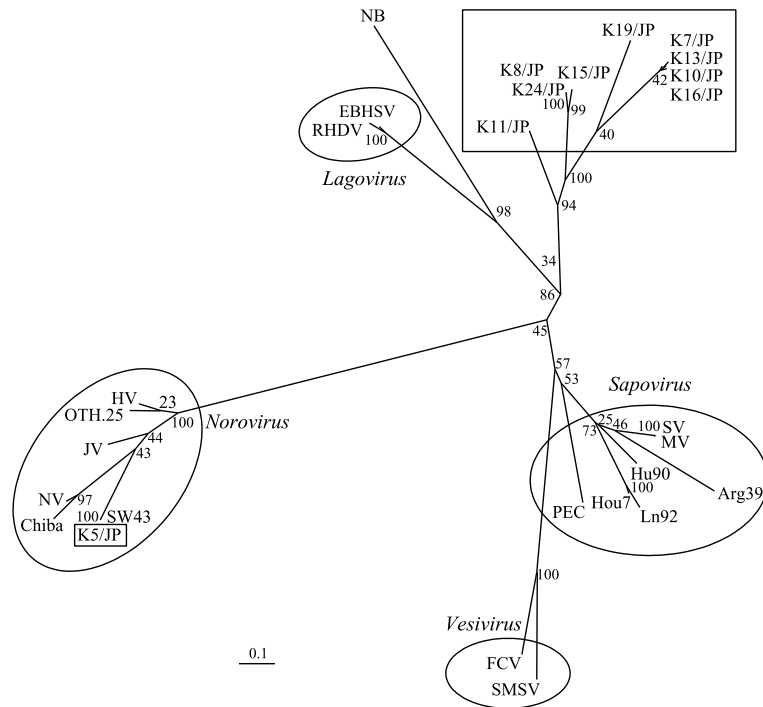

Fig. 1. Unrooted phylogenetic tree of calicivirus RNA polymerases constructed by the neighbor-joining method. The sequences of the strains used in this analysis were either from GenBank or our unpublished data. Trees were prepared using the Treeview programs and are based on 100 bootstrapped data sets. The viruses detected in this study are boxed

unclear whether the other four piglets, from which K15/JP, K16/JP, K19/JP, and K24/JP were detected, had diarrhea or not. The sizes of the RT-PCR products were 319 bp for K5/JP and 331 bp for the other positive samples, which were determined by sequence analysis. The predicted aa sequences of the products contained the GLPSG motif, which is characteristic of the RNA polymerase regions of caliciviruses and picornaviruses [12, 20]. The ten viruses were shown to be separated into two groups in the phylogenetic tree of calicivirus polymerase genes (Fig. 1). K5/JP was included in the NVs and close to swine NV SW43, which was previously detected in Japan [28], and the others appeared to form a
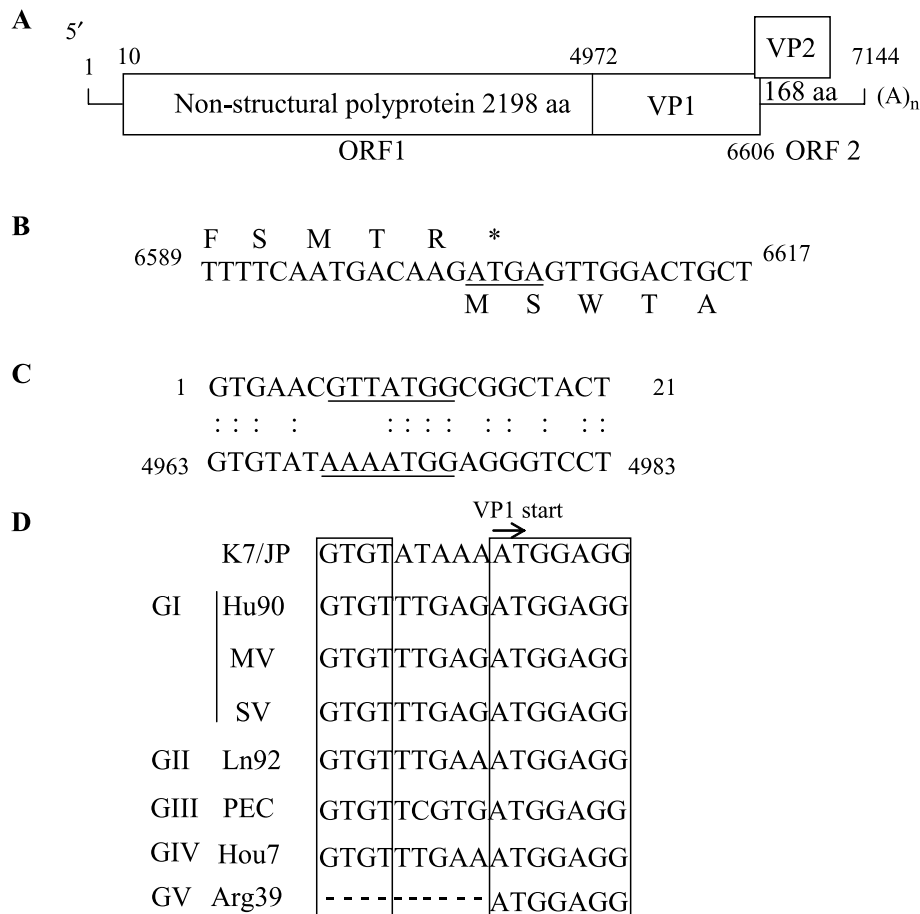

