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Department of Veterinary Pathology, College of Veterinary Medicine, Chonnam National University, Gwangju, Korea

Detection and Molecular Characterization of Porcine Enteric Calicivirus in Korea, Genetically Related to Sapoviruses

H. J. Kim[1,*], H. S. Cho[1,*], K. O. Cho[1] and N. Y. Park[1,2]

Address of authors: [1]Department of Veterinary Pathology, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Korea; [2]Corresponding author: Tel.: +82 62 530 2843; fax: +82 62 530 2847;
E-mail: nypark@chonnam.ac.kr

With 1 figure and 1 table

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Summary

Porcine enteric calicivirus (PECV) shares morphological and genetical similarities with Sapoviruses (SVs), which are the leading cause of epidemic, non-bacterial gastroenteritis in children worldwide. The aim of this study was to identify the prevalence of PECV infection in pig farms in Korea, and to compare the evolutionary inter-relationships between Korean PECVs and other caliciviruses. Among 102 diarrhoeic faecal samples of sucking ($n = 50$) and weaned ($n = 52$) piglets from 31 different farms in Korea, five samples (4.9%) were detected positive by reverse-transcriptase polymerase chain reaction (PCR), but nine (8.8%) by nested-PCR. Furthermore, we found that Korean PECVs are closely related to SVs.

Introduction

Caliciviruses are divided into four genera, namely: (i) Vesivirus; (ii) Lagovirus; (iii) Noroviruses (NVs, former Norwalk-like viruses); and (iv) Sapoviruses (SVs, former Sapporo-like viruses) (Guo et al., 1999, 2001). Viruses within one genus are phylogenetically related, have common features in genomic organization, and high degrees of sequence similarities in the RNA polymerase and capsid regions (Guo et al., 1999). Animal caliciviruses are often associated with systemic diseases and gastroenteritis in cats and chickens. Vesicular lesions, reproductive failure in swine and marine animals, and haemorrhagic diseases in rabbit have also been reported. In children, the leading cause of epidemic, non-bacterial gastroenteritis is associated with human caliciviruses (HuCV) (Murphy et al., 1999).

Caliciviruses, causing mainly viral enteritis in swine, include SVs and NVs. Porcine enteric calicivirus (PECV) was first reported in the USA and later also found in the UK, Hungary, Japan and Korea (Saif et al., 1980; Nagy et al., 1996; Sugieda et al., 1998; Park et al., 2000). Sequence analysis revealed similarities between the PECV found in the USA and SVs from the UK, while NVs, detected from four caecal contents of pigs in Japan, were closely related to HuCV. The latter finding has raised public health concerns regarding the potential of cross-species transmission, and swine as a possible host for HuCV (Sugieda et al., 1998). Moreover, reverse-transcriptase polymerase chain reaction (RT-PCR) has also detected enteric caliciviruses in foods, water, sewage and fomites. Thus HuCV has been suggested as the leading cause of food and waterborne viral gastroenteritis in humans (Mead et al., 1999; Schaub and Oshiro, 2000). RT-PCR amplification of PECV RNA from stool specimens has been described. However, there are no reports of diagnosing PECV in Korea (Guo et al., 2001). This is the first report of the molecular epidemiology of PECV outbreaks in Korea, which covers a 2-year period (2001–2002).

Caliciviruses are small, non-enveloped, 27–35 nm in size, and show icosahedral symmetry (Murphy et al., 1999). They possess a single-stranded, plus-sense genomic RNA, which is 7.4–7.7 kb in size and encodes a single structural capsid protein of 58–80 kDa. The capsid protein is a polyprotein that contains motifs indicative of a putative 2C helicase, 3C-like protease, RNA-dependent RNA polymerase 3D (RDRP), and a small basic protein of unknown function (Guo et al., 1999). The RDRP protein of PECV is conserved among calicivirus strains (Guo et al., 1999). As in SVs, the PECV capsid proteins are divided into three distinguished regions. The N-terminal region 1 (aa 1711–1984 for PECV) is highly conserved and shows higher amino acid sequence identities with those of SVs than with those of other caliciviruses. Region 2 (aa 1985–2132 for PECV) is hypervariable, and corresponds to regions C–E in feline calicivirus and to SMSV1 and SMSV4 (Neill, 1992; Liu et al., 1995; Guo et al., 1999). The C-terminal region 3 is conserved but to a lesser extent than region 1. The capsid diversity among caliciviruses of the same genogroup or genus is mainly determined by the variability within region 2. This region may contain the multiple serotype-specific determinants recognized by monoclonal antibodies (Hardy et al., 1996). Region 3 has conserved amino acid residues and a few antigenic epitopes but shows some variability (Hardy and Estes, 1996; Guo et al., 1999).

