

Isolation and evolutionary analyses of gout-associated goose astrovirus causing disease in experimentally infected chickens

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ABSTRACT Astroviruses are a common cause of gastroenteritis in humans and animals. They are also associated with extraintestinal infections, including hepatitis in ducklings, nephritis in chickens, as well as fatal meningitis and encephalitis in humans and other mammals. Since 2014, outbreaks of disease characterized by visceral gout and swelling of kidneys have been reported in goslings and ducklings in China, with the causative agent revealed to be a novel avian astrovirus designated goose astrovirus (**GoAstV**). In the present study, this novel gout-associated GoAstV was identified in diseased goslings from 2 farms in Hunan province, China. Three genomes were successfully sequenced and analyzed and were shown to have high identities of 99.7 to 99.8% between each other, with some specific amino acid alterations revealed in open reading frame 2 when compared with other gout-associated GoAstVs. Two strains were further

efficiently isolated in the DF-1 chicken fibroblast cell line with high virus titers of 10^{11} viral genomic copies per mL of culture media. A pilot virus challenge study using GoAstV in chickens demonstrated that this virus can cause clinical visceral gout in chickens, indicating its ability to cross the species barrier. Based on the phylogenetic analyses of capsid sequences, the identified GoAstVs were proposed to be classified into 2 genotypes, GoAstV1 and GoAstV2, and the novel gout-associated GoAstVs were all clustered in GoAstV2. Further Bayesian inference analyses indicated a nucleotide substitution rate of 1.46×10^{-3} substitutions/site/year for avian astrovirus based on open reading frame 2 sequences, and the time to the most recent common ancestor of GoAstVs was estimated to be around 2011. This is the first report to confirm GoAstV can infect chickens while also providing an estimation of the evolutionary rates of *Avastroviruses*.

Key words: goose astrovirus, chicken, infection, evolution, time to the most recent common ancestor (TMRCA)

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INTRODUCTION

Members of the family *Astroviridae* are small, nonenveloped, single-stranded RNA viruses, with a spherical shape of 28–30 nm in diameter and a distinctive five- or six-pointed star on the surface of about 10% of virions (Bosch et al., 2011). Their genomes are characterized by a positive sense, single-stranded RNA molecule of around 6.8 (6.2–7.8) kb which excludes the poly (A) tail and contain 3 open reading frames (ORFs), ORF1a, ORF1b, and ORF2 (Bosch et al., 2014; Cortez et al., 2017). Presently, based on the species of origin of astroviruses (**AstV**), *Astroviridae* have been classified into 2

genera, *Avastrovirus* which infect birds and *Mamastrovirus* which infect mammals (Bosch et al., 2011; De Benedictis et al., 2011; Donato and Vijaykrishna, 2017). Infection with avian astrovirus (AAstV) often presents with intestinal or extraintestinal involvement, including damage to the liver, kidney, or the immune system. In contrast, infection with *Mamastrovirus* is predominantly associated with gastroenteritis, with some recently discovered members associated with nonsuppurative meningoencephalomyelitis in humans, cattle, minks, sheep, and pigs (Bosch et al., 2011; Reuter et al., 2018). The current International Committee on Taxonomy of Viruses (ICTV) classification defines 19 species of *Mamastrovirus* and 3 species of *Avastrovirus* (<https://talk.ictvonline.org/taxonomy/>). However, as new members of the *Astroviridae* family are being discovered on a regular basis from different animals, many unassigned species have been recognized. Most recently, 33 distinct *Mamastrovirus* and 7 *Avastrovirus* species have been proposed (Guix et al., 2013).

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Since 2014, a novel goose disease characterized by visceral urate deposition, hemorrhage, and swellings of the kidneys has been reported in several provinces in China, causing 10–50% mortality in infected goslings, and a novel goose astrovirus (**GoAstV**) was identified as the causative agent (Jin et al., 2018; Liu et al., 2018; Niu et al., 2018; Yang et al., 2018; Zhang et al., 2018a; Zhang et al., 2018b). Recently, this virus has been detected in diseased ducklings with similar clinical symptoms as affected goslings (Chen et al., 2020; Wei et al., 2020), indicating the potential for cross-species transmission of this novel GoAstV from goose to duck. However, it is unknown whether this novel GoAstV could infect other domestic birds and possibly cause pathogenicity.

In May 2019, goslings located in 2 different farms from Hunan province, China, showed clinical symptoms of gout, and upon necropsy, urate depositions were found on the surface of the kidney, liver, and other internal organs. Several goose tissue samples from these 2 farms were sent to our laboratory to identify possible causative agents. The genomes of 3 astroviruses were obtained from the samples, and upon sequencing, high genomic identities to the novel GoAstV previously reported were observed. Two of these astroviruses were isolated and propagated using the chicken fibroblast cell line DF-1. Moreover, chickens were experimentally infected and developed similar clinical symptoms to those observed in goslings, demonstrating that the isolated GoAstV could infect chickens under experimental conditions. In addition, based on the ORF2 nucleotide sequences, the time to the most recent common ancestor (TMRCA) of GoAstVs and the evolutionary rate of *Avastroviruses* were also estimated.

MATERIALS AND METHODS

Ethics Statement

The chicken infection experiments described in this study were approved by the Animal Care and Use Committee of Hunan University, China, and conducted using humane procedures.

Sample Collection

In May 2019, 2 goose farms located in Liuyang city and Changsha city, Hunan province, China, were sampled, and 4 (LY-G1 to LY-G4) or 3 (CS-Z1 to CS-Z3) tissue samples were collected and shipped on ice to our laboratory. Approximately 10 to 20% of the geese in the 2 farms presented with a clinical history of gout, and postmortem examination revealed urate deposits in the ureter, on the surface of the kidneys, heart, liver, and other internal organs, as reported previously (Yang et al., 2018; Zhang et al., 2018a). Kidney, heart, spleen, and liver tissue samples were collected and used for immediate nucleic acid extraction or stored at -80°C until use.

