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BRIEF REPORT

Detection and molecular characterization of porcine toroviruses in Korea

Dong-Jun Shin · Sang-Ik Park · Young-Ju Jeong · Myra Hosmillo · Ha-Hyun Kim · Hyun-Jeong Kim · Hyung-Jun Kwon · Mun-Il Kang · Su-Jin Park · Kyoung-Oh Cho

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Abstract This study examined the prevalence and genetic diversity of the porcine torovirus (PToV) in Korea. Of 295 samples, 19 (6.4%) samples tested positive for PToVs by RT-PCR. A low nucleotide sequence identity of the partial S gene was detected among the Korean PToVs (73.5%) and between the Korean and European PToVs (74.0%). Phylogenetic analysis of the spike and nucleocapsid genes showed that the Korean PToVs form distinct branches with clusters corresponding to the farm of origin, which were separate from the other known foreign PToVs. These findings suggest that genetically diverse Korean PToV strains cause sporadic infections in Korea.

Toroviruses, which belong to the family *Coronaviridae*, are spherical, oval, elongated, or kidney-shaped enveloped viruses that possess a positive-sense, single-stranded, polyadenylated RNA genome of approximately 25–30 kb in length [3, 9, 19]. Bovine torovirus (BToV or Breda virus) was first detected in 1982 in the United States during an outbreak of neonatal calf diarrhea in which 15% of the affected animals died [23]. Since then, toroviruses have been detected in normal horses and also in humans, pigs, and turkeys with diarrhea [1, 2, 4, 5, 12, 21].

Fecal shedding of the porcine torovirus (PToV) has only been reported in the Netherlands [12], Canada [6], South Africa [15], USA [22], Hungary [13], and England [17]. Despite the impact of PToVs as enteric pathogens, there is a paucity of sequence and phylogenetic data on the PToV genes. Moreover, sequence data for PToV strains are limited to the S, M, HE, and N genes and have been reported only in European countries, including the Netherlands, Italy, and Hungary [12, 16, 18]. Therefore, it is still unclear if PToV strains circulating in other countries have distinct genetic characteristics. This paper reports the detection of PToV shedding in diarrheic piglets using RT-PCR and the genetic diversity of the spike (S), hemagglutinin-esterase (HE), membrane (M), and nucleocapsid (N) genes of PToV strains circulating in Korea.

A total of 295 diarrheic fecal specimens from 3- to 45-day-old piglets from 65 farms were collected from three provinces (Jeonnam, Jeonbuk, and Jeju) in Korea during 2007. They were examined for common enteric bacteria including *Escherichia coli* and *Salmonella* spp. using specific agar media, and suspected colonies were identified based on biochemical tests. Testing for parasite eggs (*Coccidium* spp. and *Cryptosporidium* spp.) was done using standard flotation techniques. For virologic assays, fecal suspensions of each sample were prepared by diluting the feces (1:10) in 0.01 M phosphate-buffered saline, pH 7.2. The suspensions were vortexed for 30 s and centrifuged (1,200×*g* for 20 min), and the supernatants were collected and stored at −80°C for further testing.

RNA was extracted from a 200 µl starting volume of the centrifuged 10% fecal suspensions using Trizol-LS

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D.-J. Shin · S.-I. Park · Y.-J. Jeong · M. Hosmillo · H.-H. Kim · H.-J. Kim · H.-J. Kwon · M.-I. Kang · K.-O. Cho (✉)
Biotherapy Human Resources Center, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, South Korea
e-mail: choko@chonnam.ac.kr

S.-J. Park (✉)
Bioindustry Research Center, Korea Research Institute of Bioscience and Biotechnology, Jeongeup, Jeonbuk 580-185, South Korea
e-mail: sjpark@kribb.re.kr

Table 1 Nucleotide and amino acid sequence comparisons of the partial spike protein genes of the Korean porcine torovirus strains with other known torovirus strains

