

Detection of canine astrovirus in dogs with diarrhea in Japan

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Abstract Canine astrovirus (CAstV) is the causative agent of gastroenteritis in dogs. We collected rectal swabs from dogs with or without diarrhea symptoms in Japan and examined the feces for the presence of CAstV by RT-PCR with primers based on a conserved region of the ORF1b gene. The ORF1b gene of CAstV was not detected in the 42 dogs without clinical illness but was present in three pups out of the 31 dogs with diarrhea symptoms. Based on the full-length capsid protein, the CAstV KU-D4-12 strain that we detected in this study shared high homology with the novel virulent CAstV VM-2011 strain.

Keywords Canine astrovirus · Diarrhea

Astrovirus (AstV) is an approximately 30-nm spherical virus with a star-like structure. It has a positive-sense single-stranded RNA genome comprised of three open reading frames (ORFs) [1]. ORF1a and ORF1b, which code for non-structural proteins, are present on the 5'-terminal side, while ORF2, which codes for a capsid protein is present on the 3'-terminal side. AstV infection has been reported in mammals and birds [3, 7, 8], with the main clinical symptom being diarrhea. In humans, AstV is a cause of infectious gastroenteritis in children [14]. Although the clinical symptoms of human AstV (HAstV) infection are milder than those of norovirus and rotavirus infections, fatal cases of HAstV infection have been reported in immunocompromised pediatric patients [15].

Canine AstV (CAstV) is the causative agent of gastroenteritis in dogs [10]. The amino acid sequence of the CAstV capsid protein is 18–33 % identical to those of other animal species [12]. Using electron microscopy, particles similar to AstV have been detected in the feces of dogs [11]. The CAstV gene has also been detected in feces of dogs with diarrhea symptoms, and a virus with different antigenicity from that of the prototype CAstV was recently identified in dogs with gastroenteritis [12]. CAstV infection is spreading across the world; however, in Japan, the epidemiology of CAstV infection in dogs remain unclear.

In the present study, we collected rectal swabs from dogs in Japan and examined the feces for the presence of CAstV by RT-PCR with primers based on a conserved region of ORF1b gene. The results of detection were compared between dogs with diarrhea symptoms and healthy dogs in order to investigate the relationship between CAstV infection and clinical symptoms. In addition, the amino acid sequence of the capsid protein in the detected CAstV was deduced and its genetic relationship to known CAstV isolates was examined using phylogenetic analysis.

Rectal swab samples were collected from 42 dogs without clinical illness and 31 dogs with diarrhea symptoms between 2011 and 2014. These samples were submitted by veterinary clinics in Japan (Aomori, Saitama, Tokyo, Kanagawa, and Okinawa).

Viral RNA was extracted from the rectal swabs using a High Pure Viral RNA Isolation Kit (Roche, Switzerland), following the manufacturer's instructions. RNA preparations from rectal swabs (10 ng), random primers (final concentration of 50 pM), and a dNTP mixture (final concentration of 10 mM each) were mixed, and the volume was adjusted to 10 µL with ddH₂O. This template and primer mixture was heated at 65 °C for 5 minutes and then

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rapidly cooled on ice. After mixing 10 μ L of the mixture and 4 μ L of 5 \times PrimeScript Buffer (TaKaRa, Japan), an RNase inhibitor (20 U; TaKaRa, Japan) and PrimeScript Reverse Transcriptase (100 U; TaKaRa, Japan) were added, and the volume was adjusted to 20 μ L with ddH₂O. The reaction mixture was heated at 30 °C for 10 minutes, and then at 42 °C for 60 minutes. After heating at 70 °C for 15 minutes, the reaction mixture was rapidly cooled on ice. PCR was performed using the synthesized cDNA.

