Extensive genetic heterogeneity and molecular characteristics of emerging astroviruses causing fatal gout in goslings

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ABSTRACT Since 2017, outbreaks of gosling *astroviruses* (**GoAstV**) causing the major symptoms related to gout in geese have posed a threat to China's poultry industry and caused huge economic losses. In this study, tissue samples from goslings with gout and urate deposition as the main symptoms were taken from 14 goose farms in different regions of China and screened for pathogen infection. The infection rate of GoAstV was 100%, whereas the infection rates of goose parvovirus, reovirus, Tembusu virus, and goose hemorrhagic polyomavirus were 2, 4, 0, and 0%, respectively. In total, 14 GoAstV strains were isolated and their complete genomes were sequenced. Based on the phylogenetic trees, the 14 isolated strains were classified as GoAstV (G-I) and were considered distant from strains belonging to GoAstV (G-II). The multiple sequence alignments indicated a tremendous amount of amino acid mutations in some parts of the encoding proteins of these strains; the main mutations were located in open reading frames (**ORFs**)—ORF1a and ORF2, such as M533V and F568S in ORF1a and A614T in ORF2. On the other hand, Further, 2 of the 14 GoAstV strains were possibly derived through inter-GoAstV-I recombination. Taken together, these findings indicate that GoAstV*s* are evolving in a more complex manner and have diverse transmission routes.

Key words: astrovirus, gosling gout, phylogenetic tree, mutation analysis, genetic heterogeneity

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INTRODUCTION

Astroviruses (**AstVs**) were first authenticated in the feces of human infants in 1975 (Finkbeiner et al., 2009). Thus far, AstVs belonging to the family *Astroviridae* are classified into 2 genera, *Mamastroviruses* and *Avastroviruses* (Bosch et al., 2014). AstVs are nonenveloped, positive-sense, single-strand RNA viruses. They are approximately 7.0 kb in length and have 3 open reading frames (**ORFs**; ORF1a, ORF1b, and ORF2), 3′ untranslated regions (**UTRs**), and a poly-A tail (Canelli et al., 2012). ORF1a encodes nonstructural proteins and ORF2 encodes the capsid polyprotein (Bulbule et al., 2013).

AstVs infect multiple animals, including rats, sheep, pigs, chickens, turkeys, goslings, dogs, cats, and cattle. They are closely related to viral diarrhea and intestinal diseases, such as chicken nephritis and duck hepatitis, which spread to multiple areas and result in high mortality (Todd et al., 2009). The abovementioned diseases have caused tremendous economic losses to the poultry industry (Yang et al., 2018). It is noteworthy that avian nephritis virus (**ANV**) has been detected in the embryos of geese and ducks. The fact that turkey astrovirus-1 (**Tastv-1**) has also been found in duck embryos indicates the possibility of cross-species transmission of AstVs (Biđin et al., 2012). Since 2016, novel goose-origin astrovirus (**GoAstV**) has been isolated from diseased geese in China (An et al., 2020). GoAstVs have caused a disease epidemic in a growing number of provinces of China, wherein the disease is characterized by gout, hemorrhage, and kidney swelling.

Transmission of GoAstV*s* has been proven to occur vertically as well as horizontally, and its host spectrum has broadened to ducks and Muscovy ducks (Wei et al., 2020). GoAstVs were also found to cause

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clinical visceral gout in experimentally infected chickens (Li et al., 2021). These findings indicate that the harm caused by GoAstVs is expected to increase, highlighting strengthen of the importance of continuous investigation of the viruses.

To analyze the recent evolution and mutation of GoAstVs, a systematic investigation of the pathogens was conducted using gout samples collected from 14 gosling farms across different provinces of China.

MATERIALS AND METHODS

Ethics Statement

In this survey, permission was obtained from the farm owners to collect sick goslings. The birds were sampled and autopsied in strict accordance with the recommendations of the guide for the care and use of experimental animals of from the South China Agricultural University Committee for Animal Experiments (approval ID: SYXK-2014-0136).