Strains[a]	Origin	% identity with strains					
		4 Korean strains[b] (Farm A)		2 Korean strains[c] (Farm B)		5 Korean strains[d] (Farm C)	
		nt	aa	nt	aa	nt	aa
4 Korean strains	Porcine	99.8–100	100	73.5–74.0	71.4–71.9	75.5–75.8	73.4
2 Korean strains	Porcine	73.5–74.0	71.4–71.9	98.8	98.0	93.8–94.1	95.0
5 Korean strains	Porcine	75.5–75.8	73.4	93.8–94.1	95.0	99.8–100	100
Markelo strain	Porcine	74.0–74.2	73.2	92.1	92.5	92.9–93.1	95.5
BToV strains	Bovine	64.2–66.9	59.3–62.8	63.5–65.8	57.2–60.5	61.9–64.8	55.7–59.0
Berne strain	Equine	63.0	56.0	64.5	57.4–57.9	64.5–64.6	58.4

[a] GenBank accession numbers are listed in Supplementary Table 2

[b] Strains from farm A (07-56-11, 07-56-14, 07-56-22 and 07-56-23)

[c] Strains from farm B (07-55-4 and 07-55-5)

[d] Strains from farm C (07-109-11, 07-109-12, 07-109-13, 07-109-14 and 07-109-15)

(Invitrogen, USA). The total RNA recovered was suspended in 50 µl of RNase-free water and stored at −80°C. One-step RT-PCR with a primer pair specific for a portion of the torovirus N gene (Supplementary Table 1) was performed as described previously [12] with slight modifications. To screen for coinfections with other enteric viruses, including group A, B, and C porcine rotaviruses (PRV A–C), porcine sapovirus (PSaV), porcine norovirus, transmissible gastroenteritis coronavirus, and porcine epidemic diarrhea coronavirus, one-step RT-PCR and nested PCR with primer pairs specific for each viruses (Supplementary Table 1) were performed as described previously [11].

To obtain genomic data on the Korean PToVs, partial (648 bp) and full-length S (4,722 bp) genes, full-length HE (1,275–1,284 bp), M (702 bp) and N (492 bp) genes from 11 fecal samples from three farms were amplified by RT-PCR with each corresponding primer pair (Supplementary Table 1). The RT-PCR products were then purified using a QIAEX II gel extraction kit (Qiagen, Germany) according to the manufacturer's instructions. The amplicons were sequenced directly using each corresponding primer pair. DNA sequencing was carried out using an ABI system 3700 automated DNA sequencer (Applied Biosystems, USA).

Using the DNA Basic module (DNAsis MAX, Alameda, CA), the nucleotide and deduced amino acid sequences of the partial and full-length S gene and the M, HE and N genes were compared with those selected from other known toroviruses (Supplementary Table 2). Bootstrap tests (1,000 replicates) of the phylogenetic analysis based on the nucleotide alignments without the primer sequences were carried out employing the neighbor-joining method and the Kimura 2-parameter model using the Molecular Evolutionary Genetics Analysis program (MEGA version 4.0) with pairwise distances [20]. A sequence similarity search was performed for each gene using the LALIGN Query program of the GENESTREAM network server at the Institut de Génétque Humaine, Montpellier, France (http://www.eng.uiowa.edu/~tscheetz/sequence-analysis/examples/LALIGN/lalign-guess.html).

Using the RT-PCR assay (targeting a 185-bp fragment of the N gene of PToV), 19 (6.4%) out of 295 fecal samples from four farms (6.2%) tested positive for PToV. Among the 19 samples, only five tested positive for PToV alone, while the remaining 14 samples were also positive for other enteric pathogens including PRV A–C, PSaV, and *Salmonella* spp (data not shown). This result indicates that the prevalence of PToV infections on Korean pig farms is sporadic, similar to BToV infections (6.9%) on cattle farms in Korea [14].

Pairwise comparison of nucleotide and deduced amino acid sequences of the partial S gene revealed that the genetic diversity of Korean PToV strains varied depending on the farm from which they originated; the strains from farms A and B showed the most divergent sequences, sharing only 73.5–74.0% nucleotide and 71.4–71.9% deduced amino acid identity (Table 1). The strains from farm A also shared low nucleotide (74.0–74.2%) and deduced amino acid (73.2%) identities with the Netherland strain, Markelo. On the other hand, the strains from farms B and C had a high nucleotide (93.8–94.1%) and deduced amino acid (95.0%) identity with each other as well as with the Markelo strain (92.1–91.1% nucleotide and 92.5–95.5% amino acid identity) (Table 1). Alignments of the nucleotide sequences of the partial S gene showed that all of the PToV strains could be placed in the porcine phylogenetic lineage (Fig. 1a). This agrees with a previous study in which three distinct torovirus genotypes of the S gene with apparent preferences for horses, cattle, and swine