cDNA was amplified by PCR, which was performed in a total volume of 50 μ l using the following two methods: (i) Detection of the conserved region of the ORF1b gene: 2 μ l of sample cDNA was mixed with 25 μ L of Quick Taq HS DyeMix (Toyobo, Japan), 1 μ l of 20 μ M primer mix (Astr4380F: 5'-GAYTGGRCNCNGNTWYGATGGNACIAT-3', Astr4811R: 5'-GGYTTNACCCACATNCCAA A-3'), and 22 μ l of distilled water. Using a PCR Thermal Cycler Dice (TaKaRa, Japan), DNA was denatured at 94 °C for 5 min, followed by 36 cycles of denaturation at 94 °C for 30 s, primer annealing at 45 °C for 30 s, and extension at 72 °C for 60 s, with a final extension at 72 °C for 10 min. The detection methods of RNA virus genes other than CAstV (canine distemper virus, canine rotavirus, canine calicivirus, canine norovirus, canine kobuvirus, and canine coronavirus) have been described by Chung *et al.* [2], Ellschner *et al.* [4], Erles *et al.* [5], and Martella *et al.* [10]. (ii) Preparation of PCR products for sequencing: 2 μ l of sample cDNA was mixed with 10 μ l of 5-fold PrimeSTAR Buffer (TaKaRa, Japan), 4 μ l of dNTP Mixture (TaKaRa, Japan) containing 2.5 mM of each dNTP, 1 μ l of 20 μ M primer mix (the nucleotide sequences of the primers are shown in Table 1), 0.5 μ l of PrimeSTAR HS DNA Polymerase (2.5 U/ml; TaKaRa, Japan), and 32.5 μ l of distilled water. Using a thermal cycler, DNA was denatured at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, primer annealing at 55 °C for

15 s, and synthesis at 72 °C for 1 min, with a final extension at 72 °C for 5 min.

Thirty microliters of PCR products was electrophoresed with DNA markers in a 1.5 % agarose gel. Singlet bands were excised and transferred to microtubes, and DNA was purified using a QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany). The purified DNA was subjected to TA-cloning using a Mighty TA-cloning Reagent Set for PrimeSTAR (TaKaRa, Japan), following the manufacturer's instructions. The clones were sequenced using the M13 primers from the TA-cloning vector (pMD20-T vector; TaKaRa, Japan) and the specific primers from canine astrovirus (Table 1). The purified plasmid was sent to Sigma Genosys (Japan) for sequencing. The sequences of virus genomes were determined, and phylogenetic trees were constructed using MEGA software (version 6). Phylogenetic relationships were determined using the neighbor-joining algorithm, and branching order reliability was evaluated by 1,000 replications of a bootstrap resampling analysis. The phylogenetic tree of the full-length capsid protein was prepared as described previously by Martella *et al.* [12] and the International Committee on Taxonomy of Viruses (2010.018a-cV.A.v4. Mamastrovirus.pdf: http://talk.ictvonline.org/files/ictv_official_taxonomy_updates_since_the_8th_report/m/vertebrate-official/4178.aspx).

CAstV was detected in rectal swabs collected from dogs in Japan. It was not detected in the 42 dogs without clinical illness but was present in three (D4, D19, and D21) out of the 31 dogs with diarrhea symptoms. These rectal swabs were negative for canine parvovirus antigen. CAstV-gene-positive rectal swabs were tested for CAstV and other RNA viruses (canine distemper virus, canine rotavirus, canine calicivirus, canine norovirus, canine kobuvirus, and canine coronavirus). Of the three dogs with positive rectal swabs, D4 (Toy Poodle, male, 2 months old) was positive for the CAstV gene only. In D19 (Miniature Dachshund, female, 1