Sample Processing and Nucleic Acid Extraction

Nucleic acid was extracted from one composite sample for each goose, which consisted of a pool of all the tissue samples available for one individual animal. In brief, approximately 0.1 g of the minced tissue pool was placed into a 2-mL Eppendorf tube containing 2 1.5-mm steel balls and 800 μL of Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Thermo Fisher Scientific). This was followed by grinding using a Mixer Mill MM 400 (Retsch, Germany) at 4°C for 2 min. The resulting homogenates were centrifuged at 12,000 g at 4°C for 10 min. A 200- μL aliquot of the supernatant from each tube was used for viral RNA isolation, according to the DNA/RNA extraction kit protocol (Axygen). Nucleic acid was stored at -80°C until use.

Reverse Transcription (RT)-PCR, Real-Time PCR and Viral Genome Sequencing

Viral cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Previously published degenerate semi-nested primers targeting a portion of the astrovirus RdRp gene were used to amplify any astrovirus sequence present in the samples (Chu et al., 2008). PCR positive samples, with expected fragments of about 400 bp, were sequenced using the Sanger dideoxy DNA sequencing method. After a BLAST search with the obtained sequences, primers for whole genome amplification were designed based on the conserved regions of the genomes which showed high identities ($>95\%$) to the present sequences.

To quantify the virus loads in the samples, a set of Taqman real-time PCR procedures were established, and the virus loads in the samples were quantified based on the recombinant viral plasmid as described previously (Xiao et al., 2012; Xiao et al., 2013a). Briefly, a PCR fragment from a sample which tested positive using the degenerate semi-nested primers (Chu et al., 2008) was cloned into the pMD19-T vector (TaKaRa). The plasmid was then transformed into competent *Escherichia coli* DH5 α cells. After blue-white selection, several positive clones were further proliferated and sequenced. Based on a conserved region of the sequences obtained, a pair of primers (qGoAstV-DF/DR) and a probe (GoAstV-prob) were designed (Table 1). One of the recombinant plasmids was quantified, and 10-fold serial dilutions were created as assay reference standards. Amplification and quantification were performed using the Bio-Rad CFX96 Real Time PCR System (Bio-Rad Pacific Ltd.).

Sequence Analysis

The sequences were analyzed with the DNAMAN (Lynnon Corporation) and MEGA7.0 (Kumar et al., 2016) software. To identify possible recombination events within the GoAstV genomes, the Recombination Detection Program version 4 was used (Martin et al., 2015). Only putative recombination events detected by more than 2 methods were adopted.

Table 1. Primer information for capsid expression and realtime PCR of GoAstV.

Primer/probe	Sequence (5'-3')	Size
GoAsV-Cp1F	<u>TTGGATCCAAGGTGACCAAGAAGGTTACAAAAGT</u>	591 bp
GoAsV-Cp1R	<u>TAGTCGACTTAACCAAGTGAGTCAGTGGGTGAAT</u>	
GoAsV-Cp3F	<u>TTGGATCCCTTGAAACAGCAGTCCTGCGTG</u>	549 bp
GoAsV-Cp3R	<u>TAGTCGACTCACTCATGTCCACCCTTCTCAAAG</u>	
qGoAsV-DF	<u>GGTTGAGGAGCACGCTGCCAG</u>	239 bp
qGoAsV-DR	<u>GCTTCCTCTCGGCCTTCATGGGT</u>	
GoAsV-prob	FAM-TTTCTCAAATCTGGTGAGTGCGGACCGA-BHQ1	

Underlined sequences indicate the restriction sites for cloning into the vector.

To substantiate the phylogenetic relationship of the present GoAstVs with other AAstVs and to estimate the rate of evolution as well as the TMRCA of GoAstVs, the ORF2 sequences of GoAstVs and other representative AAstV strains were selected and analyzed by the BEAST 1.10.4 (<http://beast.community/>) software package. Specifically, the General Time Reversible substitution model (GTR) with a proportion of invariant sites and gamma distributed rate heterogeneity (GTR + I + Γ) was used by the methods of Bayesian inference (BI). During the analysis of the BI, a relaxed molecular clock with an uncorrelated log-normal rate distribution and a chain length of 2×10^7 generations with sampling every 2000 generation was performed to estimate the posterior probability. An extended Bayesian skyline plot was used as a tree prior, and the burn-in was set at 10% of the sampled states. The time scaled phylogeny of GoAstVs was reconstructed by using a standard continuous-time Markov chain process over discrete sampling locations with the Bayesian stochastic search variable selection model. The results were assessed by the program Tracer and were accepted only if the effective sample size exceeded 200 (<http://beast.bio.ed.ac.uk/software/tracer/>). The tree was viewed using the FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>) software.

Virus Isolation

To isolate any viable virus from the samples, the tissue homogenates were filtered twice, first through 0.45- μ m filters, followed by 0.22- μ m filters. Then 100 μ L of the flow-through was mixed with an equal volume of DMEM and inoculated into the chicken embryo fibroblast cell line DF-1 in a 24-well plate. Before inoculation, the cells were grown in DMEM supplemented with 100 units/mL of penicillin and 100 mg/mL of streptomycin and 10% heat-inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific). After 1 h incubation at 37°C with 5% CO₂, the inoculant was discarded, and the cells were supplemented with 500 μ L of DMEM and further incubated at 37°C with 5% CO₂. The cells were checked daily for cytopathic effects. After 3 to 4 d, the plate was frozen at -80°C and thawed 3 times. A 100- μ L aliquot of the freeze-thaw solution was then inoculated into new cells as described previously. After 5 passages, nucleic acid was extracted from the cell culture supernatants to detect the presence of GoAstV,

and consecutive passages were carried out for the GoAstV positive cell cultures.

