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Research Paper

Genome analysis of newly emerging goose-origin nephrotic astrovirus in China reveals it belongs to a novel genetically distinct astrovirus

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ABSTRACT

Since 2017, a new type of goose-origin astrovirus (GoAstV) disease occurred in China. This disease can cause joint swelling of sick geese, and the anatomy shows a clear urate precipitation in the viscera. The rate of death or amputation can reach more than 30%, revealing its severe pathogenicity. One novel goose-origin astrovirus strain, designated as CXZ18, was isolated from diseased geese with a fatal infection characterized by visceral urate deposition. Similar clinical anatomy symptoms were partially reproduced by attacking infection of healthy geese. The CXZ18 has no hemagglutination with chicken erythrocyte, only reproduced in goose embryos, not in SPF chicken or duck embryos. The complete genome-encoded three open reading frames (ORFs) of CXZ18 were 7252 nt in length. BLAST-based homology analysis of viral complete genome showed that CXZ18 has only 53.0%–61.8% with other classic avian astrovirus from various hosts. Further analysis of ORF 1a, ORF 1b, and ORF 2 genes revealed that the isolate was genetically distinct from known astroviruses and belonged to a distinctive branch of avian astroviruses. To conclude, a naturally occurring novel nephrotic astrovirus, distinguished with all previously reported avian astroviruses, was derived from goose

1. Introduction

Astrovirus (AstV) is a single-stranded, positive-sense RNA virus with a whole genome length of 6.4–7.9 kb (De et al., 2011). Its genome contains a 5′-untranslated region (UTR), three open reading frames (ORFs), a 3′-UTR, and a poly (A) tail. Astroviridae can be divided into Mamastrovirus and Avastrovirus genera according to the different types of infected hosts. Mamastrovirus genus is further divided into human, cat, swine, sheep, and mink astroviruses. Avastrovirus genus includes duck astrovirus, turkey astrovirus, avian nephritis virus, and chicken astrovirus (Kang et al., 2018; Arias and Dubois, 2017).

Poultry in any growing period is susceptible to AstVs. At present, AstVs infections have been found to be associated with multiple fowl diseases as duck hepatitis, chicken diarrhea symptoms, and chicken nephritis symptoms (Kang et al., 2018; Koci and Schultzcherry, 2002; Zhang et al., 2017; Donato and Vijaykrishna, 2017). The spread of the virus is often occurring and can be transmitted in poultry, turkey, waterfowl, and guinea fowl. To date, increasing reported evidences indicate a high degree of cross species transmission of AstVs between domestic fowl (Todd et al., 2009; Pantinjackwood et al., 2006). These mentioned questions should be further investigated toward clear understanding of genetic evolution of avian AstV.

In 2017, a new type of GoAstV disease, which is characterized with urate precipitation in viscera, has rapidly spread in China, causing significant economic losses. Considering the genetic diversity and possible interspecies transmissions of GoAstV, here we reported the isolation of a goose-origin novel astrovirus. Its whole-genome analysis reveals that it belongs to a novel genetically distinct astrovirus, different with previously reported fowl astroviruses.

2. Materials and methods

2.1. Virus isolation and identification

The goose liver samples suspected AstV were inoculated via the allantoic cavity onto 11-day-old specific-pathogen-free (SPF) chicken embryos, 12-day-old duck embryos, and goose embryos. After 120 h post inoculation, allantoic fluids of all dead and alive embryos were collected and tested for the presence of hemagglutination (HA) by using a 0.5% solution of chicken erythrocytes. A total of five passages were performed in chicken embryos, duck embryos and goose embryos. In parallel, total RNAs were extracted from allantoic fluids of all embryos followed by Reverse-transcription polymerase chain reaction (RT-PCR) to amplify a 390 bp fragment of ORF 1b gene. On the basis of the

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conserved ORF1b protein sequence encoded RNA-dependent RNA polymerase (RdRP) of the GoAstV from GenBank (No:MH052598) (Yang et al., 2018), Primer ExPress 3.0 software was used to design primers, primers: F-5′CGGTGGAATACATCAGCGAGTA3′; R-5′CCTTCCTTATTGACACAAGCCTAT 3′.