In this study, we report the occurrence of PECV as one of the causative agents of diarrhoea in pig farms in Korea. Additionally, this work describes phylogenetic relationships underlying this genomic variation, and expands the knowledge of genomic diversity among SVs and other caliciviruses by characterization of additional strains.

Materials and Methods

Faecal samples

Faecal samples were collected from 102 piglets, with a history of diarrhoea, from 31 different pig production farms located in the provinces Gyunggi, Chungnam, Jeonbuk, Jeonnam and

*These authors contributed equally to this work.

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Jeju of Korea during the period of 2001–2002. The number of samples collected in one farm ranged from two to four, representing approximately 10% of the piglets with diarrhoea at the time of sampling. Faecal samples collected from 60 piglets without a history of diarrhoea (10 different pig production farms) were used as negative control.

Electron microscopy, RT-PCR and DNA sequencing

Faecal samples were examined for viruses by electron microscopy, RT-PCR and nested-PCR. One set of primers in the polymerase region, previously reported by Guo et al. (2001) was used, termed Primer 45 (antisense) and Primer 46 (sense). Primers of nested-PCR for the RDRP region were designed according to the published sequence of the RNA polymerase region of the Cowden strain. The sequence of these primers is as follows: primer nR (5′-GAGTGTCTGTTGGCTCAATG-3′, 4771–4752); primer nF (5′-CTCCTATGCTGAGGACA-CAC-3′, 4392–4411). To find out the variability of the region 2 of the capsid region, the oligonucleotide primers used in the RT-PCR and nested-PCR were designed from the published sequence of the capsid region of the Cowden strain. The sequence of primers is as follows: Primer PECVcapsidR (5′-AAAGCATGATGTTGTTAGGC-3′, 6454–6435); forward primer for RT-PCR, Primer PECVcapsidF (5′-CTCATCAA-CCCTTTTGAAAC-3′, 5698–5717); Primer PECVCnR (5′-AAAGCATGATGTTGTTAGGC-3′, 6454–6435); Primer PECVCnF (5′-GTGGTCATAGTAGGTGTGGC-3′, 5890–5909). The specificity of these primers was checked against the GenBank and EMBL Data Library. No sequence homologies to coronaviruses [porcine epidemic diarrhoea virus (PEDV) and transmissible gastroenteritis virus (TGEV)] or rotaviruses were found.

To compare the sensitivity of the nested PCR with RT-PCR, PECV (1 ng/μl) RNA concentration was determined by the absorbance at A_{260} and A_{280} on a spectrophotometer (Beckman Coulter, Fullerton, CA, USA). RNA was serially diluted 10-fold in RNase-free water. PECV RNA was extracted from raw faecal samples by using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's protocol, with the following modifications to increase the yield of viral RNA. The extraction of single-stranded RNA was repeated twice with TRI, and combined aqueous phases from the first and second extractions. One-step RT-PCR assay was performed with modifications as described previously (Guo et al., 2001). As a negative control, one-step RT-PCR and nested PCR were also performed with the extract of viral nucleic acid from Korean Field Isolate (KPEDV-9) of PEDV, and Miller strain of TGEV, cultured on vero and swine testicular cells (provided by Choong Ang Animal Disease Laboratory Ltd, Seoul, Korea) respectively. To increase the sensitivity and specificity of the RT-PCR, 5 μl of diluted RT-PCR products (1 : 100) were added to a tube containing 45 μl of the PCR mixture (final dilution 1 : 1000). If the predicted band was absent by RT-PCR, 5 μl of undiluted RT-PCR product were subject to nested PCR. The first PCR product of double distilled water with all the necessary components was used as a negative control for nested PCR.