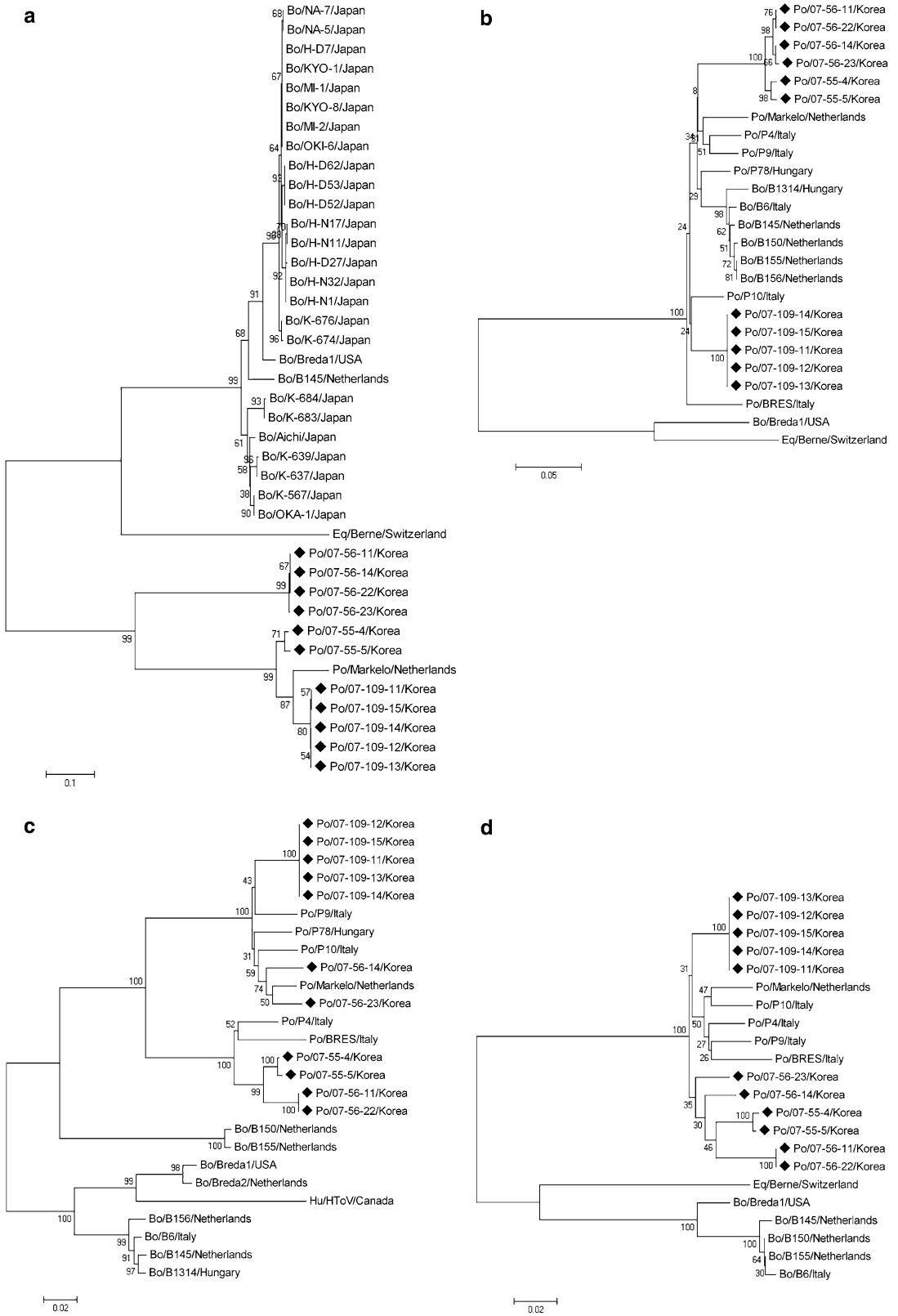

Fig. 1 Phylogenetic trees based on the nucleotide sequences of the Korean porcine torovirus, indicating its genetic relationship with the other known toroviruses. The new Korean strains are indicated by a *filled diamond*. **a** Spike protein gene. **b** Nucleocapsid protein gene. **c** Hemagglutinin-esterase gene. **d** Membrane protein gene

