BRIEF REPORT

Epidemiological and phylogenetic analysis of canine kobuviruses in Tangshan, China

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Abstract

Canine kobuviruses (CaKoV) have been found in healthy and diarrheic dogs as well as asymptomatic wild carnivores in various countries. In order to investigate the prevalence and evolution of CaKoV in Tangshan, China, 82 dog fecal samples from pet hospitals in Tangshan were subjected to RT-PCR targeting a segment of the 3D gene of CaKoV. Using this method, we identified CaKoV in 14 samples (17.07%, 14/82). Of the CaKoV-positive samples, 78.57% (11/14) and 50% (7/14) were positive for canine parvovirus and canine coronavirus, respectively. The nucleotide sequences of the 14 strains 96.6%–100% identical to each other and 77.6%–99.2% identical to representative sequences from the NCBI GenBank database. We also amplified the 14 VP1 gene sequences and found that they were 93.3%–99.6% identical to each other and 73.3%–97.8% identical to representative sequences from the NCBI GenBank database. Phylogenetic analysis revealed that the 14 CaKoV strains from Tangshan are closely related to those identified in China and Thailand and display less similarity to those found in Africa, the United States, and Europe. Our data suggest that CaKoV circulated in young pet dogs in Tangshan and displays a high co-infection rate with CCoV and CPV. However, the relationship between the three viruses and their roles in the host requires further investigation.

Keywords Canine kobuvirus · Epidemiology · Phylogenetic analysis · Molecular characterization

Introduction

Gastroenteritis is a common cause of hospitalization of dogs, and it primarily affects puppies under 1 year of age [1]. Due to the various causes of gastroenteritis, and particularly because coinfections with different viruses or bacteria are common, it can be challenging for veterinarians to determine the etiological agent causing the disease [2, 3]. Kobuviruses have been shown to be potential gastroenteritis-causing agents in dogs [4].

The genus *Kobuvirus* family *Picornaviridae* includes members of the species *Aichivirus A-F* and two unclassified viruses, according to the International Committee on Taxonomy of Viruses (ICTV) reports [5–8]. Canine kobuvirus (CaKoV) is a member of the species *Aichivirus A* [9]. The CaKoV genome is 8.1–8.2 kb in length, with a 5′ untranslated region (UTR), an open reading frame (ORF), and a 3′UTR [9]. The ORF is 7,332–7,341 nucleotides (nt) in length and encodes a single polyprotein, which is separated into a leader (L) protein, three structural proteins, and seven non-structural proteins [4, 10].

CaKoV was first detected in diarrheic dogs in the United States in 2011 [4, 10]. To date, CaKoV has also been detected in both healthy and diarrheic dogs in the United Kingdom [11], Italy [12], Korea [13], Tanzania [14], Japan [15], China [16, 17], Thailand [18], and Brazil [19]. In this study, we performed an analysis of partial 3D and VP1 gene sequences in order to compare the strains we detected with CaKoV strains reported elsewhere to determine the prevalence of CaKoV in Tangshan, China, and to improve our understanding of the relationship between different viruses and hosts.

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Materials and methods

Sampling

Eighty-two fecal samples were provided by pet hospitals in Tangshan between April and June 2019. Of the samples, 62 samples were from animals with diarrhea. All samples were stored in ultra-low-temperature freezers (− 80 °C).

DNA and RNA extraction

The fecal samples were dissolved in phosphate-buffered saline (PBS), and the supernatant was collected by centrifugation. DNA and RNA were extracted from the supernatant using an AxyPrep™ Body Fluid Viral DNA/RNA Miniprep Kit (Corning Inc., New York, USA). Extracted RNA was reverse transcribed into cDNA using an M5 HiPer First Strand cDNA Synthesis Kit (Me5 Technology Co., Ltd., Beijing, China).

Detection of pathogens

All fecal samples were tested for canine parvovirus 2 (CPV-2), canine coronavirus (CCoV), and CaKoV by PCR or RT-PCR according to previously described procedures [11, 20–22]. The purified amplification products of the CaKoV 3D gene fragment and the VP1 gene were ligated into a Blunt-Zero cloning vector using a 5 min TA/Blunt-Zero Cloning Kit (Vazyme Biotech Co., Ltd., Nanjing, China). Recombinant Blunt-Zero cloning vectors were introduced into DH5α competent cells by transformation, and individual colonies were picked three times for sequencing using the Sanger method. Fourteen partial sequences of the 3D and VP1 genes of CaKoV were deposited in the GenBank (NCBI) database (accession nos. MT0199770-MT019983 and MT465833-MT465846).