2.2. Animal regression test in geese

The standard animal infection experiment (Koch-postulate verification) was conducted to evaluate viral pathogenicity of CXZ18 isolate. Twenty 7-day-old geese were tested as negative for avian astrovirus (besides the novel goose AstV), later were infected with CXZ18 by chest intramuscular injection at a dose of 10^6 EID_{50} per 100 μL, the normal saline attacking group as control. Clinical signs and mortality of attacking geese were recorded for 10 days. The liver, spleen, and kidney tissue of dead geese and survival geese after 10 days were immediately frozen for virus identification. All the animal studies were conducted under biosafety level 2^+ conditions according to the established guidelines and regulations of the Institutional Animal Care and Use Committee of Poultry Institute, and all relevant procedures were approved by the committee under the license No: 18–02.

2.3. Electron microscopic observation

The liver tissue samples were stored with a stabilizer (3% glutaraldehyde) for morphological observation via an transmission electron microscope from WeiYa Biotechnology Co., Ltd. of China. The samples of about $1mm^3$ were fixed by 3% osmium, gradient dehydrated, then embedded by Epon812; subsequently, ultrathin sections were made, double-stained by sliced uranyl acetate and citromalic acid and JEM 1200EX electron microscope was used for viral observation.

2.4. Genome sequence

The viral genome were extracted and detected by 1% agarose gel electrophoresis. Then they were processed into fragments (about 300–500 bp) by Covaris M220 for to build cDNA library (TruSeq DNA Sample Prep Kit, Illumina, USA). In turn, the two connectors were connected at both ends of the DNA fragments.The DNA amplifiers were subsequently linearized into a single strand by Bridge-PCR (TruSeq PE Cluster Kit, Illumina, USA). By Bridge-PCR amplification, DNA clusters were produced and linearized into single strand. In Illumina sequence system, the surface of the reaction plate was scanned by laser to read the nucleotide species polymerized by the first round reaction of each template sequence. The fluorescent labels and termination labels were chemically cleaved to restore the 3′-end viscosity and continue to polymerize the second nucleotide. With the fluorescence signals were collected in each round, and the sequences of assembled template DNA fragments were obtained (Truseq SBS Kit, Illumina, USA). Eventually, the whole-genome of CXZ18 was assembled and combined by a software system SOAP v2.04 (http://soap.genomics.org.cn/). The whole-genome sequences were subsequently submitted to the NCBI database.

2.5. Homology and phylogenetic analysis

Referenced sequences of other avian AstVs were downloaded from GenBank. Their detailed informations were shown in Table 1. Nucleotide sequence editing, analysis, and deduction of amino acid sequences were performed using EditSeq program by the DNASTAR v8.0 (DNASTAR Inc., USA) by Clustal W method. Nucleotide and protein homologies of ORF 1a, ORF 1b, ORF 2 and whole-genome were analyzed using Lasergene software v8.0 (DNASTAR Inc., USA) by Clustal W method. Phylogenetic trees of three ORFs were constructed using the Maximum Likelihood method in MEGA 7.0th Ed. The associated taxa clustered together in the bootstrap test was performed with 1000 replicates.

Table 1
Homologies of ORFs and genome of CXZ18 and classic strains of avian AstV genus.

Strains (GenBank no)	GoAsTV.CXZ18 (MH807626)			
	ORF 1a (aa)	ORF 1b (aa)	ORF 2 (aa)	Genome (nt)
CAstV/ANAND (KY038163)	48.4	64.9	36.8	53.6
CAstV/CkP5 (KX397576)	48.4	65.4	36.8	53.5
CAstV/GA2011 (JF414802)	48.2	65.2	36.7	54.0
CAstV/4175 (JF832365)	48.1	58.1	31.7	53.4
DkAstV/NGB (FJ434664)	48.5	64.3	56.1	53.4
TAstV/MO/01 (EU143848)	60.0	68.5	56.9	58.2
TAstV/CA/00 (EU143844)	59.7	68.5	57.0	61.7
TAstV/VA/99 (EU143851)	59.8	65.3	57.3	61.8
GoAstV/ FLX (NC034567)	47.8	60.6	40.9	53.0
PiAstV/CHN/10 (JF422783)	–	–	26.8	–