Fig. 2. Genomic characteristics of K7/JP. **A.** Schematic of the genomic organization of K7/JP showing the two predicted ORFs: ORF1, encoding a polyprotein fused to and contiguous with the capsid protein (VP1), forming a large polyprotein; and ORF2, encoding a small basic protein (VP2) of unknown function. **B.** Aligned nucleotide and predicted amino acid sequences at the junction between ORF1 and ORF2. ORF2 overlaps the 3′ end of ORF1 by 4 nt (underlined). **C.** Schematic of the conserved nucleotide sequence motifs at the 5′ termini of the genomic and predicted subgenomic RNAs. The Kozak context, favorable for translation initiation, is underlined. **D.** Aligned nucleotide sequences at the junction between the RNA polymerase and VP1 genes of SVs

cluster independent of the four calicivirus genera. K7/JP showed 29% aa sequence identity with K5/JP, and the aa sequence identity of K7/JP with other samples was 59% for K11/JP, 61–62% for K8/JP, K15/JP and K24/JP, 65% for K19/JP, and 96–97% for K10/JP, K13/JP, and K16/JP.

Genomic organization of the K7/JP virus

The complete RNA genome of K7/JP, excluding its 3′ poly(A) tail, consisted of 7144 nt, which was shorter than that of other caliciviruses. It had a ribonucleotide composition of 24% A, 27.4% C, 25.7% G, and 22.9% U (GenBank accession No. AB221130). The 5′-untranslated region was nine bases long and began with the 5′-terminal tri-ribonucleoside sequence GTG. Similar to the genomes of SVs and LVs, the K7/JP genome was predicted to encode a VP1 gene contiguous with the large non-structural polyprotein in ORF1 and to have two ORFs (Fig. 2A). ORF1 contained 6597 bases (nt 10–6606), whose predicted translated sequence of 2198 aa included non-structural proteins and VP1 (544 aa). ORF2, consisting of 507 bases (nt 6603–7109), was frame-shifted −4 relative to ORF1 (Fig. 2B) and was

Table 2. Percentages of amino acid sequence identity of K7/JP with other caliciviruses in regions aligned for phylogeny

Genus	Virus	GenBank accession no.	Region	% Identity		
				Non-structural polyprotein	VP1	VP2
SVs	MV	X86560	All genome	30	36	34
	SV	U65427	VP1 + VP2		36	33
	Hu90	U95644	VP1 + VP2		34	30
	Ln92	U95645	VP1 + VP2		32	29
	Hou7	AF435814	VP1 + VP2		34	28
	Arg39	AY289803	VP1 + VP2		35	27
	PEC	AF182760	All genome	33	37	28
	K10/JP		VP1 + VP2		88	87
NVs	NV	M87661	All genome	20	28	21
	JV	AJ011099	All genome	20	26	15
	Chiba	AB042808	All genome	20	25	18
	HV	U07611	All genome	20	34	33
	OTH.25	L23830	VP1 + VP2		32	29
	SW43	AB074892	VP1 + VP2		28	28
	K5/JP		VP1 + VP2		28	29
VVs	FCV	D31836	All genome	28	30	25
	SMSV	AF181081	All genome	30	35	21
LVs	RHDV	M67473	All genome	26	23	30
	EBHSV	Z69620	All genome	25	24	17
Unknown*	NB	AY082891	All genome	23	27	23

*Means genus designation is unclear

predicted to encode a 168-aa protein, VP2 (Fig. 2A). The predicted polyprotein encoded by ORF1 contained the characteristic 2C helicase (GPPGIGKT), 3C protease (GDCG), and RNA-dependent RNA polymerase (GLPSG and YGDD) motifs that are highly conserved in all caliciviruses. The PPG motif was also present in the predicted VP1 (data not shown).