Phylogenetic analysis of 3D and VP1 genes

For phylogenetic analysis, partial sequences of CaKoV 3D and VP1 genes from canines were retrieved from the GenBank database for use as reference sequences. The ClustalX program was used to make multiple alignments of the sequences [23], and MEGA 6.06 [24] was used to generate a phylogenetic tree, by neighbor-joining (NJ) method with 1000 bootstrap replicates.

Results

We detected 10 (16.13%, 10/62) CaKoV-positive samples from 62 diarrheic dogs and 4 (20.00%, 4/20) CaKoV-positive samples from 20 dogs without diarrhea. Thus, the overall rate of CaKoV positivity in our sample population was 17.07% (14/82). The characteristics of the CaKoV-positive dogs are presented in Table 1, and age information about the dogs used in this study is presented in Table 2. Of the 82 samples, CPV-2 was detected in 64 (78.04%) and CCoV was detected in 14 (17.07%). Of the CaKoV-positive samples, 78.57% (11/14) were co-infected with CPV-2, 50% (7/14) were co-infected with CCoV, and 42.86% (6/14) were co-infected with both CPV-2 and CCoV I/II.

Table 1 Fecal samples from Tangshan, China, that were positive for canine kobuvirus

Number	Dog identification	Age	Gender	Diarrhea	CaKoV partial 3D gene	CPV/subtype	CCoV/subtype
1	TS-A3	3 months	F	No	+	+/2c	−
2	TS-A4	2 months	M	Yes	+	−	−
3	TS-B4	4 months	M	Yes	+	+/2c	+/II
4	TS-C2	2 months	M	Yes	+	−	−
5	TS-C5	1 month	F	No	+	−	+/II
6	TS-D18	4 months	M	Yes	+	+/2c	+/II
7	TS-E1	3 months	F	Yes	+	+/2c	+/II
8	TS-E4	2 months	F	Yes	+	+/2c	+/I
9	TS-G3	2 months	M	Yes	+	+/2c	−
10	TS-G8	3 months	M	Yes	+	+/2a/ 2c	+/II
11	TS-H5	2 months	F	Yes	+	+/2c	−
12	TS-H7	2 months	M	No	+	+/2c	−
13	TS-H8	2 months	M	No	+	+/2c	+/II
14	TS-I6	3 months	F	Yes	+	+/2c	−

+, positive; –, negative; M, male; F, female

Table 2 Age characteristics of the dogs used in this study

Age	CaKoV positive (%)		Total
	Asymptomatic	Diarrhea	
Young (< 1 year)	4/16 (25.00%)	10/54 (18.52%)	14/70 (20.00%)
Adult (1–5 years)	0/4 (0%)	0/7 (0%)	0/11 (0%)
Older (> 5 years)	0 (0%)	0/1 (0%)	0/1 (0%)
	4/20 (20.00%)	10/62 (16.13%)	

Phylogenetic analysis (NJ) of the 14 partial 3D gene sequences and reference sequences was performed using MEGA 6 software with 1000 bootstrap replicates (Fig. 1). The 14 sequences shared 96.6%–100.0% nt sequence identity with each other and 77.6%–99.2% nt sequence identity with other previously reported representative CaKoV sequences. The TS-C2, TS-G8, TS-A3, and Gifu0226 strains formed a small cluster, with nt sequence identity between 98.2% and 100.0%. TS-G3 was located between two small branches and exhibited the closest relationship to strain SMCQ-S4 (98.4% nt sequence identity). Strain TS-E1 was most closely related to strain UK003 from the United Kingdom (96.6% nt sequence identity). The strains TS-H8, TS-C5, TS-I6, and SMCQ-M9 formed a small branch with 97.8%–98.6% nt sequence identity. The strains TS-A4 and TS-B4, form an independent small branch with the Tokyo1173 strain and the CU_125 strain from Thailand with 97.4%–99.8% nt sequence identity. The highest nt sequence identity was observed between the strains TS-H5 and SMCQ-M20 (99.2%). The strains TS-E4, TS-D18, TS-H7, and Y12 form a small branch with 98.2%–99.0% nt sequence identity.