Mouse Polyclonal Antibody Preparation of Capsid Protein

To detect GoAstV in cells, a mouse polyclonal antibody was produced in mice. An *Escherichia coli* expression system was used as previously described to express 2 fragments (cp1 and cp3) of the ORF2 capsid gene, which correspond to the partial amino and carboxyl terminal of the capsid protein (Catanzariti et al., 2004; Xiao et al., 2013b). The primer information is listed in Table 1. The recombinant vectors and the expressed proteins were confirmed by sequencing and a Western blot assay based on His-tag antibody, respectively. The expressed proteins were purified by Ni-column and checked by SDS-PAGE. A total of 100 μ g of each protein was inoculated subcutaneously into 2 eight-week-old BALB/c mice. Two additional weaker inoculations were performed by injecting 50 μ g of the protein at 2-week intervals. Blood was collected after 2 wk, and serum was obtained through centrifugation.

Immunofluorescence Assay and Virus Titration

At 48 h after inoculation, DF-1 cells were fixed with cold acetone and methanol (1:1, v/v) at -20°C for 20 min. After 3 washes with 1 \times phosphate buffered saline, they were then blocked with 1% bovine serum albumin at 37°C for 1 h. The primary and secondary detection antibodies used were the mouse anti-GoAstV capsid protein polyclonal antibody (1:100 dilution), produced and described in this study, and a fluorescent isothiocyanate-conjugated goat antimouse IgG (1:250 dilution; Bioworld Technology Inc., St. Louis Park, MN), respectively. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and the plates were then examined using an inverted fluorescence microscope (EVOS-fl, AMG).

In addition to using quantitative real-time PCR to estimate the amount of virus present in cell culture, virus titration was also performed using 10-fold serial dilutions in 96-well plates. After 60 h of inoculation, the plates were subjected to immunofluorescence assay (IFA) staining as described previously, and the virus titers were determined according to the Reed and Muench

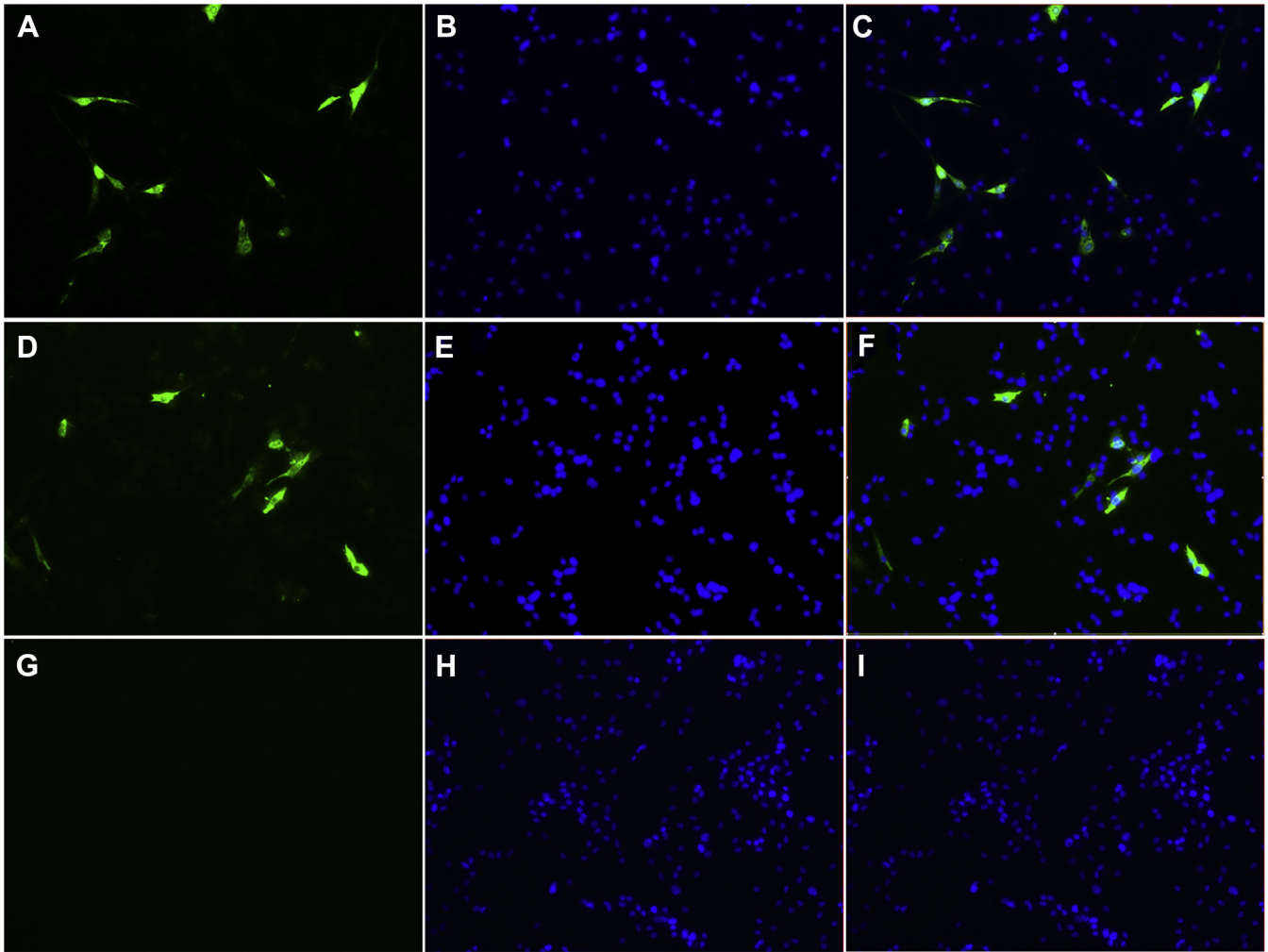


Figure 1. Indirect immunofluorescence assay demonstrating DF-1 cells infected with GoAstV isolated in the present study. (A) GoAstV-infected cells immunostained with mouse serum against partial capsid cp1 (green). (B) GoAstV-infected cells immunostained with DAPI (blue). (C) Merge of (A) and (B). (D) GoAstV-infected cells immunostained with mouse serum against partial capsid cp2 (green). (E) GoAstV-infected cells immunostained with DAPI. (F) Merge of (D) and (E). (G–I) Mock infection. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; GoAstV, goose astrovirus.

method (Reed and Muench, 1938) and expressed as the 50% tissue culture infectious dose ($TCID_{50}$)/mL.