3. Results

3.1. Isolation of GoAstV in goose embryos

In January 2018, the goose liver samples suspected AstV were inoculated in SPF chicken embryos, duck embryos, and goose embryos for viral isolation. In goose embryos, through five passages no deaths and pathological changes were observed, but for the 40 allantoic fluids of inoculated goose embryos after three passages, the positive rates of RT-PCR method were 82.5% (33/40). Further sequencing confirmed that the virus was astrovirus, designated as CXZ18, was isolated from a goose liver sample, without hemagglutination with 0.5% solution of chicken erythrocyte. Meanwhile, after five passages no deaths or pathological changes were found in chicken embryos and duck embryos, correspondingly, the results of RT-PCR test were all negative. These data showed that goose embryos are feasible for viral isolation.

3.2. Animal regression experiment in geese

Animal regression tests in geese that tested as negative for astrovirus abide by the Koch-postulate verification. In experimental infections with GoAstV.CXZ18, two geese died at 3,4 dpi, resulting in a mortality rate of 20%, without a significant peak of death as the disease observed in the field breeding. After the 10-day experimental period, clear urate precipitation in the kidney (Fig. 1A), heart and liver capsules (Fig. 1B) can be seen; symptoms of joint swelling of sick geese is shown in Fig. 1C (as indicated by the green arrows). The results of anatomic tissues were similar to those found in the clinical infected cases. Using RT-PCR based on ORF 1b gene, the GoAstV was consistently detected in the kidney, spleen, and liver of infected geese. Following goose embryo inoculation, the inoculated virus was re-isolated successfully from the infected kidney, spleen, and liver.

3.3. Electron microscopic observation of infected liver tissue

Some scattered virus particles of approximately 30 nm in diameter were detected in infected liver tissue under the transmission electron microscope. The viral particles magnified at 50,000 times are shown in Fig. 2.

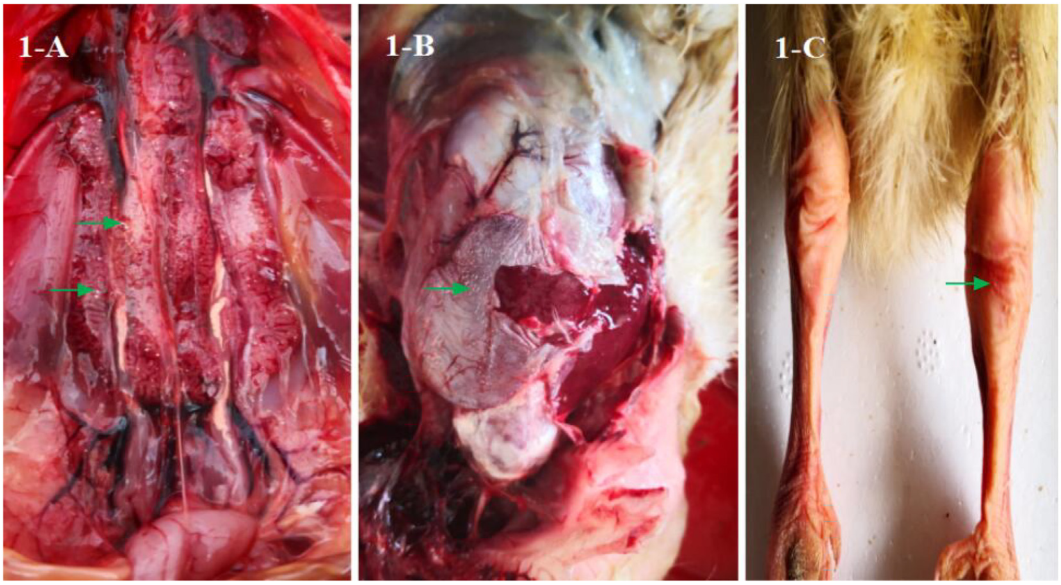

Fig. 1. Results of anatomic tissues.
1-A: Urate precipitation in the kidney; 1-B: Urate precipitation in the heart and liver capsules; 1-C: Joint swelling of sick goose.