Phylogenetic analysis was also performed as described above using VPI gene sequences (Fig. 2). The 14 sequences shared 93.3%–99.6% nt sequence identity with each other and 73.3%–97.8% nt identity with other previously reported representative sequences. The strains CM-G8, CM-H8, CU_53, CU_83, CU_101, and CU_273 formed a small cluster with 96.8%–99.5% nt sequence identity. The strains CM-A3, CM-E4, CM-C2, CM-I6, and AH-1 formed a small cluster with 97.7%–98.7% nt sequence identity. CM-E1 was located between two small branches and displayed the closest relationship to strains SMCD-59 and SMCD-M9 (97.0% nt sequence identity). Strains CM-B4, CM-H7, CM-D18, and CM-H5 formed a small branch with 96.0%–99.6% nt sequence identity. CM-G3 was located between two small branches and displayed the closest relationship to strain HLJ2015-7 (94.9% nt sequence identity). The strains CM-A4, CM-C5, and HLJ2015-7 formed a small branch with 95.8%–98.3% nt sequence identity. Overall, the 14 sequences from our study samples, nine sequences from other regions of China, and 12 sequences from Thailand formed an independent larger branch.

Discussion

The CaKoV-positive rate observed in this study (16.13%) was similar to those reported previously in diarrheic dogs in northeast China (17.91%) [17], South Korea (19.0%) [25], and Thailand (17.59%) [18] and lower than those reported in southwest China (50.46%) [22] and Japan (37.2%) [15]. The CaKoV-positive rates in diarrheic dogs observed in the United States, Italy, and Brazil were lower (3%, 4.4%, and 5.7%, respectively) than that observed in our study [4, 12, 19]. These data indicate that CaKoV-positive rates may differ between different countries or regions. Some studies have found CaKoV not only in dogs with gastroenteritis but also in healthy animals [15, 18, 22, 25]. In this study, CaKoV was detected in 20% (4/20) of dogs without diarrhea symptoms. These results indicate that CaKoV is present in both healthy and diarrheic dogs. However, the relationship between the virus and diarrhea remains unclear and requires further study.

In this study, all CaKoV-positive dogs were between 1 and 4 months old, similar to the ages of those in the report by Soma et al. [15]. However, Miyabe et al. [19] detected CaKoV in 2-year-old adult dogs. Further analysis by Charoenkul et al. [18] showed that the infection rates of puppies (< 1 year) and old dogs (> 5 years) are similar (25.45% and 18.18%, respectively) and are much higher than those in adult dogs in the 1–5 year age group (4.08%). This indicates that CaKoV can infect dogs of all ages and that puppies and elderly dogs may be infected at higher rates. Diarrhea in dogs is often caused by simultaneous infection with viruses, bacteria, and parasites as well as multiple viruses [1, 26, 27]. In order to detect coinfections with CCoV and CPV, we tested for the presence of the S gene of CCoV and the VP2 gene of CPV in the CaKoV-positive materials. The CaKoV-CCoV coinfection rate was 50% (7/14); CCoV I and CCov II were found in 7.14% (1/14) and 42.86% (6/14) of these coinfections, respectively. The CaKoV-CPV coinfection rate was 78.57% (11/14); most of these coinfections were with CPV 2c subtypes. Interestingly, a mixed CPV subtype 2a and 2c infection was detected in one sample (sample no. 10, Table 1). One sample was positive for CaKoV, but not for CPV or CCoV, suggesting that CaKoV alone may cause diarrhea, which is consistent with findings from northeastern China [17].