Chicken Challenge

Eight 7-day-old chickens, hatched from commercial fertilized eggs, were randomly divided into 3 groups (A, B, and C). Groups A and B ($n = 3$ chickens each) were used for experimental infections while group C ($n = 2$ chickens) served as the negative control group. The chickens tested negative for GoAstV by real-time PCR on serum samples. Group A was inoculated with 200 μ L of GoAstV strain LY-G3 (passage 7) while group B was inoculated with 200 μ L of GoAstV strain LY-G3 (passage 21) using combined intramuscular (100 μ L) and oral (100 μ L) routes. Similar to groups A and B, group C was inoculated with 200 μ L of sterile saline. The chickens were checked daily for clinical signs, and any chicken found dead was dissected immediately. The experiment was terminated at 8 d post challenge (dpc), and all remaining chickens were humanely euthanized and

necropsied. Sections of the small intestine (jejunum, ileum), spleen, kidneys, liver, and heart were collected, pooled, and stored at -80°C for virus detection and isolation.

GenBank Accession Numbers

The genome sequences of GoAstV obtained in the present study have been deposited in GenBank under the accession numbers MT934437 to MT934439.

RESULTS

Virus Detection

Three samples (LY-G2, LY-G3, and LY-G4) from Liuyang and 2 samples (CS-Z1 and CS-Z2) from Changsha were PCR positive for GoAstV by the degenerate semi-nested primers. The PCR products were sequenced directly with the same primers used for PCR analysis. A BLAST search on the NCBI website was performed to

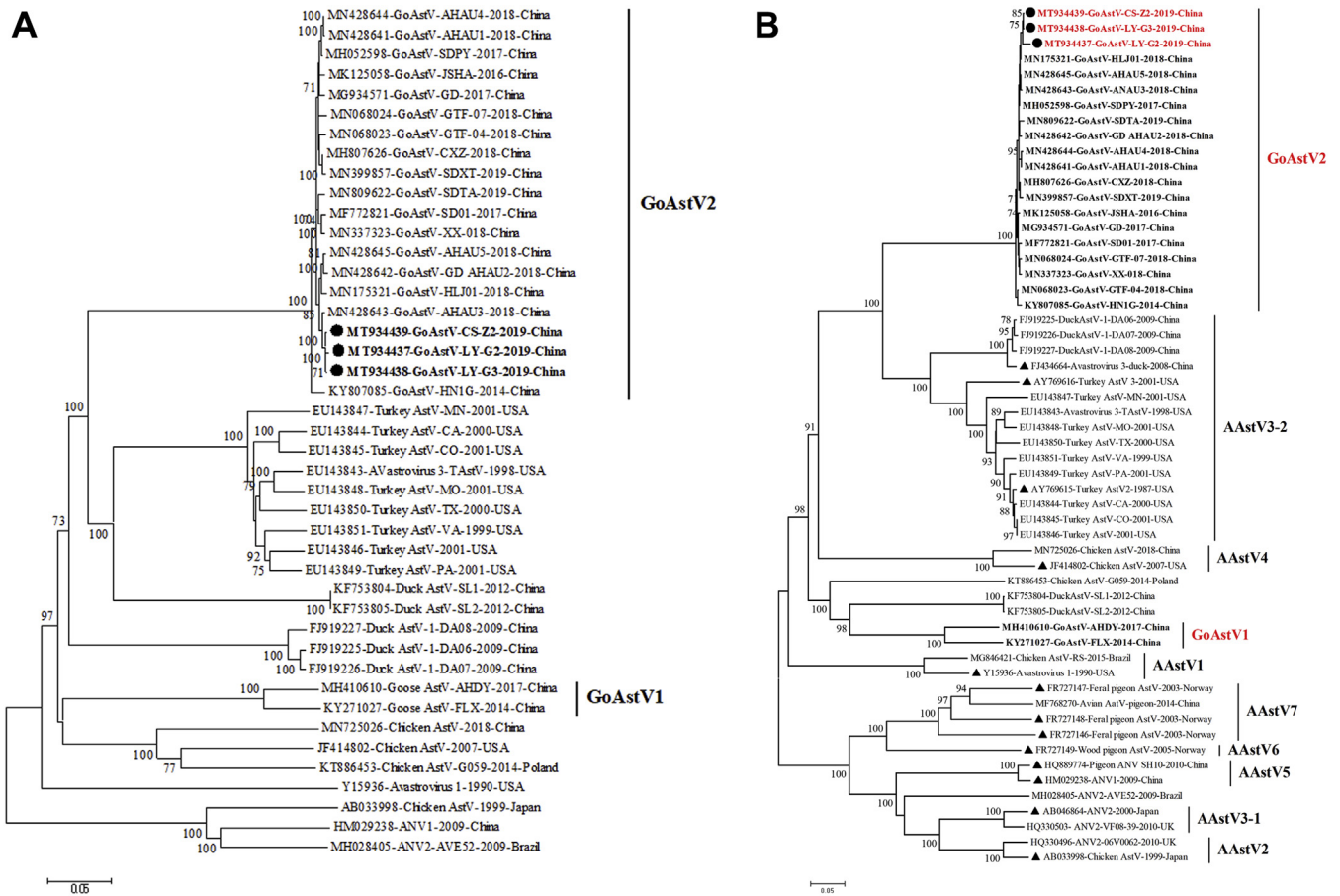


Figure 2. Phylogenetic analyses based on genomes (A) or the complete amino acid sequences of ORF2 (B) of the present 3 GoAstV strains and other representative AAstV strains obtained from GenBank. The tree was constructed by using the Neighbor-Joining method based on the p-distance model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (only >70% are shown). The tree is drawn to scale, with branch lengths measured in numbers of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 5,600 and 549 positions in the final dataset for (A) and (B), respectively. Evolutionary analyses were conducted in MEGA7. The strains of AAstV species with a solid triangle in (B) were previously defined by ICTV and others (Bosch et al., 2011; Guix et al., 2013). The sequences indicated with solid circles are GoAstV2 strains obtained in the present study. Abbreviations: avian astrovirus, AAstV; ICTV, International Committee on Taxonomy of Viruses; GoAstV, goose astrovirus; ORF2, open reading frame 2.