Sample Processing and Pathogen Screening

Samples of liver, spleen, and kidney tissues were collected from dead goslings (morbidity 45−80%; mortality 27−52%) exhibiting typical symptoms of gout. The goslings were collected from 14 goose farms in Hubei, Henan, Jiangsu, and Anhui provinces from January 2020 to October 2021. Spleen, liver, and kidney samples from the same bird were pooled for the detection of GoAstV, goose parvovirus (**GPV**), reovirus (**REOV**), goose hemorrhagic polyomavirus (**GHPV**), and Tembusu virus (**TMUV**) using PCR or RT-PCR analysis following previously reported methods (Chen et al., 2020; Zhang et al., 2021). The primers used for the initial virus screening are listed in Supplementary Table 1.

The virus filtrate sterilized from GoAstV-positive samples was inoculated onto the Leghorn Male chicken hepatocellular-carcinoma cell line (**LMH**) obtained from ATCC (Manassas, VA) according to previously described procedures (Zhang et al., 2018a). Complete genome sequencing of the isolated virus strains was performed using the RNAs extracted from cell-culture isolates using previously reported overlapping primer sets (Zhang et al., 2018a). PCR analysis and sequencing were performed at least 3 times.

Sequence Analysis

The phylogenetic relationships between the 14 new GoAstVs and the existing GoAstVs and AstVs were determined through sequence similarity alignment with pair-wise comparisons performed using Clustal W software. The complete genome data of 71 AstVs (including all the available GoAstVs and those of the representative AAstVs) were retrieved from GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis was performed using the maximum-likelihood method (complete genome) in MEGA X with 1,000 bootstrap replicates, and the sequence alignments were visualized and implemented by ESPript (https://espript.ibcp.fr/ESPript/ESPript/index.php) and HemI softwares (Tamura et al., 2013). The online Simple Module Architecture Resource Tool (http://smart.embl.de/) was used to predict the GoAstV coding-protein structures, such as transmembrane segments, signal peptides, and Pfam domains, and visualized by IBS software. Epitope regions for ORF2 of GoAstVs were predicted by using SVMTtiP and SOPMA (https://prabi.ibcp.fr/htm/site/web/home). PyMol and SWISS-MODEL (https://swissmodel.expasy.org/) were used to predict and construct the ORF2 structure of protein HB06. The recombination events of GoAstVs were analyzed by SimPlot and Recombination Detection Program (**RDP**) 4, with default parameters, such as Maxchi and RDP, being used in RDP4 (Martin et al., 2015).

RESULTS

Sample Detection

A total of 269 pooled tissue samples were collected from goslings with gout. The positive rates of GoAstV, GPV, REOV, GHPV, and TMUV among these samples were 100% (269/269); 11.52% (31/269); 9.67% (26/269); 0% (0/269); and 0% (0/269), respectively. Moreover, the positive infection rates of these viruses for all farms were 100% (14/14), 21.43% (3/14), 21.43% (3/14), 0.00% (0/7), and 0.00% (0/7), respectively.

Genome Sequencing and Mutation Analysis

The complete genomes of the 14 strains were deposited into GenBank (accession numbers are as follows: AH03: OM100590; AH04: OM100591; AH05: OM100592; AH06: OM100593; HB03: OM100594; HB04: OM100595; HB05: OM100596; HB06: OM100597; HN04: OM100598; HN05: OM100599; JS01: OM100600; JS02: OM100601; JS03: OM100602; and JS04: OM100603). The full-length genomes of these strains are 7.0 kb long and have a 5′-untranslated region (UTR), a 3'-UTR, a poly-A tail, and ORFs (ORF1a, ORF1b, and ORF2) (Figure 1). *ORF1*a was 3255 nt (loci 14−3,268) in length and included 4 predicted transmembrane domains, a nuclear localization signal (loci 773−788), a trypsin-like serine protease, and a zinc finger motif. The open reading frame of ORF1b was 1551 nt (loci 3,259−4,809), and started with highly-conservative heptameric nucleotides (5′-AAAAAAC-3′) in the overlapping region between ORF1a and ORF1b. ORF2 (loci 4,828−6,942; 2,115 nt) was located 18 nt away from ORF1b and showed genomic diversity and encoded a capsid protein.