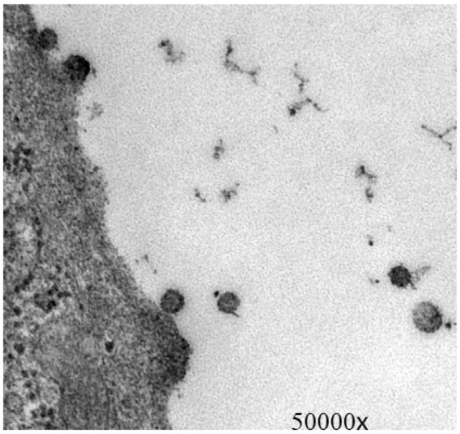

Fig. 2. Virus particles under the transmission electron microscope.

3.4. Genome sequence analysis

Full genome sequence of CXZ18 was determined with a total size of 7252 nt and subsequently submitted to the NCBI database (No: MH807626). The genome consists of a 5′UTR of 245 nt, three open reading frames (ORF 1a: 3105 nt, ORF 1b: 1551 nt, and ORF 2: 2115 nt), and a 3′UTR of 228 nt. ORF 1a, ORF 1b, and ORF 2 of the isolate encoded a nonstructural polyprotein of 1034 aa, RNA-dependent RNA polymerase of 516 aa, and capsid protein of 704 aa. Seven conserved nucleotide sequences (3341-AAAAAAC-3347) as previous AstV isolates were observed between ORF 1a and ORF 1b.

The lengths of amino acids of different strains were also quite different. For ORF 1a, ORF 1b, and ORF 2, the longest were as follows DkAstV/NGB (FJ434664) with 1240 aa, CAstV/ANAND (KY038163) with 519 aa, DkAstV/CPH (KJ020899) with 745 aa; the shortest were as follows the novel CXZ18 only with 1034 aa, GoAstV/ FLX (NC034567) with 514 aa, CAstV/4175(JF832365) with 644 aa, respectively.

Interestingly, novel CXZ18 strain had longer 5′UTR region (245 nt) than those of other avian AstVs (13–22 nt). And another similar isolate (GenBank No:MH052598) with the longer 5’UTR as CXZ18 was isolated in the different district.

3.5. Homologies and phylogenetic analysis of the isolate with representative avian AstVs

Amino acid homologies of ORF 1a, ORF 1b, ORF 2, and nucleotide homologies of whole genome of the isolate were analyzed using Clustal W method. All comparison results are summarized in Table 1. ORF 1a, ORF 1b, and ORF 2 genes of CXZ18 were 1034 nt, 516 nt, 704 nt, respectively, with 47.8%–60.0%, 58.1%–68.5%, and 26.8%–57.3% homologies to those regions of other classic avian AstVs from different hosts. Based on the analysis of complete genome, CXZ18 had merely 53.0%–61.8% with other classic avian AstVs in Table 1. In summary, the above data revealed that the CXZ18 strain shared low homologies with other reference avian AstVs regardless of any single gene or whole genome.

Phylogenetic trees of three ORFs were constructed using the Maximum Likelihood method in MEGA 7.0th Ed with 1000 bootstrap replicates. As shown in Fig. 3, further amino acid phylogenetic analysis of ORF 1a (Fig. 3A), ORF 1b (Fig. 3B), and ORF 2 (Fig. 3C) showed that this new virus was in the independent small branch, not clustered into any clade with these classic avian AstVs even GoAstV/FLX from the same host. According to the classification standard of AstV from the International Committee on Taxonomy of Viruses (ICTV), as for the full-length ORF2 encoding the capsid proteins, the mean amino acid divergent distances between different AstV species range between 0.378 and 0.750(Woo et al., 2015). Thus based on amino acid divergent distance (p-distance) of capsid proteins, the isolate had 0.504–0.532 (p-distance) with most closely TAstVs and DkAstVs (Duck Hepatitis Virus) as shown in Fig. 3C. Thus, even the divergent isolate was closely related to the members of TAstV-2 and DkAstV (Duck Hepatitis Virus) lineage, the genetic distances between them were still relatively large. In summary, the above data reveals that the CXZ18 belongs to a novel genetically distinct astrovirus.