check the homology of the obtained sequences with other published sequences. The present 5 sequences (around 400 bp long) showed high identities (99.2–99.9%) to GoAstVs which were recently associated with gout in goslings (Jin et al., 2018; Liu et al., 2018; Niu et al., 2018; Yang et al., 2018; Zhang et al., 2018a; Zhang et al., 2018b). Other pathogens, including influenza virus

and goose parvovirus, were not detected in the present samples (data not shown).

Virus Isolation, IFA and TCID₅₀

The inocula obtained from 2 samples (LY-G2 and LY-G3) from Liuyang caused moderate cytopathic

Table 2. The p-distance of the complete amino acid sequence of ORF2 between and within the GoAstVs and other AAstV genotype/species.

p-distance within AAstVs (mean ± SE)	AAstV species	Average p-distance between AAstV species (mean ± SE)							
		GoAstV1	GoAstV2	AAstV1	AAstV2	AAstV3-1	AAstV3-2	AAstV4	AAstV5
0.173 ± 0.014	GoAstV1								
0.011 ± 0.002	GoAstV2	0.584 ± 0.019							
0.13 ± 0.014	AAstV1	0.552 ± 0.019	0.602 ± 0.02						
0.074 ± 0.011	AAstV2	0.717 ± 0.018	0.724 ± 0.018	0.585 ± 0.019					
0.067 ± 0.01	AAstV3-1	0.721 ± 0.017	0.724 ± 0.017	0.594 ± 0.019	0.263 ± 0.016				
0.155 ± 0.009	AAstV3-2	0.612 ± 0.018	0.408 ± 0.019	0.613 ± 0.019	0.741 ± 0.016	0.732 ± 0.017			
0.131 ± 0.013	AAstV4	0.623 ± 0.019	0.62 ± 0.019	0.638 ± 0.019	0.727 ± 0.018	0.724 ± 0.017	0.611 ± 0.019		
0.038 ± 0.008	AAstV5	0.712 ± 0.018	0.722 ± 0.018	0.62 ± 0.019	0.365 ± 0.019	0.391 ± 0.02	0.742 ± 0.017	0.715 ± 0.018	
0.249 ± 0.014	AAstV7	0.723 ± 0.015	0.723 ± 0.017	0.68 ± 0.017	0.538 ± 0.019	0.533 ± 0.019	0.744 ± 0.016	0.732 ± 0.017	0.548 ± 0.02

Abbreviations: AAstV, avastrovirus; GoAstV, goose astrovirus; ORF2, open reading frame 2.