To further analyze the evolutionary history and trends of these newly isolated GoAstV strains, phylogenetic trees based on complete genomes and the

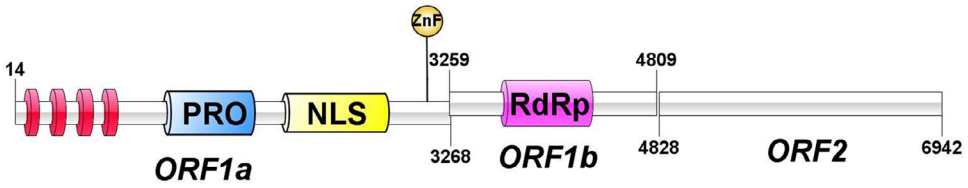

Figure 1. Predicted genome organization of newly identified GoAstVs. Three nucleotide positions of ORFs in the genome are shown. Abbreviations: GoAstVs, gosling *astroviruses*; ORFs, open reading frames.

individual viral proteins (ORF1a and ORF2) were constructed and their whole genomes were compared with other AAstV strains (Figures 2 and 3). The 14 isolated strains representing the most recently isolated GoAstV strains were categorized into the G-I group; these strains were closely related to TAstV-2 and DAstV-2. Other GoAstV strains showing a distant relationship with G-I group, such as AHDY and FLX, were clustered into the G-II group. On the other hand, the phylogenetic tree based on individual viral proteins demonstrated that the G-I and G-II groups were undoubtedly distantly related (Figure 2). Meanwhile, the phylogenetic tree based on the ORF1a protein demonstrated that strains in the G-I group were closely related to DAstV-2 and TAstV-2 (Figure 3A). However, the phylogenetic tree based on the ORF2 protein showed that the 14 isolated strains were more closely related to TAstV-2 and DAstV-1 than TAstV-1, DAstV-2, or ANV (Figure 3B).

Genetic and Antigenic Epitope Analysis

The three ORFs and whole-genome sequences of the 14 isolated strains were compared with those of other AAstV strains to investigate the mutation and evolution of the novel GoAstV strains. The nucleotides among the genomes of the 14 isolated strains shared identities of 96.3 to 99.5%. The amino acid identity sequence identities were also relatively high among the G-I strains (ORF1a: 93.3−99.5%; ORF1b: 90.9−100%; and ORF2: 96.9−99.5%). However, the nucleotide sequence identity between the strains belonging to the G-II group were only 53.6 to 54.2% and the amino acid sequence identities were 44.6 to 45.4% for ORF1a, 59.8 to 60.9% for ORF1b and 38.1 to 39.1% for ORF2. In addition, all 14 newly isolated GoAstV strains shared nucleotide sequence identities of 44.9 to 60.7% with DAstV, CAstV, TAstV, and ANV for complete nucleotide sequences.

