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Letter to the editor Open Access

Identification of a novel astrovirus from intestinal tissue of a donkey foal with severe diarrhea in China

DEAR EDITOR,

Astroviruses are known to cause gastroenteritis and diarrhea symptoms in various hosts. However, no information is currently available on any donkey astrovirus genome. In this study, we collected six intestinal samples from donkey foals that died of severe diarrhea on a donkey farm in Shandong Province, China, in 2021. Based on metagenomic next-generation sequencing, a nearly complete donkey astrovirus 1 (DAstV-1) sequence was detected as the only possible diarrhea-related mammalian virus. Real-time polymerase chain reaction revealed the presence of DAstV-1 in one of the six samples. Using Megablast, DAstV-1 exhibited almost no nucleotide similarity to any currently known astroviruses, although it exhibited several conserved sequence features of such viruses, including a frameshift between ORF1a and ORF1b. Phylogenetic tree construction indicated that DAstV-1 is a novel species of the *Mamastrovirus* genogroup II of the genus *Mamastrovirus*. To the best of our knowledge, this study is the first to report on a donkey astrovirus genome and highlight its risks to the donkey breeding industry and public health. Further studies are necessary to determine the epidemiology and pathogenicity of DAstV-1.

Astroviruses, members of the family Astroviridae, are non-enveloped and unsegmented viruses, with a genome composed of single-stranded positive-sense RNA (6.4–7.7 kb in size) (Wohlgemuth et al., 2019). Their genomes consist of three overlapping open reading frames (ORF1a, ORF1b, and ORF2), encoding viral protease, polymerase, and structural protein, respectively. Astroviruses were first identified in the feces of infants (Madeley & Cosgrove, 1975), but are widely distributed in marine and terrestrial habitats. To date, more than 80 species of animals, including mammals (humans, cats, cattle, deer, dogs, mice, pigs, sheep, minks, bats, cheetahs, rabbits, marine mammals, gerbils, and pangolins) and birds (turkeys, chickens, ducks, guinea fowl, pigeons, and crows), have been identified as natural hosts of astroviruses (Nie et al., 2022; Wohlgemuth et al., 2019). The Astroviridae family consists of two genera: *Mamastrovirus* (*MAstV*) and *Avastrovirus* (*AAstV*), which infect mammals and birds, respectively. *MAstV* includes 19 species (*MAstV-1*–*MAstV-19*) and *AAstV* includes three species (*AAstV-1*–*AAstV-3*), although new species are still being discovered (Bosch et al., 2014).

Astroviruses can cause fever, vomiting, and severe diarrhea in juvenile and elderly mammals and in immunocompromised individuals (Cortez et al., 2017). Astroviruses usually cause self-limiting diseases with mild gastrointestinal symptoms in adult humans and animals, with a lower prevalence in patients with diarrhea compared to that of rotaviruses and noroviruses (Wang et al., 2022). As such, little attention has been paid to treating astrovirus infections and limiting their transmission. However, in severe cases, these infections can be fatal in infants and juvenile animals. Furthermore, astroviruses can cause serious diseases in birds and mammals, including encephalitis, meningitis, ganglionitis, and polioencephalomyelitis (Wildi & Seuberlich, 2021). Recently, an increase in the occurrence of various emerging and re-emerging zoonotic infectious diseases have been reported, leading to significant impact on human and animal health (Zhai et al., 2022). Consequently, monitoring the emergence of new pathogens is crucial for preventing and controlling zoonotic viral breakouts. In this study, we discovered a novel astrovirus in the gastrointestinal tract of donkeys. To the best of our knowledge, this is the first documented report of a donkey-associated astrovirus.

In 2021, a considerable number of donkeys from a donkey farm in Shandong Province (China) developed diarrhea. Their symptoms were not relieved by the administration of antibiotics and several foals died of severe diarrhea and dehydration in the absence of effective treatment. The cause of the disease could not be identified.