Table 1 Nucleotide sequences of primers used for sequencing

Primer	Usage application	Orientation	Nucleotide sequence (5' to 3')	Location	Reference
Astr4380F	RT-PCR, sequencing of ORF1b gene	Sense	GAYTGGRCNCNGNTWYGATGGNACIAT	3354-3379 ^{a)}	NC_024701 ^{c)}
Astr4811R	RT-PCR, sequencing of ORF1b gene	Antisense	GGYTTNACCCACATNCCAAA	3765-3784 ^{a)}	NC_024701 ^{c)}
CAstV375f	RT-PCR, sequencing of ORF2 gene	Sense	TCCGGATCTTGAATCACTCC	375-394 ^{b)}	JN193534 ^{d)}
CAstV781f	Sequencing of ORF2 gene	Sense	GGCAGGAGTTACAGGACCAA	781-800 ^{b)}	JN193534 ^{d)}
CAstV1318f	RT-PCR, sequencing of ORF2 gene	Sense	GTGCACCTGGCAGTTCACCTA	1318-1337 ^{b)}	JN193534 ^{d)}
CAstV1841f	Sequencing of ORF2 gene	Sense	GAACCAATGCCTCCAAGTGT	1841-1860 ^{b)}	JN193534 ^{d)}
CAstV1858r	RT-PCR, sequencing of ORF2 gene	Antisense	ACTTGGAGGCATTGGTTTCAG	1839-1858 ^{b)}	JN193534 ^{d)}
CAstV3000r	RT-PCR	Antisense	TGAACCTGTACCCTCGATCC	2981-3000 ^{b)}	JN193534 ^{d)}

a) Nucleotide numbering refers to the genome of the feline AstV-D1 strain

b) Nucleotide numbering refers to the genome of the CAstV-VM2011 strain

c) GenBank accession no. NC_024701

d) GenBank accession no. JN193534

KU-D4-12	1	KLRWSLVNAE	QRKKYQKLHD	WYVDNLVDRY	VLLPSGEVTE	QHRGNPSGQF	STMDNNMIN	60
KU-D19-12		
KU-D21-12		
Dog/1068/BRA/2012/AGR40415	RR.....	...E..N.T	
Dog/915/BRA/2013/AGR40414	R.....	...E..N.T	
Dog/905/BRA/2013/AGR40413	R.....	...E..N.T	
Dog/1109/BRA/2013/AG40417	RR.....	...E..N.T	
Dog/1110/BRA/2013/AG40418	RR.....	...E..N.T	
Dog/Bari-08/ITA/2008/ADH01686	R.....	...E..N.T	
Dog/Unknown/CHN/2012/AFI71936	R.....	...N.T	
KU-D4-12	61	TWLQAFEFAY	IHGPKELWA	SYDTLVYGDD	RLSTPKLPD	DYNTKVVAMY	RDA	113
KU-D19-12		
KU-D21-12		
Dog/1068/BRA/2012/AGR40415		F...Q..L	N.....	...I.I..	N.VER..L..	K.V	
Dog/915/BRA/2013/AGR40414		F..N.Q..L	N.....	...L.I..	N.VER..L..	K.V	
Dog/905/BRA/2013/AGR40413		F..N.Q..L	N.....	...L.I..	N.VER..L..	K.V	
Dog/1109/BRA/2013/AG40417		F..N.Q..L	N.....	...L.I..	N.VER..L..	K.V	
Dog/1110/BRA/2013/AG40418		F..N.Q..L	N.....	...L.I..	N.VER..L..	K.V	
Dog/Bari-08/ITA/2008/ADH01686		F..N.Q..L	N.....	...L.I..	N.VER..L..	K.V	
Dog/Unknown/CHN/2012/AFI71936		F..N.Q..M	N.....	...L.I..	N.VER..L..	K.V	

Fig. 1 Alignment of the deduced sequences of the C-terminal 113 amino acids of the RdRp of CAstV strains detected in this study and other CAstV. Dots indicate identical amino acids

month old) and D21 (Pug, male, 2 months old), canine coronavirus and canine kobuvirus genes were detected as well as the CAstV gene. These samples were collected from different places during the same year. The newly detected CAstVs were designated as strains CAstV KU-D4-12, KU-D19-12, and KU-D21-12, respectively.

Amino acid sequences were deduced from the CAstV ORF1b gene fragments detected in the three dogs (partial RNA-dependent RNA polymerase [RdRp] gene) and subjected to phylogenetic analysis (Figs. 1 and 2). The deduced sequence of the 113 amino acids of the C-terminal of RdRp was identical in all CAstV strains detected in this study.