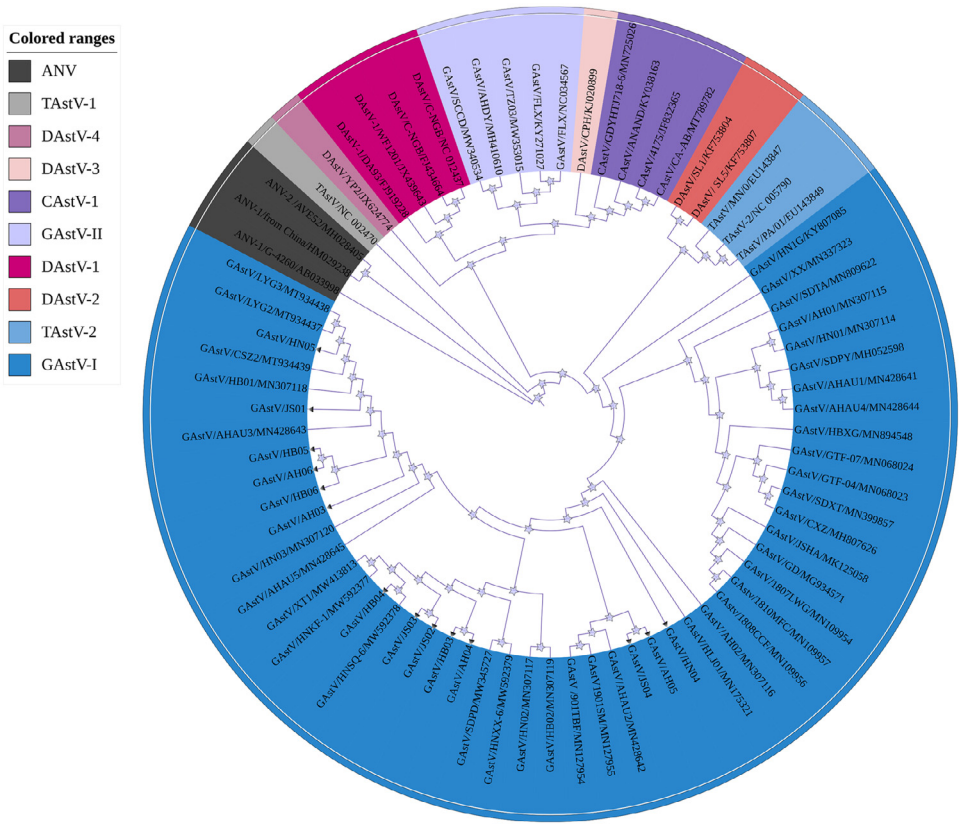

Figure 2. Phylogenetic analysis of the novel goose-origin astrovirus sequence on MEGA X using the maximum-likelihood method. The 14 GoAstV isolates are indicated using black triangles. Abbreviation: GoAstVs, gosling *astroviruses*.