The nested PCR products were purified using GeneClean II kit (Bio 101 Inc., La Jolla, CA, USA) according to the manufacturer's instructions. DNA sequencing was performed using an ABI-3700 automated sequencer (Applied Biosystems, Foster City, CA, USA). All primary readings were edited to remove vector sequences and unreliable data using the Factura program (Perkin-Elmer Life Sciences, Boston, MA, USA). Sequences longer than 100 nucleotides were further analysed, and all samples were sequenced three times.

Molecular analyses

DNA homology searches were carried out using the National Center for Biotechnology Information (NCBI) databases, and applying the BLAST network service. Local homology searches were performed using the BLAST suite of programs. BLAST searches, against the NCBI non-redundant protein database, were performed locally with a nucleotide database. To define the genetic relationship between Korean PECVs and other calicivirus representatives of different genera, human and animal calicivirus strains, for which the RDRP region and capsid regions have been characterized, were used for phylogenetic comparison. The GenBank accession numbers, genus designations, and virus strains are summarized in Table 1. Phylogenetic trees were generated for sequences in a part of the RNA polymerase region and the capsid region. Sequences in either region were aligned, and trees were generated with PhyloDraw (Choi et al., 2000).

Results

The lowest detection limit of nested PCR with PECV in DEPC-treated water was 100 fg/μl of RNA, whereas the lowest detection limit of the RT-PCR was 10 fg/μl. When PECV was diluted, RT-PCR showed 10-fold decrease in sensitivity, whereas approximately 10-fold increase in sensitivity was noted in nested PCR. We detected PECV from diarrhoeic faecal samples of sucking and weaned piglets in Korea by RT-PCR and nested PCR. Amplicons of both investigated regions were identified after RT-PCR, one for a part of the RDRP gene (572 bp) and the other for a part of the capsid gene (757 bp). Predicted fragments of the RDRP gene (380 bp) and the capsid gene (565 bp) were amplified by nested PCR with their RT-PCR products. Five samples (4.9%) were detected positive for PECV by RT-PCR, whereas nine samples (8.8%) by nested PCR, performed for a part of the RDRP and capsid region. To confirm the specificity of the primers, one-step RT-PCR and nested PCR were also performed with the extraction of viral nucleic acid from Korean Field Isolate (KPEDV-9) of PEDV, and Miller strain of TGEV, cultured on vero and swine testicular cells, where no specific band was present.

Aggregates of typical calicivirus-like particles were observed in four porcine faecal samples by transmission electron microscopy (TEM). Viruses were 30–35 nm in diameter. The six-pointed star-like appearance and stain-filled, cup-shaped depressions characteristic of caliciviruses were apparent. Typical coronaviral particles and rotaviral particles were detected in two of faecal specimens containing calici-like viruses. There were no data on bacterial infection gathered in the 102 diarrhoeic faecal samples. The 60 faecal samples, from piglets with no history of diarrhoea, were negative for PECV detected by TEM and RT-PCR.

Sequence analysis showed that Korean PECVs contained the GLPSG and YGDD consensus amino acid motifs characteristic for this genomic region. The three specimens (10702, 10802 and 15802) of nested-PCR products (380 bp), directly sequenced for a part of the RDRP region of PECV, shared 100% of the

Table 1. Calicivirus strains and GenBank accession numbers, tree notation, and a part of RNA-dependant RNA polymerase (RDRP) protein and capsid sequences used for phylogenetic comparison

