Phylogenetic analysis of kobuviruses and astroviruses from Korean wild boars: 2016–2018

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Abstract
Between 2016 and 2018, the prevalence of porcine kobuvirus (PKoV) and porcine astrovirus (PAstV) in Korean wild boars (n = 845) was 28.0% and 10.7%, respectively. Coinfection by both viruses was detected in 5.1% of boars. Phylogenetic analysis revealed that 134 PKoV isolates belonged to diverse lineages within the species Aichivirus C; however, one strain (WKoV16CN-8627) clustered with bovine kobuvirus (Aichivirus B). Forty-seven PAstVs belonged to lineage PAstV4, and only one strain (WAst17JN-10931) was a novel addition to lineage PAstV2. The two viruses were more prevalent in boars weighing ≤ 60 kg than in boars weighing > 61 kg.

Kobuviruses (family Picornaviridae) are small, non-enveloped viruses with a single-stranded, positive-sense RNA genome approximately 8.2–8.4 kb in length. The polyprotein includes a non-structural leader (L) protein, three structural (VP0, VP3, and VP1) capsid proteins, and seven non-structural (2A–C, 3A–D) proteins [1, 2]. The genus Kobuvirus comprises six species: Aichivirus A (Aichi virus) [3], Aichivirus B (bovine kobuvirus, BKoV) [4], Aichivirus C (porcine kobuvirus, PKoV) [5], and Aichivirus D-F. PKoV was first identified in farm pigs in 2008, and the strain was named S-1-HUN [1]. Since then, PKoV has been detected in fecal samples from healthy and diarrheic pigs in Japan [6], China [7], Thailand [8], South Korea [9], Italy [10], and the USA [11]. Aichi virus (strain A846/88), which only infects humans, was first isolated in 1991 from stool specimens obtained from Japanese patients with acute gastroenteritis [3]. Although pathogenic effects associated with diarrhea have not been clearly linked to PKoV, it is thought that PKoV has the potential for zoonotic transmission [12].

Astroviruses (family Astroviridae, genus Mammastrovirus) are small non-enveloped RNA viruses with a single-stranded, positive-sense RNA genome of approximately 6.1–7.9 kb in length; its polyprotein contains three open reading frames (ORFs). ORF1a and ORF1b encode non-structural proteins, and ORF2 encodes structural capsid proteins [13]. Porcine astrovirus (PAstV) was first detected in 1980 by electron microscopy analysis of feces from diarrheic piglets and was isolated later from cell culture [14]. PAstV has been detected in feces from domestic pigs in various countries, including Hungary [15], Slovakia [16], Canada [17], Thailand [18], China [19], and South Korea [20], and it is classified into five genetic lineages: PAstV-1 to PAstV-5.

As the population density of wild boars (Sus scrofa) increases worldwide, there are concerns that they may transmit infectious pathogens to domestic pigs and other animal species [21]. Indeed, wild boars in Asia and Europe may act as a reservoir for viruses that can cause disease in commercial livestock. Such viruses include classical swine fever virus, African swine fever virus, porcine parvovirus, porcine reproductive and respiratory syndrome virus, and hepatitis E virus [22]. In this study, we examined the circulation of PKoV and PAstV in wild boars in South Korea and investigated their prevalence and genetic diversity.

Of the 845 wild boars captured by hunters during 2016–2018 (a 3-year period), 729 weighed between 6 kg and 300 kg. Based on weight, we classified the wild boars as ≤ 10 kg, 11–30 kg, 31–60 kg, and ≥ 61 kg (Table 1). The remaining 116 boars lacked information and were classified as “unknown”. None of the fecal samples obtained from the captured wild boars were diarrheic. Boars were hunted and captured randomly in the mountainous areas of nine provinces: Gyeonggi (n = 75), Gangwon (n = 112), Chungbuk

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BRIEF REPORT
For analysis, fecal samples (1 g) were resuspended in 10 volumes of phosphate-buffered saline containing 1% gentamicin (Gibco Life Technologies, Paisley, UK) and then centrifuged (5,000 × g for 10 min) to obtain a clarified supernatant. Viral RNA was extracted from 0.2 ml of fecal supernatant using an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA), followed by reverse transcription into viral cDNA using random hexanucleotide primers (NanoHelix, Daejeon, Korea). To detect enteric viruses (PKoV and PAstV), PCR was carried out using primers targeting conserved regions within the 3D gene of PKoV and the RNA-dependent RNA polymerase (RdRp)-ORF2 region of PAstV as described previously [9, 20]. The amplified genes were sequenced at CosmoGenetech (CosmoGenetech Co., Daejeon, Korea) using specific primers and an ABI Prism 3730xi DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Clustal X 1.83 software was used to align these sequences with reference sequences obtained from the GenBank database [23]. Phylogenetic trees for PKoV and PAstV were constructed [24] by the neighbor-joining method [25] using MEGA 6.0 software, with the Kimura 2-parameter model as the measure of genetic distance.