A sequence analysis of the full-length capsid protein gene was performed to determine the genogroup of the detected CAstV strains, and amino acid sequences were deduced. Of the three CAstV strains, the sample volumes of the CAstV KU-D19-12 and KU-D21-12 strains were insufficient to perform a sequence analysis of the full-length capsid protein gene. Thus, only the CAstV KU-D4-12 strain was analyzed, and its amino acid sequence was deduced. The CAstV KU-D4-12 strain was found to belong to the clade corresponding to the species *Mamastrovirus 5*, as were the other CAstV strains. In addition, the full-length capsid protein of the CAstV D4-12 strain showed more than 90 % sequence identity to that of the CAstV VM-2011 strain (Fig. 3). The CAstV VM-2011 strain is a novel CAstV that differs from the prototype CAstV detected in dogs with gastroenteritis in Italy [12].

In this study, we detected CAstV for the first time in dogs in Japan. The CAstV infection rates were 0 % in dogs without clinical illness and 9.7 % in dogs with diarrhea

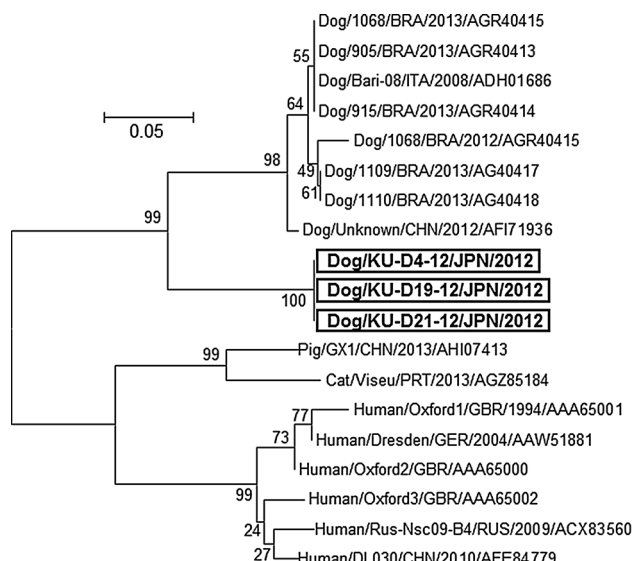


Fig. 2 Phylogenetic tree based on the C-terminal amino acid sequence of RdRp. The phylogenetic analysis was based on the deduced sequence of the C-terminal 113 amino acids of the RdRp. Phylogenetic relationships were determined using the neighbor-joining algorithm, and branching order reliability was evaluated by 1,000 replications of a bootstrap resampling analysis

symptoms, which were lower than those reported in Italy (24.5 % in dogs with diarrhea symptoms) [13], and the age of the dogs investigated may have been the reason for this difference. The dogs in the study conducted in Italy were pups younger than 3 months old, whereas our study included pups aged 1.5 months old to elderly dogs aged 15 years old, with a mean age of 3.9 ± 0.6 years old (mean \pm S.E.). Furthermore, CAstV-positive dogs were