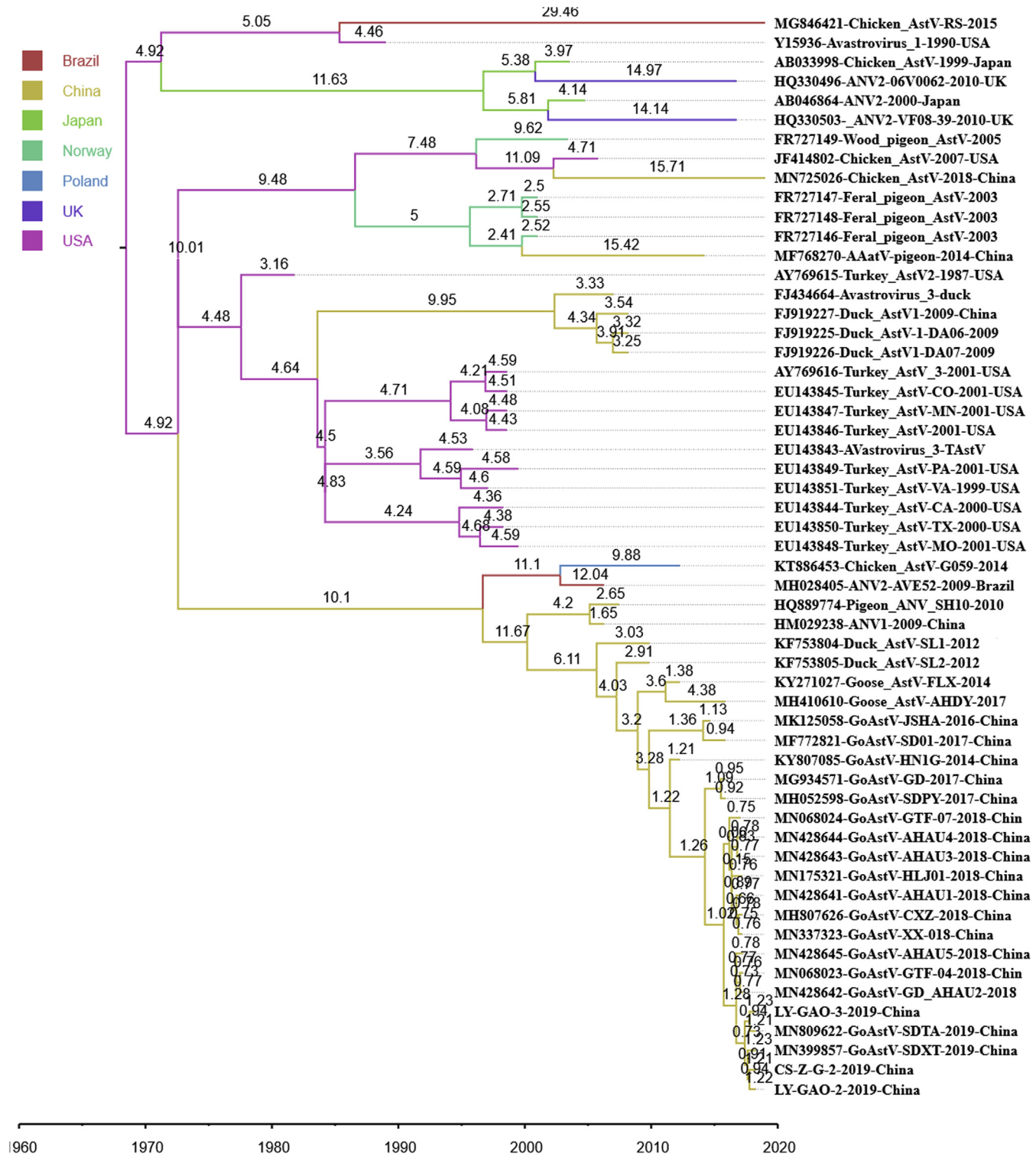


Figure 3. Bayesian MCC tree based on the ORF2 sequences of GoAstVs and other reference AAstVs. The branches are colored according to the most probable ancestral location country of their descendent nodes. The branch length of each taxon is shown above the branches. Abbreviations: Avian astroviruses, AAstVs; GoAstV, goose astrovirus; maximum clade credibility, MCC; ORF2, open reading frame 2.

effect in DF-1 cells after passage 5. Specifically, affected cells showed mild shrinking with detachment of the monolayer. The RNA of the cell culture supernatants was extracted, and sequencing of RT-PCR positive products revealed the successful isolation of GoAstV. Moreover, the TaqMan real-time PCR results of strains LY-G2 (passage 21) and LY-G3 (passage 29) revealed

high viral genome loads of $10^{11.2}$ copies/mL and $10^{11.8}$ copies/mL in the freeze-thaw solution, respectively. This indicates that DF-1 cells are permissive to the present strains. PCR testing on the DF-1 cell cultures from all the other samples investigated yielded negative results, indicating that viral isolation from those samples failed.

Table 3. Estimated time to the most recent common ancestor (TMRCA, year before present) of avian astroviruses (AAstVs) based on the analysis on the nucleotide sequences of ORF2.

Taxon ¹	TMRCA (95% HPD)	Year of estimated origin (95% HPD)
AAstV1	33 (29–42)	1985 (1976–1989)
AAstV2	23 (20–33)	1995 (1985–1998)
AAstV3-1	23 (19–33)	1995 (1985–1999)
AAstV3-2	37 (32–49)	1981 (1969–1986)
AAstV4	16 (12–27)	2002 (1991–2007)
AAstV5	11 (10–15)	2007 (2003–2008)
AAstV6 ²	14 (Not available)	2005 (Not available)
AAstV7	22 (16–35)	1996 (1983–2002)
GoAstV1	6 (5–9)	2012 (2009–2013)
GoAstV2	7 (5–11)	2011 (2007–2013)

Abbreviations: HDP, highest posterior density; ORF2, open reading frame 2.

¹The taxons were inferred from ICTV and other published data (Bosch et al., 2011; Guix et al., 2013) and the phylogenetic results of the present study as shown in Figure 2b.

²Only one reference sequence for AAstV6 was available, so the 95% HPD could not be calculated.

Furthermore, the IFA based on the 2 capsid antibodies confirmed that DF-1 cells inoculated with LY-G2 and LY-G3 were infected with GoAstV. Specific fluorescent signals were mainly detected in the cytoplasm, while there was no signal in negative control cells (Figure 1). These results demonstrate for the first time that GoAstV can be isolated in chicken DF-1 cells. Moreover, from the IFA staining results based on CP3 antibody, the infectious titers of isolate LY-G3 (passage 29) was $1.25 \times 10^{6.29}$ TCID₅₀/mL.

Genome Sequencing and Sequence Analysis

The genomes of 3 GoAstV positive samples (LY-G2, LY-G3, and CS-Z2) were successfully sequenced. All had the same genomic size of 7,154 bp, excluding the ploy(A) tail, and typical AstV genome organization. The sequences showed identities of 99.7 to 99.8% between each other and identities of 97.4 to 99.3% to other GoAstV genomes reported to be associated with gout and kidney lesions in geese and duck as described previously (later named GoAstV2; Figure 2B). However, they only had identities of 50.5 to 52.2% to other 2 GoAstV genomes (MH410610 and KY271027, later named GoAstV1, Figure 2B) reported in geese with enteritis (Zhang et al., 2017). The predicted ORF1a of the present strains is 1,084 amino acids (aa) in size, with identities of 99.4–99.6% between each other and identities of 98.9 to 99.7% to other gout-associated GoAstV2, while an identity of only 46.5% was detected with the 2 GoAstV1 strains. The predicted ORF1b of the present strains is 516 aa in size, with identities of 99.2 to 100% to other gout-associated GoAstV2 and identities of 61.2 to 61.4% to the 2 GoAstV1 strains. The capsid amino acid sequences have the same size of 704 aa across all 3 strains reported here and showed identities of 99.01 to 100% between each other. Identities between 97.8 and 99.4% were observed with other GoAstV2 strains, while identities of only 37.1 to 38.3% were seen with the capsid sequence of GoAstV1 strains.

Moreover, the amino acid sequence alignments of the GoAstV2 strains capsid protein highlighted 3 specific amino acid changes (A228 T, T376 A, and E/G610D) in the present 3 strains (Supplementary Figure 1). Interestingly, 7 amino acids alteration E653D, K663 N, E671 K, E673 V, K678 R, A679 G, and E685D were seen solely in the strain LY-GAO-2-P11 and not in the other GoAstV2 strains. However, these substitutions did not change the polarity of the amino acids except for E673 V and A679 G (Supplementary Figure 1). The significance of the amino changes in the present strains is unknown and needs further investigation.