Overall, 28.0% (237/845, including unknown samples) were PKoV positive, and the prevalence rates were 33.8% (89/263) in boars weighing 0–60 kg and 26.2% (122/466) in those weighing > 61 kg (95% CI, 0.22–0.301) (Table 1). In addition, 10.7% (90/845, including unknown samples) were positive for PAstV, with prevalence rates of 16.7% (44/263) in boars weighing ≤ 60 kg and 9.2% (43/466) in those weighing > 61 kg (95% CI, 0.065–0.118) (Table 1). Coinfection with PKoV and PAstV was detected in 5.1% (43/845) of samples (including those from boars of unknown weight). Coinfection rates were 4.1% (3/74) in boars weighing 11–30 kg, 9.9% (18/181) in those weighing 31–60 kg, and 4.3% (20/466) in those weighing ≥ 61 kg (95% CI, 0.024–0.061) (Table 1).

Table 1 Prevalence of porcine kobuviruses (PKoV) and astroviruses (PAstV) among wild boars of different weight

| Wild boar weight (kg) | PKoV Positive (%) | 95% CI | PAstV Positive (%) | 95% CI | Coinfection (PKoV + PAstV) Positive (%) | 95% CI |
|-----------------------|------------------|-------|-------------------|-------|----------------------------------------|-------|
| 0–60                  | 33.8a (89/263)b   | 0.281–0.395 | 16.8 (44/263) | 0.122–0.212 | 7.98 (21/263) | 0.047–0.112 |
| 0–10                  | 50.0 (4/8)        | 0.153–0.846 | 0 (0/8)         | 0.000–0.000 | 0 (0/8)      | 0.000–0.000 |
| 11–30                 | 33.8 (25/74)      | 0.230–0.445 | 14.9 (11/74)   | 0.067–0.229 | 4.1 (3/74)   | 0.004–0.085 |
| 31–60                 | 33.1 (60/181)     | 0.262–0.400 | 18.2 (33/181)  | 0.126–0.238 | 9.9 (18/181) | 0.055–0.143 |
| > 61                  | 26.2 (122/466)    | 0.221–0.301 | 9.2 (43/466)   | 0.065–0.118 | 4.3 (20/466) | 0.024–0.061 |
| Unknown               | 22.4 (26/116)     | 0.148–0.300 | 2.6 (3/116)    | 0.003–0.054 | 1.7 (2/116)  | 0.006–0.040 |
| Total                 | 28.0 (237/845)    | 0.250–0.310 | 10.7 (90/845)  | 0.085–0.127 | 5.1 (43/845) | 0.036–0.065 |

CI confidence interval associated with the prevalence estimate

aPercent positive

bNumber of positive/number of samples tested

The emergence of PAstV in Korean wild boars was first reported in 2011; however, the prevalence was only 0.7% (1/146) [11]. Recently (between 2016 and 2018), PAstV infections have increased: from 3.2% in 2016, to 8.0% in 2017, to 15.9% in 2018 (Fig. 1B). These findings indicate that the enteric viruses PKoV and PAstV are being transmitted at an increasing rate within the wild boar population of South Korea. Therefore, additional investigations of farm pigs will be required to clarify whether the increase in the number of virus-infected wild boars threaten domestic pigs.

Of the 90 PAstV isolates detected in Korean wild boars, 48 were used for phylogenetic analysis; redundant sequences were excluded. The phylogenetic tree, which included 31 global PAstV strains, showed that the 48 PAstV strains from wild boars grouped into two lineages: PAstV-2 and PAstV-4 (Fig. 2A). Lineage PAstV-4 was found in 47 boars, whereas lineage PAstV-2 (strain WAst17JN-10931) was identified in only one boar (Fig. 2A). PAstV has been isolated from wild boars in Hungary [26], Slovakia [27], and Korea [20]. In particular, PAstVs isolated in Slovakia have been typed to the genetic lineages PAstV-2 and PAstV-4 as well as lineages found in other species (chickens and bats) [27]. Korean wild boar PAstVs isolated in 2013 were limited to the PAstV-4 lineage [20]. In the present study, we found that two Korean wild boar PAstV strains (WAst18GB-11875 and WAst18GB-11876) were genetically distinct from the 2013 strains, indicating a possible change in the genetic diversity of PAstV in Korean wild boars.
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and WAst18JB-11889) clustered with a wild boar PAstV-4 strain (WAst17GB-10149, WAst17GB-10880, WAst18GB-11497, and WAst18JB-11455) clustered with the PAstK-31 strain detected in Korean wild boars in 2011 [20]. Other PAstV-4 strains from Korean wild boars belonged to a PAstV-4 lineage that included isolates from domestic pigs in Hungary [28], Belgium, the USA, China, and Korea [20]. Interestingly, strain WAst17JN-10931, belonging to the PAstV-2 lineage, was identified for the first time in Korean wild boars (Fig. 2 A). AstV-2 sequences detected previously in domestic pigs in South Korea (PAstK-76 and KNH14-07) [20, 29] may be closely related to the wild boar PAstV-2 identified in the present study.