In this study, we collected intestinal samples from six deceased donkey foals. The samples were stored at −80 ℃ and subsequently transported to our laboratory for pathogen detection based on virome analysis. The samples were homogenized with steel beads and total RNA was extracted using an Omega Viral DNA/RNA Kit (Omega, USA). A mixture of six nucleic acid samples was tested using next-generation sequencing. A sequencing library was constructed using a NEBNext Ultra II Directional RNA Library Prep Kit (NEB, USA). The library was sequenced on the Illumina NovaSeq 6000 (PE150) sequencing platform (San Diego, USA). In total, 52.85 million reads were obtained. Using previously described methods (Tian et al., 2022), low-quality bases were filtered out using the fastp program (v0.20.0) and the remaining reads

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were assembled *de novo* using SPAdes (v3.13.0). The assembled reads were then compared with the National Center for Biotechnology Information (NCBI) non-redundant (nr) protein database using Diamond BLASTx (v0.9.7) to identify virus-associated contigs. Accuracy of the virus-associated contigs was confirmed by comparing them with the NCBI nucleotide database using the Nucleotide Basic Local Align Search Tool (BLASTN), and a read-mapping approach was used to estimate the confirmed viral abundance (Supplementary Figure S1). We identified the family Herelleviridae, genus *Pbunavirus*, unclassified viruses in the order Caudoviricetes, family Deltaflexiviridae, which infects plants, family Bornaviridae, which is associated with neurological diseases, and family Retroviridae. Notably, astrovirus was the most abundant and only mammalian intestinal virus detected in the samples. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to bridge the sequence gaps using primers based on the obtained viral contigs. The complete sequence of the 3′ terminal was successfully determined using rapid amplification of cDNA end (3′-RACE) (TaKaRa), although 5′-RACE was unsuccessful and several amino acids at the N-terminal of ORF1 could not be recognized. Finally, a donkey astrovirus genome (6 317 nucleotides (nt) in length) was obtained, named DAstV-1. We designed two pairs of specific primers for DAstV-1 (DAstV-1-P1F: 5′-CGTCGAGGACCTTTGTTCTT-3′, DAstV-1-P1R: 5′-GATGTCGTAGGGCTTGTTAGAG-3′; DAstV-1-P2F: 5′-AACAAGCGTGGTGGTAACT-3′, DAstV-1-P2R: 5′-AATTCGGGTCAGTCCTACATTC-3′) and determined its expression level in the six intestinal samples via RT-PCR. Only one sample tested positive for DAstV-1.

Three open reading frames (ORFs) were identified in the DAstV-1 genome: ORF1a, ORF1b, and ORF2. The genome structure of DAstV-1 was most similar to that of mink astrovirus 1 (MAstV-1) (Figure 1A). A frameshift was observed between ORF1a and ORF1b, but no overlap was detected between ORF1 and ORF2. The Protein Basic Local Align Search Tool (BLASTP) revealed approximately 44% amino acid similarity between the ORF1a of DAstV-1 and that of the fox astrovirus (AGK45542.1), feline astrovirus 4 (QPI69530.1), and raccoon dog astrovirus 3 (ULF48000.1). ORF1b exhibited approximately 62% amino acid similarity to human astrovirus BF34 (YP_009047078.1) and astrovirus BF34 (QQM16389.1, MAG), while ORF2 exhibited the highest amino acid identity (48.45%) to the fox astrovirus (AGK45544.1).

Five transmembrane domains in ORF1a were predicted using TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) at genomic positions 315–383, 537–605, 639–707, 765–833, and 852–920, and trypsin-like peptidase domain, RNA-dependent RNA polymerase, and capsid protein precursor were predicted using the NCBI conserved domain database (CDD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Figure 1B). BLASTN revealed that the genome of DAstV-1 exhibited almost no nucleotide identity with any known astrovirus, except for a ribosomal frameshift signal in the overlap of ORF1a and ORF1b (AAAAAAAC, Figure 1B), and a highly conserved stem-loop II motif (s2m, 43 nt) at the 3′ end of the DAstV-1 genome (Figure 1C). This stem-loop II motif (Figure 1C) was conserved at both the nucleotide sequence and RNA structure levels, and exhibited the highest nucleotide identity to the *Hipposideros* bat coronavirus (MZ293757.1, 100% identity) and Zaria bat coronavirus (HQ166910.1, 95.35% identity). In addition, the highly conserved stem-loop II motif also exhibited a high level of similarity with the sequences of the 3′ end of the genomes of bat picornavirus, pigeon picornavirus, norovirus, coypu hunnivirus, and bat, squirrel, rodent, human, and fox astroviruses (Figure 1D). While the function of the stem-loop II motif remains unknown, this conserved region is considered a suitable target for universal and specific pathogen detection (Monceyron et al., 1997) and a potential common therapeutic target for multiple pathogens. Thus, detection and treatment strategies based on this locus may also be applicable to DAstV-1.