Phylogenetic Analysis and Classification of GoAstVs

To further infer the evolutionary relationship of the present GoAstVs with other GoAstVs and other AAstVs, the whole genomes and amino acid sequences of ORF2 of all GoAstVs and representative strains of other AAstVs available in GenBank were downloaded and analyzed. The results showed that GoAstVs were divided into 2 distant clades, named GoAstV1 and GoAstV2, according to the time of the discovery (Figure 2). As described previously, GoAstV1 is reported in geese with enteritis (Zhang et al., 2017), while GoAstV2 is reported to be associated with gout and kidney lesions in geese and ducks. From the phylogenetic analysis of genomes and amino acid sequences of ORF2, the present 3 GoAstVs are clustered into the same clade with other GoAstV2 strains, with a high bootstrap support (100%). Moreover, they showed a relatively close relationship to AAstV3-2 (Strains in group AAstV3 were later classified into AAstV3-1 and AAstV3-2.) identified in turkeys and ducks but distant to the other 2 GoAstV1 strains (Figure 2).

Mean amino acid genetic distances (p-distance) between and within GoAstV1, GoAstV2, and representative strains of other AAstVs were also calculated (Table 2). A considerable large distance of 0.584 ± 0.019 between GoAstV1 and GoAstV2 was revealed, which is comparable to the genetic distance (0.576–0.741) between virus species (genotypes) of AAstV, as defined by the ICTV (Bosch et al., 2011). This further suggests that GoAstV1 and GoAstV2 could be regarded as 2 independent virus species within the genus *Avastrovirus*. In addition, from the present evolutionary tree, strains in group AAstV3, which is defined by the ICTV and others (Bosch et al., 2011; Guix et al., 2013), could be divided into 2 separate clades, AAstV3-1 and AAstV 3-2, also supported by the large genetic distance of 0.732 ± 0.017 between them (Figure 2; Table 2). No recombination event was detected in the present GoAstV strains.

TMRCA and the Substitution Rate of GoAstVs

To estimate the rate of evolution and the TMRCA of the GoAstVs, the ORF2 nucleotide sequences of the present 3 GoAstV strains and 53 additional reference

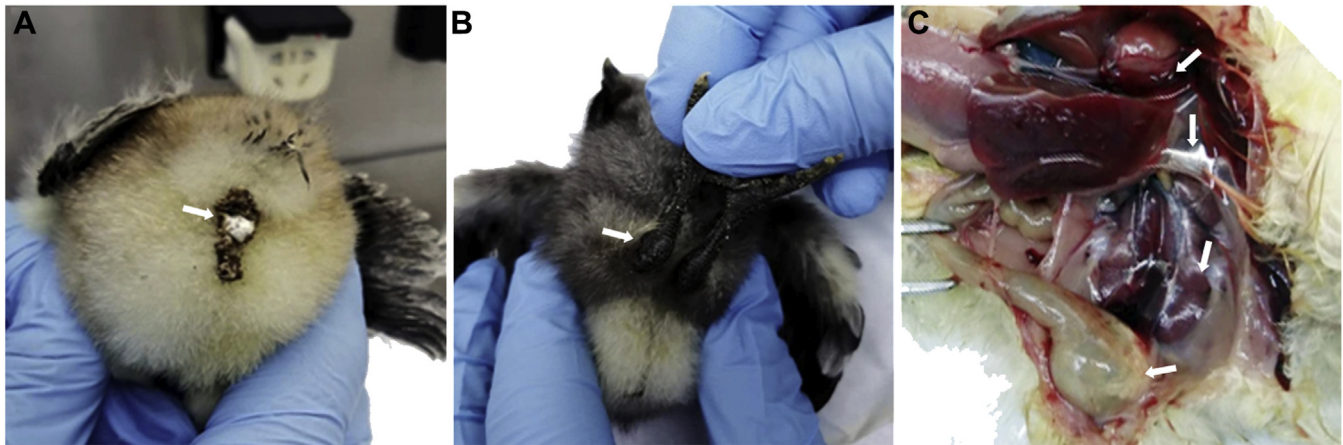


Figure 4. Clinical signs and gross lesions indicated by white arrows in chickens experimentally infected with GoAstV. (A) Diarrhea with brown-white feces. (B) Mild swollen leg joint. (C) Mild swellings of kidneys and urate deposition on the renal surface and air sacs.

sequences, including 19 GoAstV sequences, were obtained from GenBank and analyzed. The TMRCA for GoAstV1 and GoAstV2 was estimated to be 6 yr and 7 yr before present (ybp) with a 95% highest posterior density of 5 to 9 and 5 to 11 ybp, respectively, and the origin/emergence year of GoAstV1 and GoAstV2 was estimated to be 2012 and 2011, respectively (Figure 3; Table 3). Moreover, the TMRCAs and the estimated origin years of other AAstVs were also estimated based on the present data (Table 3). The mean substitution rate for the ORF2 data set was 1.46×10^{-3} nucleotide substitutions per site per year, with a 95% highest posterior density that ranged from 0.96 to 2.09×10^{-3} substitutions/site/year, which is similar to the variation rate of human astrovirus of approximately 3.7×10^{-3} nucleotide substitutions/site/year (Babkin et al., 2012).

GoAstV Challenge in Chicken

To further investigate whether the present GoAstVs could infect chickens and to evaluate the possible trans-species infection of the virus, a pilot virus challenge experiment was performed using seven-day-old chickens. On the fifth day after challenge (dpc 5), 2 chickens in group A and 2 chickens in group B were found dead, and at dpc 8, all remaining chickens were euthanized using a 10% chloral hydrate injection and dissected. The chickens in both infected groups showed similar clinical signs, including shedding white feces and enlarged leg joints from around dpc 3. At necropsy, swollen kidneys and slight to mild urate deposits on the liver, ureter,

kidneys, and other visceral organ surfaces was observed (Figure 4). Further real-time PCR results confirmed that the chickens were successfully infected with GoAstV and that the virus persisted in the body for at least 8 d. There was no obvious difference in viral loads between group A and B, indicating the virulence between passage 7 and passage 21 was similar. The saline control chickens tested negative for GoAstV (Table 4).