Table 2 Comparison of nucleotide and deduced amino acid identities of the full-length hemagglutinin-esterase genes of the Korean porcine strains with other known torovirus strains

Strains[a]	Origin	% identity with strains							
		Five strains[b] (Farm C)		Two strains[c] (Farm A)		Two strains[d] (Farm B)		Two strains[e] (Farm A)	
		nt	aa	nt	aa	nt	aa	nt	aa
5 Korean strains	Porcine	99.8–100	99.5–100	92.5–93.3	92.9–93.6	80.0–80.3	78.8–79.5	79.1–79.2	76.4–76.6
2 Korean strains	Porcine	92.5–93.3	92.9–93.6	95.2	96.2	80.1–81.4	79.5–81.2	78.8–79.8	77.4–77.8
2 Korean strains	Porcine	80.0–80.3	78.8–79.5	80.1–81.4	79.5–81.2	99.5	99.1	95.5–95.7	93.4–93.6
2 Korean strains	Porcine	79.1–79.2	76.4–76.6	78.8–79.8	77.4–77.8	95.5–95.7	93.4–93.6	100	100
Markelo strain	Porcine	92.9	93.4–93.6	94.1–96.0	94.8–95.6	80.1–80.3	80.7–81.2	78.9	78.3
P10 strain	Porcine	92.9	92.2–92.5	93.1–94.3	93.9–94.1	80.0–80.3	80.5–80.9	78.9	77.8
P4 strain	Porcine	80.2–80.3	77.3–77.5	80.4–80.8	78.3–79.5	91.6	91.5–91.8	92.1	93.4
P78 strain	Porcine	93.4–93.5	93.4–93.6	92.9–93.8	93.4–93.6	80.7–81.0	80.0–80.4	79.9	78.0
P9 strain	Porcine	93.4–93.5	92.2–92.5	91.8–92.7	91.7–92.2	80.9–81.1	80.0–80.4	79.4	77.1
BRES	Porcine	78.5–78.6	76.1–76.4	78.3–78.6	76.7–77.1	90.7–90.8	89.6–89.9	91.1	90.8
BToV strains	Bovine	69.6–74.1	65.5–70.3	68.8–74.8	64.3–71.3	70.7–73.9	66.7–71.3	70.9–74.0	68.1–72.7
HToV strain	Human	64.9	53.5	64.1–65.0	51.8–52.8	65.7–65.9	52.2	65.9	52.8

[a] GenBank accession numbers are listed in Supplementary Table 2

[b] Strains from farm C (07-109-11, 07-109-12, 07-109-13, 07-109-14 and 07-109-15)

[c] Strains from farm A (07-56-14 and 07-56-23)

[d] Strains from farm B (07-55-4 and 07-55-5)

[e] Strains from farm A (07-56-11 and 07-56-22)

were identified [7, 8, 10, 18]. Moreover, the PToV lineage could be divided into two large clusters (Fig. 1a), with the first cluster consisting of only Korean PToV strains from farm A and the other porcine phylogenetic cluster being composed of the three subclusters (strains from farms B and C and the Markelo strain) (Fig. 1a). These results indicate that different PToV strains are circulating in Korea. In addition, nucleotide and deduced amino acid identities were considerably lower between the PToV S genes and those of equine Berne virus (63.0–64.6% nucleotide and 56.0–58.4% deduced amino acid identity) and between the PToV and BToVs strains (61.9–66.9% nucleotide and 55.7–62.8% deduced amino acid identity) (Table 1), supporting the idea that PToV strains are phylogenetically and genetically distinct from the BToVs and EToVs [7, 10, 18].