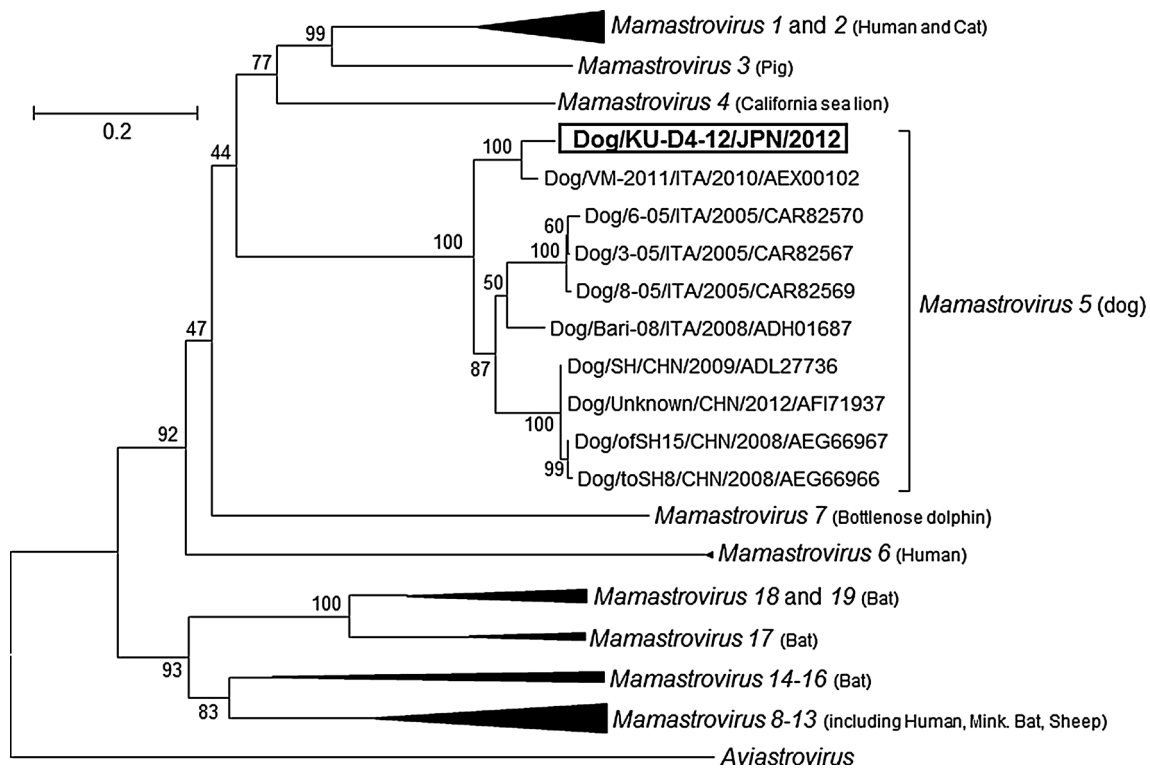


Fig. 3 Phylogenetic tree based on the amino acid sequence of full-length VP1. The phylogenetic analysis was based on the deduced amino acid sequence of the full-length VP1. Phylogenetic

relationships were determined using the neighbor-joining algorithm, and branching order reliability was evaluated by 1,000 replications of a bootstrap resampling analysis

2 months old or younger. The CAstV infection rate in pups younger than 3 months old with diarrhea symptoms was 33.3 % (3/9), which was slightly higher than that in the pups in Italy. In this study, we examined samples from dogs of various ages, but CAstV was detected only in young pups, suggesting that CAstV infection occurs primarily in young pups in Japan, as has been observed in Europe and China [6, 13, 16].

We did not find CAstV in non-diarrheic dogs, although the presence of astrovirus is commonly reported in the young of different species without association with clinical signs [6, 13]. However, healthy young pups (1 to 3 months old) in which the CAstV gene was not detected have also been reported [16]. The maintenance conditions of the dogs used may have influenced the results, as a possible reason for this contradiction. In our study and that reported by Zhu et al. [16], dogs brought to animal hospitals in major cities such as Tokyo and Shanghai were used, but dogs maintained in a group, such as those in an animal shelter, were not used. It is necessary to investigate the state of CAstV infection under different animal maintenance conditions.

Despite several attempts, we could not identify the full-length ORF2 gene sequence of the KU-D19-12 and KU-D21-12 strains. The following reasons were considered for this result: i) the detection sensitivity of the ORF2 gene was lower than that of the ORF1 gene because the gene

contains many mutations, or ii) the total amount of sample was insufficient because rectal swab samples, not fecal samples, were used.

Recently, it was reported that the AstV 1637F strain, which is similar to human astrovirus, has been detected in cats [9]. This finding indicates that new human AstV may emerge through recombination between animal AstV and human AstV. Therefore, it is necessary to periodically investigate the presence of AstV in animals and humans.

We collected rectal swabs from dogs in Japan and examined the feces for the presence of CAstV by RT-PCR with primers based on conserved region of the ORF1b gene. The CAstV infection rate was 33.3 % in pups with diarrhea symptoms. Based on the full-length capsid protein, the CAstV KU-D4-12 strain shared high homology with the novel virulent CAstV VM-2011 strain.

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