The ORF1a, ORF1b, and ORF2 sequences of DAstV-1 were aligned with the same fragments of various other astroviruses using MAFFT v7.505 (https://mafft.cbrc.jp/alignment/software/). The optimal model was predicted using IQ-TREE v2, and phylogenetic trees were constructed using the maximum-likelihood method (Figure 1E, F, G). The capsid of DAstV-1 was clustered with astroviruses detected in foxes and humans, indicating that DAstV-1 should be classified into the genus *Mamastrovirus* and the *Mamastrovirus* genogroup II. The capsid of DAstV-1 was most closely related to that of fox astrovirus 5 (KC692365), followed by human and porcine astroviruses in the phylogenetic tree. In the ORF1a and ORF1b phylogenetic trees, the evolutionary branches closest to DAstV-1 consisted of fox, cat, mink, and canine astroviruses, while the porcine and human astroviruses were distantly related to DAstV-1. Taken together, these results confirm that DAstV-1 is a novel astrovirus species.

In this study, we reported the presence of a novel astrovirus (DAstV-1) in donkey foals suffering from severe diarrhea on a donkey farm in China. Thus far, DAstV-1 is the only virus known to be associated with diarrheal symptoms in the donkey intestine. However, our study was limited due to the small sample size and detection of only one positive sample of DAstV-1. We found no evidence suggesting that DAstV-1 played a role in the death of the donkey foals with severe diarrhea, with the symptoms subsiding in live donkeys over time. Although our detection primers were specific to DAstV-1, relying on its low genomic similarity to other known viruses, new rapid detection methods for this virus need to be developed and optimized. Moreover, continuous surveillance and prevention strategies are crucial for reducing the economic losses caused by DAstV-1 and other pathogens. The identification of the DAstV-l genome in our study contributes to the diversity of mammalian viruses in donkeys and the Astroviridae family. Additionally, the identified sequence can be used for the detection of other pathogens. Therefore, our study provides a scientific basis for future investigations into animal-borne infectious diseases caused by astroviruses.

DATA AVAILABILITY

The data reported in this paper were deposited in GenBase of the National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences/China National Center for Bioinformation under accession number C_AA001300.1 (https://ngdc.cncb.ac.cn/genbase), in the NCBI GenBank database under accession number OQ693700, and in the Science Data Bank under https://doi.org/10.57760/sciencedb.07856.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