DISCUSSION

Since 2014, a gout disease in goslings emerged in China, and the causative agent was revealed to be associated with a novel goose astrovirus (Jin et al., 2018; Liu et al., 2018; Niu et al., 2018; Yang et al., 2018; Zhang et al., 2018a; Zhang et al., 2018b). Recently, a similar disease in ducklings was also associated with this novel astrovirus, and similar clinical symptoms were reproduced upon experimental infection (Chen et al., 2020; Wei et al., 2020). Moreover, this virus has been isolated in goose embryos (Zhang et al., 2018a), goose embryo kidney cells (Niu et al., 2018), chicken embryos (Yang et al., 2018), chicken liver cells (Zhang et al., 2018b), duck embryos, duck embryo hepatocytes, and duck embryo kidney cells (Chen et al., 2020). In the present study, the chicken embryo fibroblast DF-1 cell line was successfully used to isolate GoAstV from 2 samples taken from goslings suffering from gout. After serial passages up to 29 generations, the virus was able to grow to a titer greater than 10^{11} copies/mL in the freeze-thaw cell culture solution and an infectious titer of $1.25 \times$

Table 4. Design and result of chicken challenge with GoAstV.

Group (passage of virus in cells)	Number	Challenge (μ L)		No. of deaths (day post challenge)	Viral loads in pooled tissues of each chicken (\log_{10} copies g^{-1})			
		IM	Orally		1	2	3	Average
A (Passage 7)	3	100	100	2 (dpc 5)	5.1	8.7	6.6	6.8
B (Passage 21)	3	100	100	2 (dpc 5)	7.4	6.8	5.6	6.6
C (Saline control)	2	100	100	0	Neg	Neg	-	-

Abbreviations: dpc, days post challenge; GoAstV, goose astrovirus; IM, inoculated intramuscularly.

$10^{6.29}$ TCID₅₀/mL. This indicates that DF-1 is a highly permissive cell line to the present GoAstV isolates, which also provides an additional useful cell model to investigate the pathogenesis of GoAstV as well as a convenient viral culture system for the development of an effective vaccine.

As demonstrated, gout-associated GoAstV2 can cause significant disease in domestic waterfowls (goslings and ducklings) and has led to high economic losses for farmers. The present pilot challenge experiment evidenced for the first time that, in addition to waterfowls, GoAstV2 can also infect domestic chickens and can cause disease in this species. Broad tropism and the potential for cross-species transmission of this virus cannot be excluded, and the possibility that this virus is able to infect other domestic landfowls such as turkeys, guinea fowl, and pigeons is likely. Therefore, prophylactic measures to control this virus may need to be investigated and implemented by animal health companies and poultry farmers. Further investigations using field chicken samples, especially from chicken farms in close proximity to goose or duck farms known to suffer from GoAstV, are needed to investigate the possible field prevalence of GoAstV in chickens.

The further phylogenetic analyses based on the genomes and ORF2 amino acid sequences of GoAstV and other reference AAstVs indicated that GoAstV could be classified into 2 distinct clades GoAstV1 and GoAstV2 according to the time of discovery. Consequently, the gout-associated GoAstV strains are all clustered in GoAstV2. The present classification will be helpful to avoid confusion and will provide more convenience when comparing data from different researchers working on different GoAstV strains, especially within the GoAstV1 or GoAstV2 clades. Interestingly, GoAstV2 was more closely related to AAstV3-2. However, these 2 GoAstV clades showed a large genetic difference between each other and with other astroviruses, supporting the proposed classification as 2 independent species or genotypes, rather than being classified as AAstV3 (Chen et al., 2020) (Table 2; Figure 2B). Accordingly, canonical criteria for demarcating AAstV species/genotypes, ideally created by the ICTV, are necessary to consistently and efficiently determine the taxonomic classification of these novel GoAstVs.

Based on Bayesian inference analyses, the TMRCA of AAstVs, including GoAstVs, was estimated in the present study for the first time. By disregarding GoAstV1 or GoAstV2 genotypes because their TMRCA are similar, the results indicated that these novel GoAstVs emerged very recently, around 2011, and the possible ancestral country of origin for this virus is China (Figure 3). Moreover, the estimated mean substitution rate of AAstVs determined in the present study was 1.46×10^{-3} nucleotide substitutions per site per year, which is typical of RNA viruses with high evolutionary rates, as reported previously (Babkin et al., 2012). However, more sequences from additional regions may need to be analyzed to strengthen the estimated results of the TMRCA and the nucleotide substitution rate of the other AAstVs.

In conclusion, in the present study, we identified several GoAstV strains from goslings suffering from gout. The genomic analyses of the 3 genomes obtained were included in the same clade, with the GoAstVs reported to cause gout in goslings. Two GoAstV strains were further successfully isolated in DF-1 cells to a high titer. A pilot virus challenge study demonstrated that the virus could infect chickens and cause similar symptoms to those seen in goslings, which provides a useful cell model to study the pathogenesis of this virus. It was additionally confirmed that, in addition to waterfowls, this virus is also capable of infecting landfowl. Moreover, to avoid confusion and based on the analyses of the genome and the ORF2 amino acid sequences, the classification into 2 distinct species or genotypes (GoAstV1 and GoAstV2) was proposed. The estimated TMRCA and substitution rate of AAstVs provided useful data for the first time to infer the origin and evolution rate of AAstVs, including GoAstVs. However, the mechanism responsible for GoAstV2 cross-species transmission and its pathogenesis in different bird species needs further investigation.

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DISCLOSURES

The authors declare that they have no competing interests.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2020.11.003>.

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