In order to investigate the genetic relationship between CaKoV strains in dogs in Tangshan, China, partial 3D and VP1 gene sequences of the 14 CaKoV-positive strains were compared and used for phylogenetic analysis. The VP1 gene encodes the kobuvirus capsid protein, which is the most variable gene among kobuviruses [28, 29]. The 14 VP1 sequences formed a large branch with previously
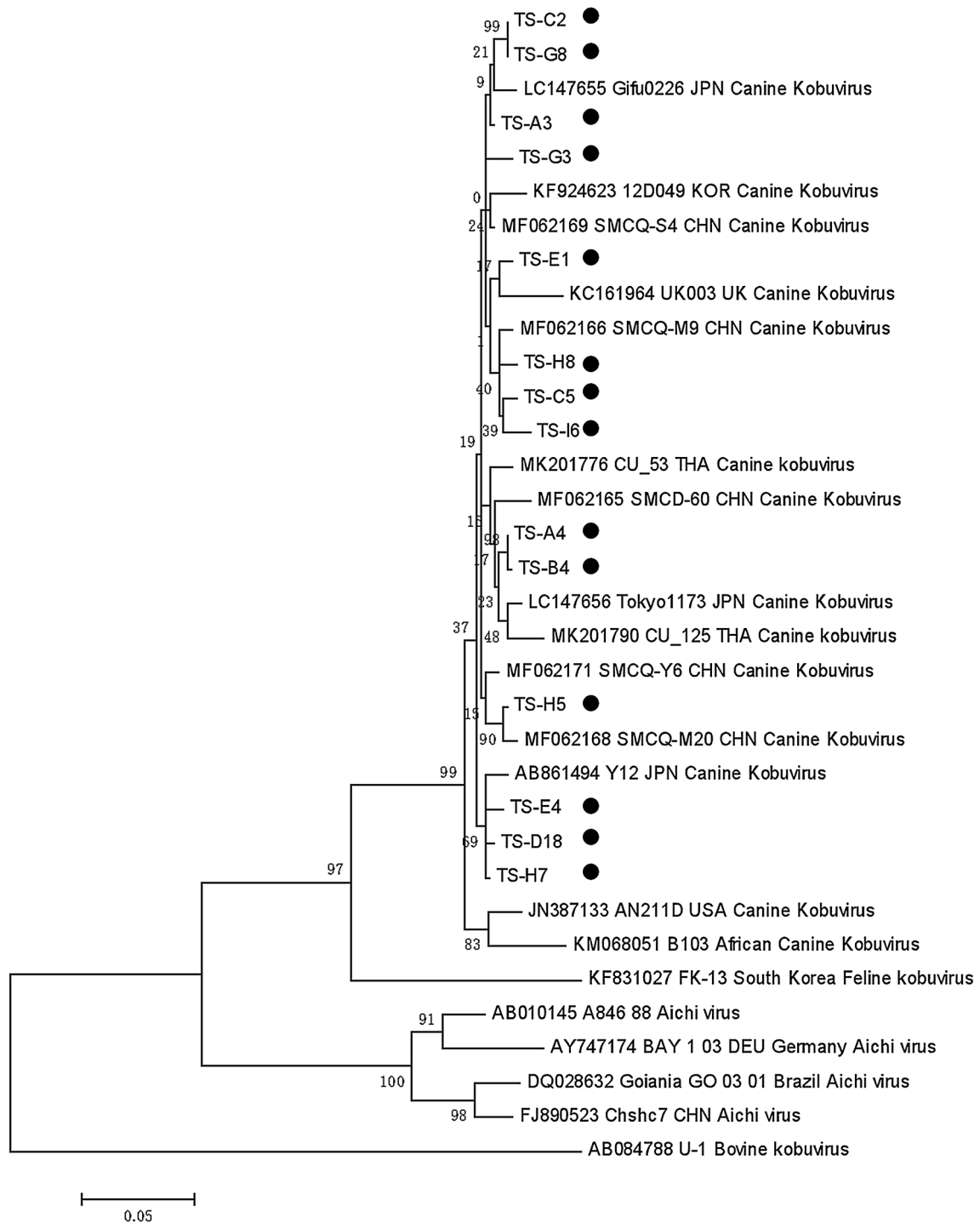
Fig. 1 Phylogenetic tree based on partial 3D gene sequences (504 bp) from CaKoVs, constructed using the neighbor-joining (NJ) method in MEGA 6 software. Bootstrap analysis was performed with 1,000 replicates. ● represents the CaKoV strains identified in this study

published CaKoV sequences from China and Thailand, which is consistent with previous research and indicates that kobuvirus strains may have strong geographical distributions [18].