Through phylogenetic trees based on multiple sequence alignments of strains using Clustal W method, the divergences in genetic alignments among different strains were also observed. As for amino acid analysis of ORF 1a and ORF 1b (Fig. 3A and B), TAstV and DkAstV-2 were in one same clade, which is that clade of newly CXZ18 belonged to, but DkAstV-1, GoAstV and CAstV were grouped into a distinct clade among Avastrovirus genus.The alignment distributions of almost all strains based on amino acids of ORF 2 were consistent with those of ORF 1a and ORF 1b, except the two strains (GoAstV.FLX and

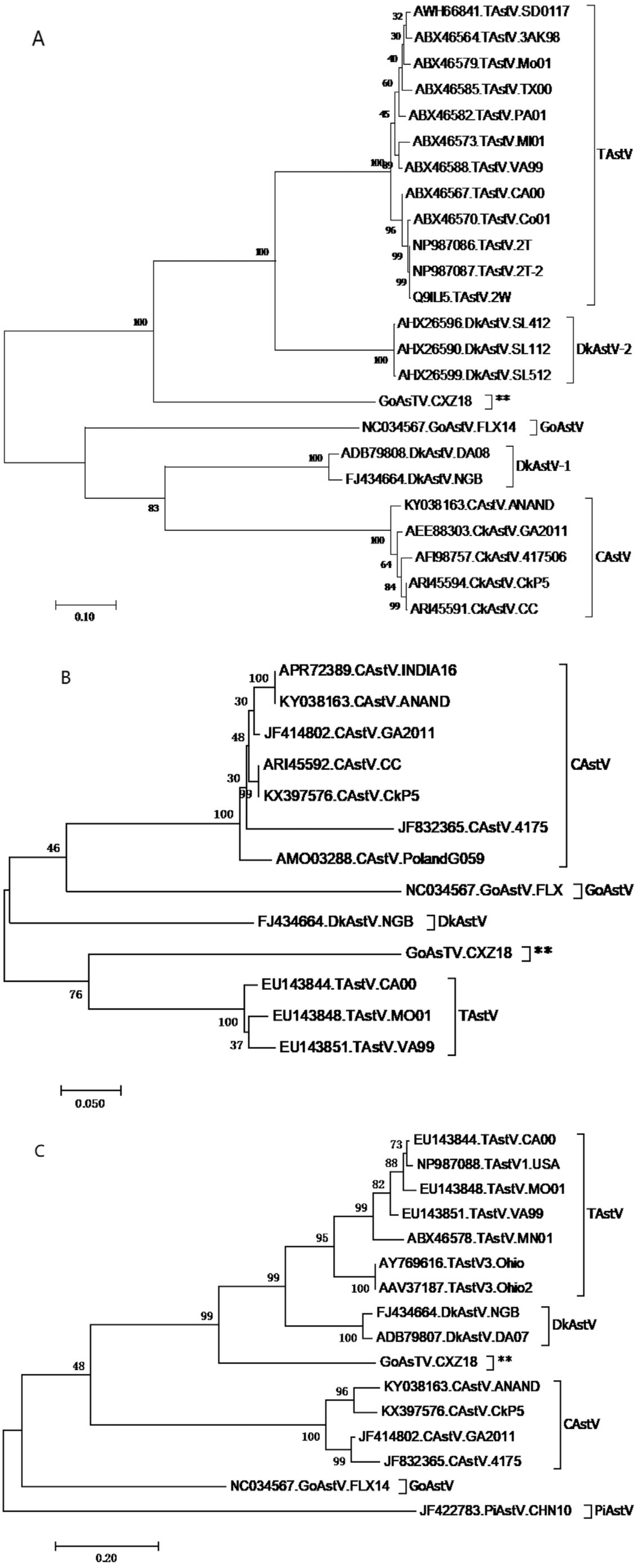

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Fig. 3. Phylogenetic trees derived from analysis of amino acids encoded by ORF 1a gene (A), ORF 1b gene (B), and ORF 2 gene (C). Amino acid sequences were analyzed using Mega 7.0, and phylogenetic trees were constructed using the Maximum Likelihood method with 1000 bootstrap replicates.The goose-origin astrovirus CXZ18 in this study were indicated with double stars **.
GoAstV, goose AstV; PiAstV, pigeon AstV; CAstV, chicken AstV; TAstV, turkey AstV; DkAstV, duck AstV.