Calicivirus genus	Virus strain	GenBank accession no.	Tree notation*
Sapoviruses	HuCV/Houston/90	U95644	Houston/90
	HuCV/Potsdam	AF294739	Potsdam/2000
	HuCV/Lyon	AJ271056	Hu/Lyon
	Human enteric calicivirus /Plymouth isolate	X86559	MV
	Human enteric calicivirus /London/29845	AAC40584	London
	Porcine enteric calicivirus /tissue culture-adapted	AF182760	TC PECV
	Porcine enteric calicivirus /Cowden strain/wild type	NC000940	WT PECV
	Porcine enteric calicivirus /Korean strain/6802	RDRP: AY289186 Capsid: AY280339	6802
	Porcine enteric calicivirus /Korean strain/8402	RDRP: AY289187 Capsid: AY280341	8402
	Porcine enteric calicivirus /Korean strain/10802	RDRP: AY289188 Capsid: AY280340	10802
	Porcine enteric calicivirus /Korean strain/15802	RDRP: AY289188 Capsid: AY280342	15802
Noroviruses	HuCV/Desert Shield virus	U04469	DSV
	HuCV/Southampton	L07418	SHV
	Bovine enteric calicivirus/Jena	AJ011099	BECJV
	HuCV/Melksham virus	X81879	MeV
	HuCV/Toronto	U02030	TV
	HuCV/Mexico virus	U22498	MxV
	HuCV/Lordsdale virus	X86557	LV
	HuCV/Neustrelitz260/2000/DE	AY772730	HuDE
	Swine/Norovirus	AB074893	SW
Vesiviruses	Feline calicivirus	AF479590	FeCV
	San Miguel sea lion virus-1	U15301	SMSV1
	Canine calicivirus	AB070225	CC
	Walrus calicivirus	NC004541	Walrus
	Feline calicivirus	AY560118	FeUS
Lagoviruses	Rabbit haemorrhagic disease virus	M67473	RHDV
	Rabbit haemorrhagic disease virus	AF454050	RHDV1
	Rabbit haemorrhagic disease virus	AF454049	RHDV2
	Rabbit haemorrhagic disease virus	NC001543	RHDV-FRG

*PECV cowden strain wild type was retrieved from reference (Guo et al., 2001)

nucleotide sequence investigated, suggesting the same original source for viruses in these faecal samples. However, the nucleotide sequences of the nested-PCR products from two other specimens (8402 and 6802) were to 99.4% and 92.6% identical with the above three (10702, 10802 and 15802). This finding suggested that the two viruses with the less sequence identities originated from different sources. In addition, Korean PECVs had high nucleotide (90.1–92.6%) and amino acid (98.3–99.1%) identities in a part of the putative RNA polymerase region, but low nucleotide (82.3–83.4%) and amino acid (85.2–86.3%) identities in a part of the capsid region, in comparison with those of the Cowden strain of tissue culture-adapted (TC) PECV and wild-type (WT) PECV. The Cowden strain PECV has higher overall amino acid sequence identity in the predicted RDRP protein with SVs rather than with vesiviruses, lagoviruses and NVs (Guo et al., 1999). Similarly, the Korean PECV has a higher overall amino acid sequence identity (73%) with SVs than with vesiviruses (46.2–52.5%), lagoviruses (50.4%) or NVs (30.8–36.4%) (Fig. 1).

The two specimens (10702 and 10802) of nested-PCR products (565 bp) directly sequenced for a part of the capsid region of PECV shared 100% nucleotide sequence identities, suggesting the same original source for viruses in these faecal samples, as determined by sequence comparison of a part of the RDRP region above. The other nested-PCR products (15802, 8402 and 6802), directly sequenced for a part of the capsid region of PECV, shared 99.8%, 99.8% and 85.5% nucleotide sequence identities, respectively, with the two specimens (10702 and 10802). In addition, the four specimens (8402, 10702, 10802 and 15802) of nested PCR products differ in their TAA nucleotide insertion from TC PECV, WT PECV and 6802. Due to this finding, Korean PECVs are classified into two groups when compared with the TC PECV and WT PECV regarding differences in the hypervariable capsid region. The nucleotide and amino acid changes between Korean PECV and that of the Cowden PECV may reflect Korea's different environmental condition, including climate and growing systems. The amplicons shared only limited amino acid sequence identities with a part of the capsid region of vesiviruses (20.5–21.6%), lagoviruses (15.8–23.4%) and NVs (18.0–24.9%). Moreover, these amplicons shared low amino acid identities with those of SVs (22.1–23.9%) (Fig. 1). The reduced virulence could probably be due to the result of a potential change in tissue tropism or binding that may be related to the amino acid substitutions in the hypervariable capsid region. Thus, the amino acid changes in the capsid hypervariable region may be associated with both the cell culture adaptation and the attenuation of the virulence of the TC PECV in piglets.