Sequence comparison

In addition to the entire genome of K7/JP, we cloned and sequenced the 3′ regions (from the RNA polymerase region to the 3′ end) of the K5/JP and K10/JP genomes (GenBank accession Nos. AB221132 and AB221131, respectively). We compared the aa sequence identities of K7/JP with those of other caliciviruses, including K5/JP and K10/JP (Table 2). The non-structural protein in K7/JP ORF1 showed 20–33% aa identity with the other viruses examined; the highest identity was shown with the SVs (30–33%), and the lowest identity was with the NVs (20%). K7/JP shared a higher aa identity in the VP1 region with PEC Cowden (37%) than with other SVs (32–36%), VVs (30% and 35%), NVs (25–34%), LVs (23% and 24%), and NB (27%). The VP2 region also showed the highest aa sequence identity (27–34%) with SVs. A phylogenetic tree generated from the VP1 sequences showed that K7/JP and K10/JP were more closely related genetically to the SVs than to the other human and animal caliciviruses (Fig. 3). K5/JP was
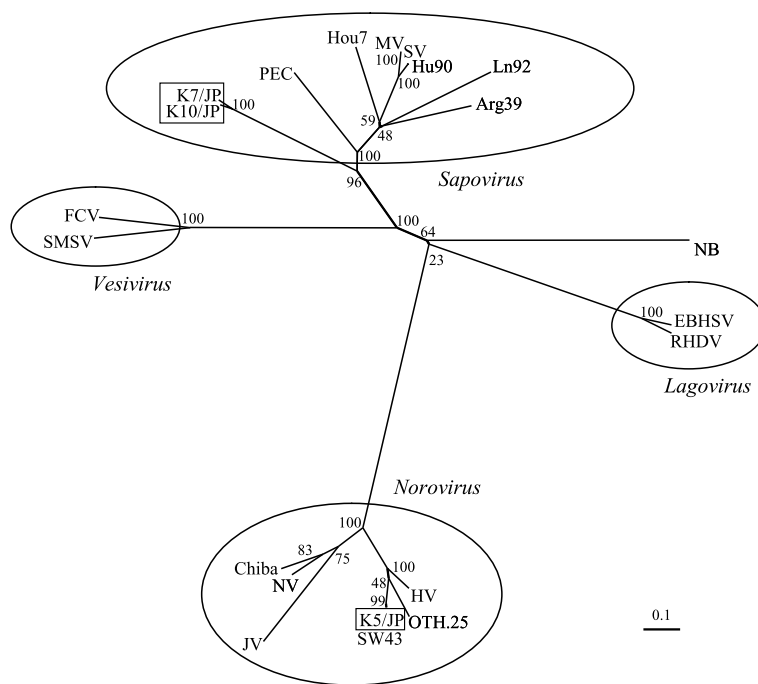

Fig. 3. Unrooted phylogenetic tree of calicivirus VP1s constructed by the neighbor-joining method. The sequences of the strains used in this analysis were either from GenBank or our unpublished data. Trees were prepared using the Treeview programs and are based on 100 bootstrapped data sets. The viruses analyzed in this study are boxed

clearly shown to belong to the NVs and to be closely related to SW43 [29]. Similar results were obtained for phylogenetic trees generated from non-structural proteins and VP2 sequences (data not shown).

The 5′ termini of the genomic and predicted subgenomic RNAs of K7/JP had leader sequences possessing a Kozak structure (G/A NN ATGG), similar to that of PEC Cowden [11], which is favorable for translation initiation of eukaryotic mRNA [16] (Fig. 2C). The analysis of the aligned SV sequences showed that a nucleotide stretch located at the junction of the RNA polymerase and VP1 genes was genogroup-specific (GI to GIII) [25]. The junction of K7/JP had a unique sequence compared with those of other SVs, including GIV, which were recently proposed by Farkas et al. [7] (Fig. 2D). The VP1 regions (544 aa) of K7/JP and K10/JP were the same length as that of PEC and slightly smaller than those of SVs of human origin. The GI, GIV, and GV strains of SVs were found to have an additional ORF overlapping with the VP1 gene [7]. K7/JP and K10/JP did not possess the overlapping frame, resembling instead the SV GII and GIII groups. In the genus SV, the ORF1/2 junction (OOJ) consists of a 1- or 4-nt overlap between the stop codon of ORF1 and the first AUG codon of ORF2 [10]. MV (GI), PEC (GIII), and Arg39 (GV) have 4 nt, but Ln92 (GII) and Hou7 (GIV) have only 1 nt in the OOJ. The OOJs of K7/JP and K10/JP consisted of 4 nt (Fig. 2B), which differed from the OOJ of LVs (frame-shifted −20) and NB (not overlapping, frame-shifted +2).