Since the Korean PToV strains from farm A showed relatively low nucleotide and deduced amino acid identity to other Korean strains and the Markelo strain, we sequenced the full-length S gene of one strain (07-56-22) from farm A and compared it with other known strains. The S gene of the 07-56-22 strain contained an open reading frame of 4722 nucleotides, which encoded a predicted S protein of 1573 amino acids. The 07-56-22 strain had comparatively low nucleotide and deduced amino acid identity to the PToV Markelo strain (86.4% nucleotide and 89.2% deduced amino acid identity). Phylogenetic analysis of the nucleotide sequence of the S gene showed that the 07-56-22 strain belonged to the porcine lineage but formed a different branch, separate from the Markelo strain and other known bovine and equine strains (data not shown).

A comparison of the nucleotide and deduced amino acid identities of the full-length N gene among the Korean PToV strains showed the highest sequence identities within farms, but they also showed relatively high sequence identities regardless of the farm of origin, sharing 91.5–100% nucleotide and 92.6–100% deduced amino acid identity (Supplementary Table 3). Phylogenetic analysis based on the nucleotide sequence of the full-length N gene showed that all PToV strains were grouped with the BToV strains [18] (Fig. 1b). Among the PToV strains, 5 Korean PToV strains from farm C clustered most closely with the porcine P10/Italy strain, and strains from farms A and B clustered with the porcine Markelo/Netherlands, P4/Italy, and P9/Italy strains (Fig. 1b). Moreover, the Korean PToV strains formed different branches, separated according to the farm of origin. These results support the hypothesis that different PToV strains are circulating in Korea.

Comparison of the nucleotide and deduced amino acid sequences of the full-length HE genes among the Korean PToV strains showed that the Korean PToV strains had 78.8–100% nucleotide and 76.4–100% deduced amino acid identity with each other (Table 2). Within a farm, the strains showed relatively higher nucleotide and deduced

amino acid identity except for those from farm A (Table 2). Unlike other Korean PToV strains, Korean PToV strains, 07-56-14 and 07-56-23, originating from farm A, had the highest nucleotide (94.1–96.0%) and deduced amino acid (94.8–95.6%) sequence identities with the PToV Markelo/Netherlands strain but relatively lower nucleotide (78.8–79.8%) and deduced amino acid (77.4–77.8%) identity with other strains (07-56-11 and 07-56-22) from the same farm (Table 2). Phylogenetic analysis based on the nucleotide sequences of the full-length HE genes showed that all of the Korean PToV strains belonged to the porcine PToV lineage and were distinct from other species [18]. This PToV lineage was separated largely into two sublineages, the first including Korean PToV strains from farms C and A (07-56-14 and 07-56-23) clustered with other known PToV strains (Markelo/Netherlands, P10/Italy, P78/Hungary and P9/Italy), and the second including other Korean strains from farms B and A (07-56-11 and 07-56-22), which formed a separate sublineage with strains PToV P4 P4/Italy and BRES/Italy (Fig. 1c). Korean PToV strains originating from farms B and C formed their own clusters. As with sequence homology analysis, strains 07-56-14 and 07-56-23 from farm A were placed distantly with the other strains from same farm but clustered most closely with the PToV Markelo/Netherlands strain (Fig. 1c). These results indicate that different PToV strains are circulating within the same farm in Korea.

A comparison of nucleotide and deduced amino acid sequences of the full-length M gene revealed that all Korean PToV strains have high nucleotide (93.9–100%) and deduced amino acid (99.1–100%) identity with each other (Supplementary Table 4). The sequence identities of all Korean PToV strains were relatively high within each farm. A phylogenetic tree based on the nucleotide sequence of the full-length M gene showed that all PToV strains belonged to the porcine PToV lineage (Fig. 1d), consistent with a previous report [18]. The Korean PToV strains of the porcine PToV lineage branched into two clusters; the strains from the farm C formed a separate branch and were closely related to other known foreign strains (Markelo, P10, P4, P9 and BRES strains), whereas the strains (07-55-4 and 07-55-5) from farm B formed a separate branch and were closely related to two strains (07-56-11 and 07-56-22) from farm A (Fig. 1d). These findings indicate that different PToV strains are circulating within the same farm in Korea.

In summary, genetically diverse Korean PToV strains cause sporadic infections in Korea. Molecular characterization of PToVs in Korea is needed for vaccine development efforts and evaluation and also for clarifying the ecology and evolution of PToV. Sequence data on the PToV genes from many different countries would provide the fundamental data necessary for the development of more sensitive and specific diagnostic tools that could be used to determine the worldwide distribution of this virus.

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