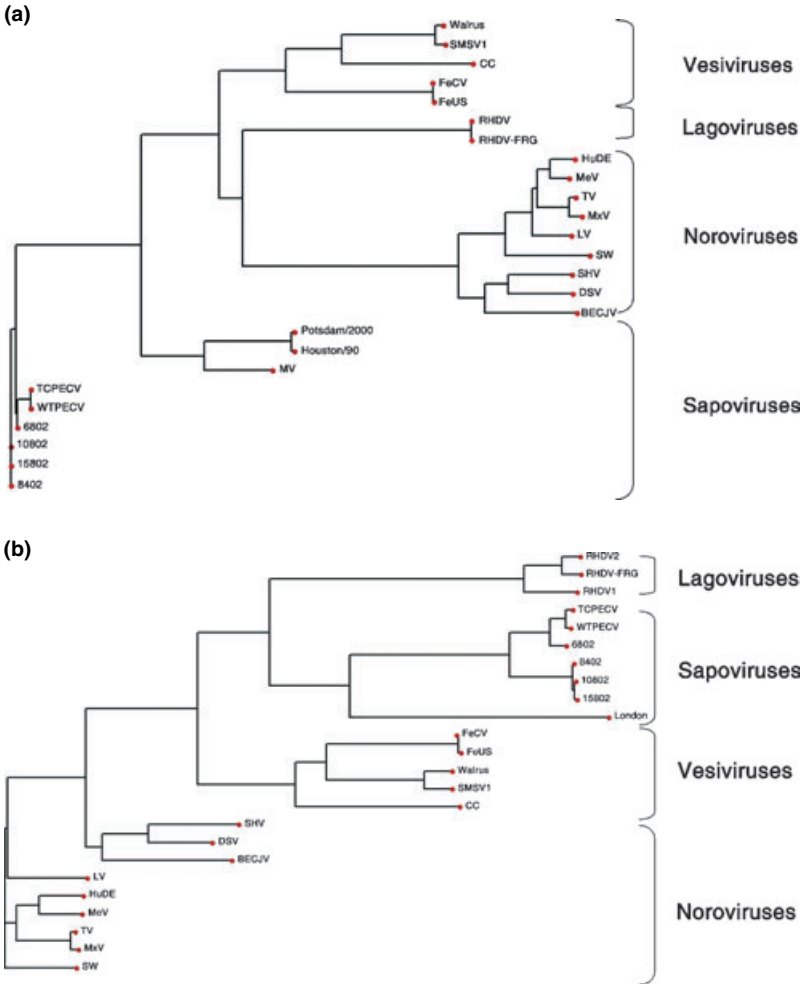

Fig. 1. Phylogenetic tree generated for the sequences in a part of the RNA-dependent RNA polymerase (RDRP) region (a) and the capsid region (b). Alignments were generated from the part of the RDRP region that includes the GLPSG and YGDD regions. Strain names and abbreviations (GenBank accession numbers) are indicated in Table 1.

Discussion

The caliciviruses that mainly occur in swine causing viral enteritis are SVs and NVs. SVs were identified in the USA, while NVs were detected in Japan (Sugieda et al., 1998; Guo et al., 1999). NVs were detected in four caecal contents (0.09%) of 1117 clinically normal pigs slaughtered in Japan. In this study, SVs have been identified via sequence analysis as the type of caliciviruses that can cause porcine viral enteritis in Korea. Although there are no cases reported on the prevalence of SVs in Japan (Sugieda et al., 1998), viruses were detected in nine of the collected faecal samples of 102 diarrhoeic pigs located at different pig farms in Korea. Among the 60 faecal samples without a history of diarrhoea, there were no positive samples by TEM and RT-PCR. These results conclude that SVs are associated with diarrhoea in piglets.

Genetic analysis revealed that the TC Cowden PECV has one distant and three clustered amino acid substitutions in the capsid region, and two amino acid changes in the RNA polymerase region, when compared with the WT PECV (Guo et al., 1999). All the Korean PECVs, which had been naturally transmitted, are more closely related to WT PECV than TC PECV.

In conclusion, we speculate that PECV may be present in most of Korean pig farms, as approximately half the Korean territory is covered by sampling in the provinces of Gyunggi, Chungnam, Jeonbuk, Jeonnam and Jeju. Moreover, most of the breeding pigs were imported from the USA and Europe where this type of enteric virus is omnipresent. In addition, the Korean PECV was closely related to HuCV from UK and USA. PECV is the only virus, among caliciviruses to cause gastrointestinal diseases that is presently being exploited in an *in vitro* model system (Chang et al., 2005). The study of the functional correlation of mutated genes, virulence and cultural system of PEVC might help to understand viral replication strategies, pathogenesis and host immunity.

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