Discussion

Sapporo virus, the prototype SV, was identified in 1982 from an outbreak of diarrhea in an orphanage in Sapporo, Japan [19]. Schuffenecker et al. [25] classified three major genetic groups. Furthermore, the genus *Sapovirus*, including viruses of human and pig origin, has been divided into five genogroups based on the genetic diversity of VP1 or the viral polymerase [7]. By molecular detection and analysis in this study, strains K7/JP and K10/JP were shown to have not only genetic characteristics common to SVs but also unique positions in the phylogenetic tree, suggesting that K7/JP and K10/JP comprise a new SV genogroup. This is the first report to show the existence of SVs other than PEC in a pig population. The sequence identity of VP1 between K7/JP and K10/JP was 88%, and the two new swine SVs were considered to be in the same genetic cluster. We detected pol genes in the other samples, which showed 59–97% identity with K7/JP. According to the phylogenetic analysis, the pol genes were grouped in a branch independent of the four genera of caliciviruses (Fig. 1). The amplification of the VP1 regions of such samples has been unsuccessful or is ongoing, but the genetic diversity of swine enteric caliciviruses may be confirmed by analyzing the samples.

K5/JP had a 547-aa capsid protein, as long as that of SW43, and the two proteins showed 98% identity (date not shown). Sugieda et al. [28] first detected the NV genes SW43 and SW913 in 1117 cecum samples from healthy pigs on 26 farms in Shizuoka prefecture, Japan, in 1997. Five years later, we found

an additional swine calicivirus strain, K5/JP, which was genetically related to the original swine strains, in the intestinal contents of piglets from a south-western prefecture of Japan, situated more than 800 km from Shizuoka prefecture. In The Netherlands, NVs were detected in two stool samples from pig farms; partial pol gene sequences of these NVs strongly resembled (100% for aa) those of SW43, although the VP1 sequences were unknown [30]. As similar NV strains were detected from pigs in apparently unrelated situations and were grouped into a similar cluster, it was suggested that the NV in this cluster of GII might be unique to pigs. Recently, high prevalence of antibodies against swine NV SW918 in pigs in Japan and the United States was shown by an enzyme immunoassay based on baculovirus-expressed SW918 virus-like particles [6]. In that study, antibodies against human NVs of GI as well as GII were also detected in pigs in the US, indicating that not only GII NVs but also GI NVs may exist in pigs. Further research is required to show the epidemiological significance of swine NVs detected in the pig population.

PEC, the first SV of pig origin, is known as an enteropathogenic virus, and its wide distribution in the US has been described [11]. In Japan, calicivirus-like particles, as well as coronavirus- and astrovirus-like particles, were detected in acute porcine gastroenteritis by electron microscopy [26]. However, the relationship between the enteric caliciviruses detected in this study and diseases manifested by the piglets from which the samples were obtained is unclear at present. To elucidate the role of swine enteric caliciviruses other than PEC, intensive clinical and virological monitoring will be needed. The nucleotide sequence data on enteric caliciviruses obtained in the present study will be useful for such monitoring in the future. In addition, the positive samples in this survey may have a use in experimental infections using specific-pathogen-free pigs, although it is necessary to establish monitoring methods for virus shedding and antibody response, as well as to eliminate other enteropathogenic agents that may exist in the inoculum.

In summary, we detected calicivirus genomes in the intestinal contents of piglets by RT-PCR using the primer pair P290/289. Phylogenetic tree analysis, based on the sequence data of VP1, suggests that K7/JP and K10/JP belong to a new SV genogroup and that K5/JP belongs to a cluster of swine NVs. It is speculated that several genetic types of enteric caliciviruses may circulate simultaneously in pigs. As the VP1 genes of these new swine caliciviruses were isolated as cDNA clones, virus-like particles can be produced by recombinant expression of VP1 using the baculovirus expression system. These particles may be used in further studies, such as serological surveys of the swine population and antigenic comparison with related viruses of human origin.

Acknowledgments

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