PiAstV.CHN10) were respectively clustered into two separate clades (Fig. 3C).

4. Discussion

Generally, AstVs infections have been found to be associated with duck hepatitis, fowl entertis and chicken nephritis (Chu et al., 2012; Han et al., 2017). In the present study, we report the discovery of a novel distinct goose astrovirus. The rare GoAstV case can cause clear joint swellings and a typical precipitation of urate in the kidney and liver in geese.

According to ICTV (https://talk.ictvonline.org/), both the genetic analysis of the full-length ORF 2 encoding the capsid protein and the host of viral origin should be considered in classification of AstVs (Woo et al., 2015). Thus, in consideration of the p-distance index between the isolate and its most closely classic strains, together with its distinct host different from duck origin or turkey origin, the goose astrovirus CXZ18 should represent a distinct AstV branch in the avian AstVs genus.

Complete genome of CXZ18 strain has been sequenced with a total size of 7252 nt encoded ORF1a, ORF1b and ORF 2. And the length of ORF 1a was shorter than other avian astroviruses, only with 1034 aa. The novel CXZ18 strain had longer 5′UTR region (245 nt) than those of other avian AstVs (13–22 nt) (Cattoli et al., 2005; Chen et al., 2017; Cattoli et al., 2007). Diversity of the 5′UTR is known to cause adjustable changes in secondary structure of RNA (Buchko et al., 2017; Bravo et al., 2018; Patiño Galindo et al., 2018), but whether this longer region is related to the novel CXZ18 viral virulence remains to be further confirmed.

The genetic homologies between ORF 1a, ORF 1b, ORF 2 and whole genome of the CXZ18 isolate, and those of the other representative strains were relatively low. It had merely 47.8%–60.0%, 58.1%–68.5% and 26.8%–57.3% with other classic avian AstVs homologies to those regions of other classic avian AstVs from different hosts. Based on the analysis of complete genome, CXZ18 had merely 53.0%–61.8% with other classic avian AstVs. Thus, initially, we screened the conserved regions of previous avian AstVs and attempted to conduct PCR amplification and sequencing (Zhang et al., 2018; Wan et al., 2018; Liao et al., 2015), but the amplifications of multiple primers were unsuccessful through a multitude of trials. These unsuccessful operations also verified indirectly that the homologies of the isolate are very low with the other previous avian AstVs. This finding was consistent with the whole genome sequence analysis obtained by the later Illumina method.

Electron microscopy is one of the main methods for the early virus detection. Here some scattered virus particles of about 30 nm in diameter were detected in the ultrathin sections of goose liver. In fact, in these ultrathin sections the specific star image of astroviruses can not be clearly seen. Thus an intracellular accumulation of virus particles would be more reliable for virus detection.

In our study, CXZ18 strain inoculation of the allantoic cavity into goose embryos is feasible for goose-origin viral isolation. But the same operation failed in the chicken embryo and the duck embryo. And this result whether to isolate the GoAstV by goose-origin cell line inoculation needs to be further verified.

In goose experimental infections with the GoAstV CXZ18, the high mortality rate observed in the field was not reproduced. Thus, the goose-origin astrovirus described here, if only as the solo critical pathogen or mixed infection with other pathogens still needs further studies.

Here we have described the discovery of a novel goose-origin nephrotic astrovirus. Based on the results of comparative genome and phylogenetic analyses, we proposed the isolate as a novel species in the genus avian AstVs which would be of great clinical significance for the Astroviridae.

Conflicts of interest

The authors declare no conflicts of interest.

Contributions

XY Yuan and YL Wang designed the details of the experiments and assisted in the experimental operations and writing of the manuscript, K Meng and YX Zhang contributed to viral isolation and the clinical trials, ZJ Yu and W Ai helped in the sample collection procedures. All authors read and approved the final manuscript.

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