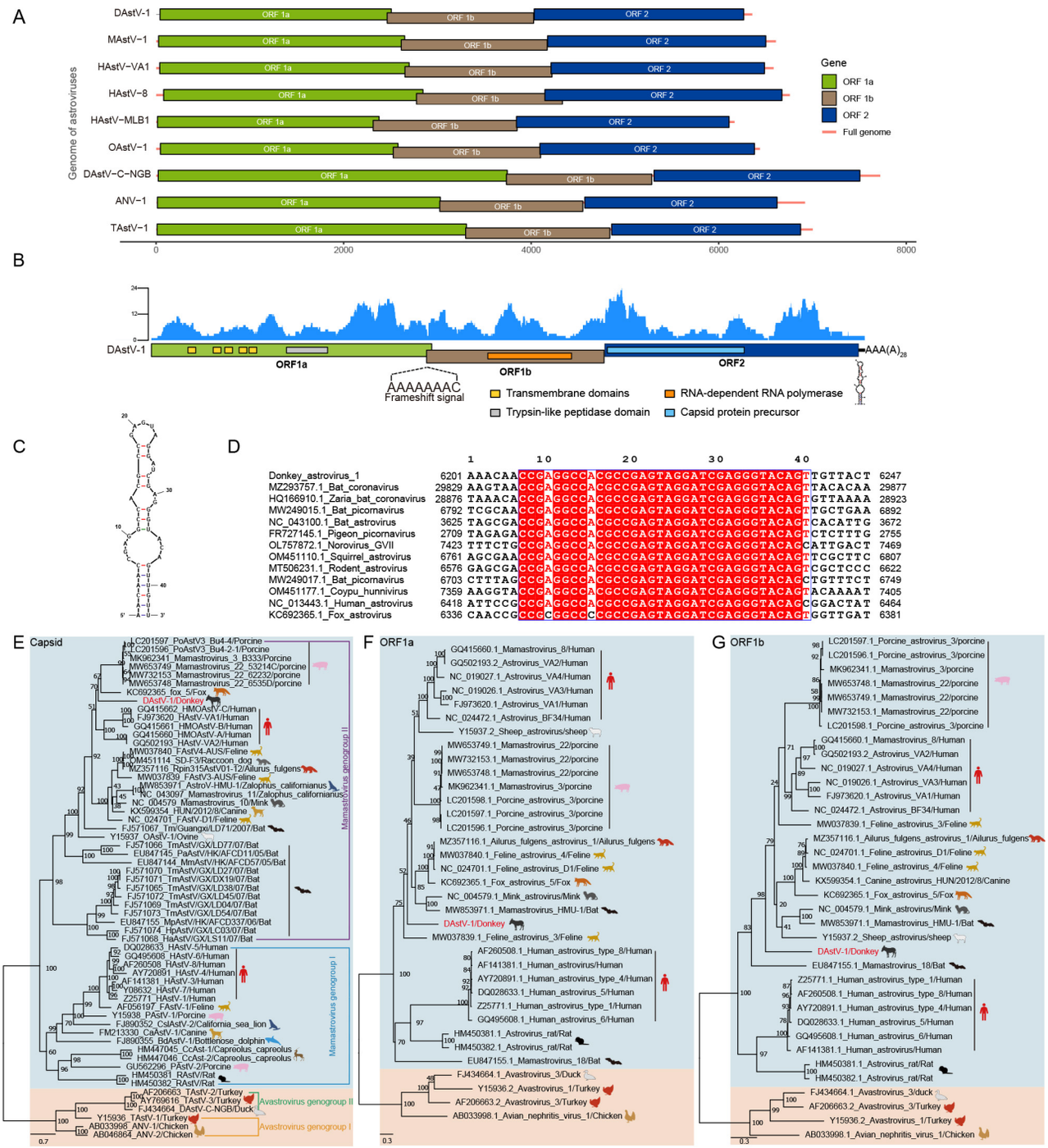

Figure 1 Genomic characterization of DAstV-1 identified in this study

A: Genomic organization of selected astroviruses in representative host species, including mink astrovirus 1 (MAstV-1), human astrovirus VA1 (HAstV-VA1), human astrovirus 8 (HAstV-8), human astrovirus MLB1 (HAstV-MLB1), ovine astrovirus 1 (OAstV-1), duck astrovirus C-NGB (DAstV-C-NGB), avian nephritis virus 1 (ANV-1), and turkey astrovirus 1 (TAstV-1). B: Overview of genome structures of DAstV-1. ORF1a, ORF1b, and ORF2 are marked in green, brown, and dark-blue, respectively. Transmembrane domains, trypsin-like peptidase domain, RNA-dependent RNA polymerase, and capsid protein precursor are marked in yellow, gray, orange, and light blue, respectively. Blue graph shows coverage of DAstV-1 reads. C: Secondary structure of conserved stem-loop II motif at 3′ end of DAstV-1 genome. D: Alignment of RNA sequences of stem-loop II motif at 3′ end of DAstV-1 and different viruses, showing high similarity with DAstV-1. Highly conserved part is marked in red. E–G: Phylogenetic relationships between DAstV-1 from this study and other astrovirus species based on amino acid sequence of capsid protein (E), ORF1a (F), and ORF1b (G). Optimal models were predicted using IQ-TREE 2, and trees were constructed using maximum-likelihood method, with branch scale bar shown as 0.7, 0.3, and 0.3 substitutions per site, respectively. *Mamastrovirus* and *Avastrovirus* genera of family Astroviridae are distinguished by different background colors (blue and orange, respectively). Novel DAstV-1 is marked in red and others are marked in black. Genogroups are listed in different colors on the right, and GenBank accession numbers are listed on the left.

AUTHORS' CONTRIBUTIONS

Y.G.T. and H.Y.R. conceived and designed the study. F.J.T. and J.L. analyzed the data and prepared the initial draft of the manuscript. Q.H.T. performed the sequencing experiments. S.X., W.L.L., Y.L., and Y.B. contributed to sampling work. J.Y., W.H.L., and Y.Q.X. contributed to investigation and case collection. All authors read and approved the final version of the manuscript.

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