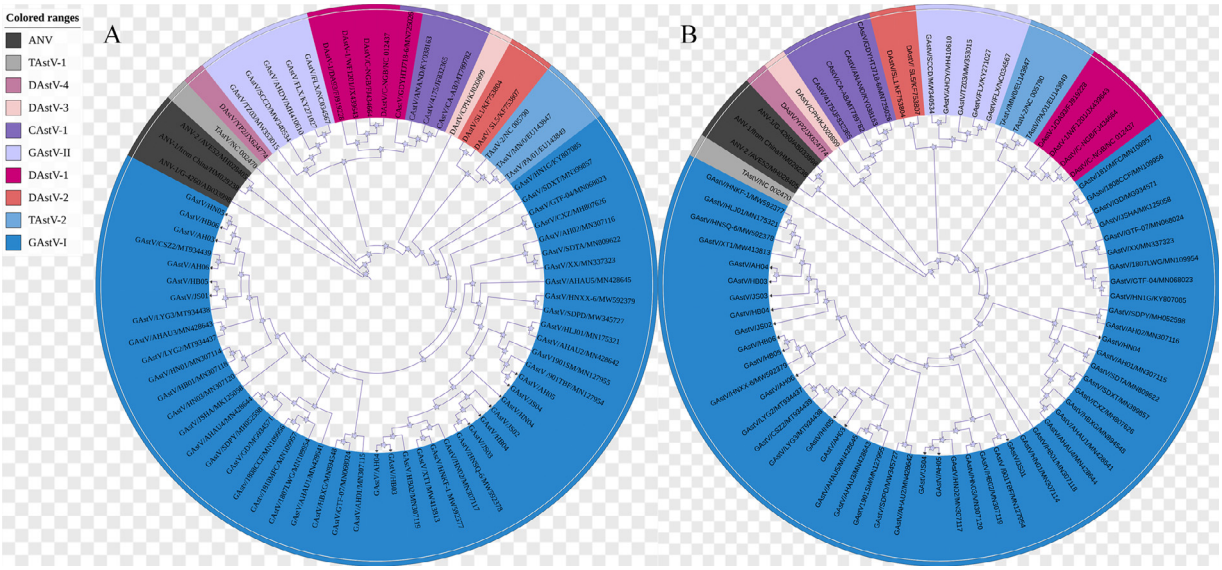

Figure 3. Phylogenetic analysis of the novel goose-origin astrovirus ORF1a (A) and ORF2 (B) sequences on MEGA X using the maximum-likelihood method. The 14 GoAstVs isolates are indicated using black triangles. Abbreviations: GoAstVs, gosling *astroviruses*; ORF, open reading frame.

The amino acid sequences of the 14 isolates were compared according to the alterations in the sequences; identities between the newly isolated strains and reference strains were 53.9 to 98.7%. The mutation sites of ORF1a, ORF1b, and ORF2 are presented in detail in Supplementary Tables 2–4. The amino acid polymorphism analysis indicated that hypermutations mainly occurred in ORF2. The following mutations were observed: V387A, N428T, F568S, and A782T in ORF1a (shown in red) and T218A, D456E, S608T, and A614T in ORF2. Additionally, Figure 4 reveals that the GoAstV-JS01 strain contained specific consecutive mutations in ORF1a (V357E, G375V, K376N, V387N, and A391P).

Figure 5 and Table 1 reveal the predicted antigen epitopes of ORF2 in GoAstV-JS01, and the 6 epitope regions and confidence levels ranging from 0.66 to 1.00 were predicted using SVMTrip and SOPMA. Furthermore, the tertiary structure model of ORF2 was predicted using SWISS-MODEL and PyMol in (Figure 6); loci 446 and 540 remarkably affected the tertiary structure of the ORF2 protein.

Recombination Analysis of GoAstVs

The recombination events in the 14 GoAstV strains predicted using RDP4 and SimPlot software are displayed in Figure 7. The results show that there were two recombination events in the 14 isolated strains; one recombination event at loci 3,256–6,146, originating from GoAstV-HNKF (a major parent with 99.3% identity) and recombining with GoAstV-LYG2 (a minor parent with 99.6% identity), and other event at loci 3,264–6,247 originating from GoAstV-HNKF (a major parent with 99.5% identity) and recombining with GoAstV-HB01 (a minor parent with 99.8% identity).

DISCUSSION

In recent years, gout has become one of the most serious diseases for goslings affecting goslings, putting immense burden on the poultry industry in recent years (Liu et al., 2020). In this study, the screening investigation of tissue samples obtained from diseased goslings proved that GoAstV is the causative viral pathogen for gout (Mandal and Mount, 2015; Tang, 2018). To date, GoAstV has caused substantial great economic losses to the goose industry in China (Jin et al., 2018), and currently, there is no effective vaccine for prevention and control (Xu et al., 2019). Various dynamic factors lead to the rapid spread of the GoAstV, thereby causing continued harm to the goose industry in China which is expected to persist in the foreseeable future (Yuan et al., 2019; Wang et al., 2021).

According to the phylogenetic tree analysis, two major GoAstV groups have different evolutionary histories. The results sensibly showed that the 14 isolated strains were most similar to other isolated GoAstV strains belonging to the G-I group, and the highest sequence identity for complete genome was 99.2% (99.5% for ORF1a, 100% for ORF1b, and 99.5% for ORF2). The evolutionary trees also showed that there were obvious differences between the 14 newly isolated strains and the FLX and AHDY strains from the G-II group (Zhang et al., 2017). To date, the TZ03 strain isolated from Taizhou, Jiangsu Province, China shared the highest identity (96.6%) with the GoAstV-2 strain FLX, indicating that G-II strains are circulating and causing ongoing goose gout disease in China (Wang et al., 2021).