Of the 237 PKoV isolates detected in Korean wild boars, 135 were used for phylogenetic analysis (again, redundant sequences were excluded). To investigate the genetic relationships among kobuvirus sequences, we constructed a phylogenetic tree based on 195 partial 3D gene sequences of PKoV, including 60 global PKoV strains (Fig. 2B). The neighbor-joining tree showed that the 134 Korean wild boar PKoV strains clustered within diverse lineages belonging to the species Aichivirus C. Most Korean wild boar PKoVs clustered with domestic pig strains identified in the USA, Japan, Thailand, China, Korea, Spain, and Hungary. Two Korean wild boar strains (WKoV17GN-11321 and WKoV18GN-11819) were closely related to the WB-1-HUN strain isolated from Hungarian wild boars [28] (Fig. 2B). We speculate that the geographical mobility of wild boars results in wide circulation and genetic diversity of Korean PKoVs. Comparison of partial 3D nucleotide sequences suggests that most PKoV strains detected in Korean wild boars, except WKoV16CN-8627, show a high degree of sequence similarity (83.5–94.5% identity) to viruses isolated from Korean domestic pigs and to reference PKoV strains reported in Hungary [30], Spain, the USA, China [2, 31, 32], Japan, and Thailand [33] (84.6–99.7%). Interestingly, another wild boar PKoV strain, WKoV16CN-8627, showed low sequence identity (50.2–54.9%) to sequences from Korean domestic pigs but showed higher identity (74.2–75.7%) to sequences from bovine kobuvirus (BKoV) strains (EGY-1 and U-1) [4, 34]. This suggests that interspecies transmission of kobuviruses between pigs and cows may have occurred naturally.

In conclusion, we show here that PKoV and PAstV are circulating nationwide in Korean wild boars, and that there is no correlation between prevalence of the virus and the province or the weight of the animals. PKoV strain WKoV16CN-8627 from Korean wild boars showed the most similarity to bovine kobuvirus strains, suggesting natural
Fig. 2  Phylogenetic tree based on partial 3D gene sequences (488 bp) of kobuvirus (KoV) strains and partial RdRp-ORF2 gene sequences (799 bp) of astrovirus (AstV) strains. (A) Comparison of partial 3D sequences of 135 KoV strains isolated from wild boars to those of 60 global KoV strains available in the GenBank database. (B) Comparison of partial RdRp-ORF2 sequences of 48 AstV strains isolated from wild boars to 31 global AstV strains available in the GenBank database. The trees were constructed using the neighbor-joining method (MEGA v6.0), with bootstrap values calculated from 1000 replicates. The PKoV WKoV16CN-8627 and PAstV WAst17JN-10931 strains detected in Korean wild boars are indicated by red and blue arrows, respectively. The PKoV and PAstV strains detected in Korean wild boars in this study are indicated by red dots.
transmission of kobuviruses between pigs and cattle. The data also suggest that PAstV-2 infecting Korean wild boars could be involved in interspecies transmission to domestic pigs.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00705-021-05164-1.

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Data availability The partial 3D gene (488 bp) sequences of 135 PKoV strains (accession numbers MW297847-MW297981) and the partial RdRp-ORF2 gene (799 bp) sequences of 48 PAstV strains (accession numbers MW297982-MW298009, MW298011-MW298014, MW298018-MW298019, MW298023, MW298027, MW298034, MW298039-MW298041, MW298044, MW298047, MW298049-MW298053, and MW298056) detected in Korean wild boars have been deposited in the GenBank database.

Declarations
Conflict of interest The authors declare no conflicts of interest.

Ethical statement All animal experiments were approved by the Animal and Plant Quarantine Agency, Ministry of Agriculture Food and Rural Affairs.

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