Fig. 2 Phylogenetic tree based on VP1 gene sequences (834 bp) from ▶ CaKoVs, constructed using the neighbor-joining (NJ) method in MEGA 6 software. Bootstrap analysis was performed with 1,000 replicates. ● represents the CaKoV strains identified in this study
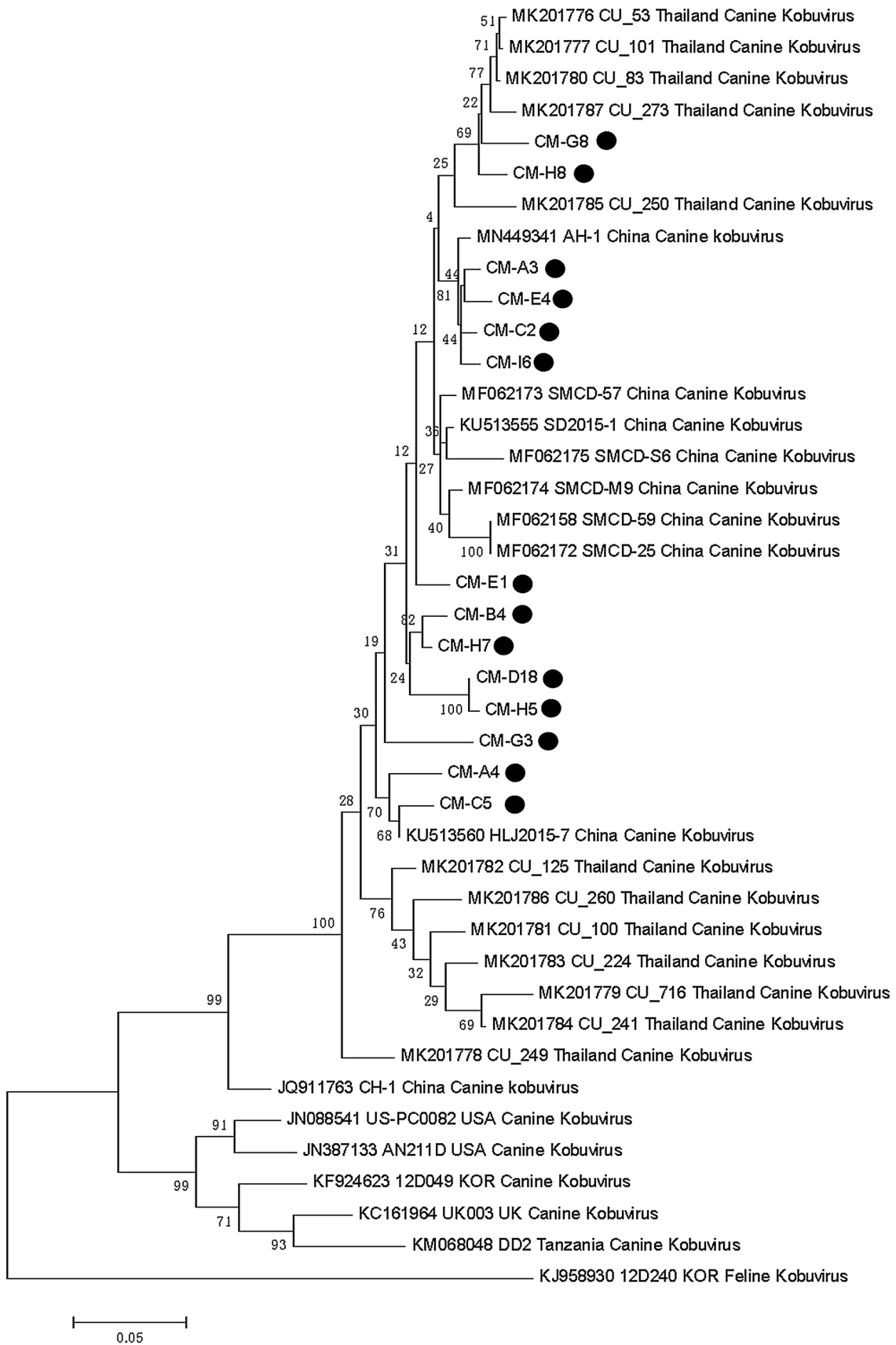
In conclusion, we report for the first time an epidemiological and phylogenetic analysis of canine kobuvirus (CaKoV) in Tangshan, China. Phylogenetic analysis showed that the distribution of the virus displays regional differences. Compared with other enteroviruses, the strain of the CaKoV virus in Tangshan has greater genetic diversity and a higher coinfection rate. Virus positivity appears to be associated with age; puppies and elderly dogs may be infected at a higher rate than adult dogs.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Animal experiments (sampling) were approved by the Animal Care and Use Committee of the Institute of Animal Sciences of the Chinese Academy of Agricultural Sciences. Sampling and data release were approved by the animals' owners.

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