The recombination analysis revealed the reorganization events in the 14 strains with GoAstV-HNKF, GoAstV-LYG2, and GoAstV-HB01 as parents. Most notably, the existence of recombination events may indicate that GoAstVs were constantly evolving during transmission, thereby accelerating the probability of

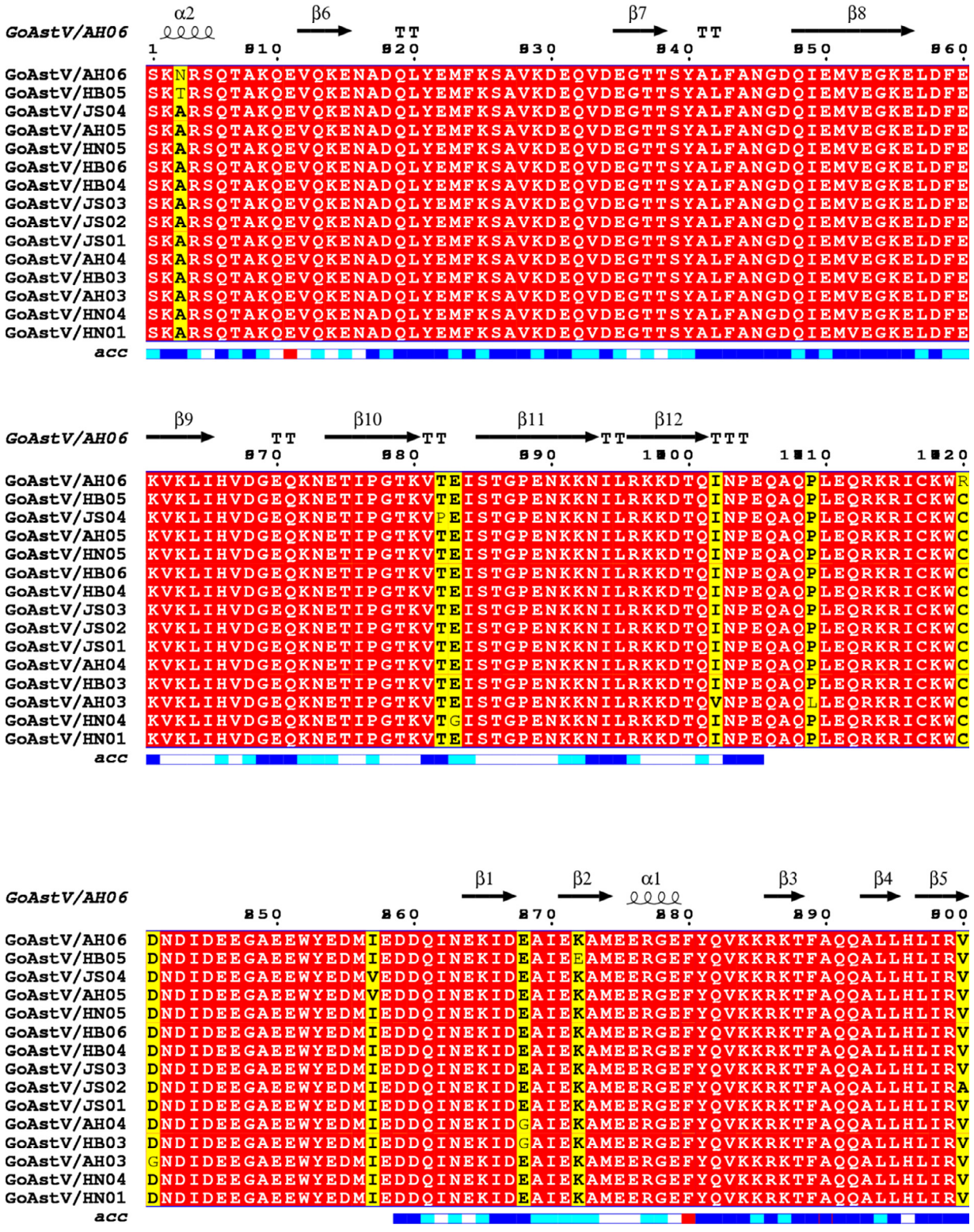

Figure 4. Sequence alignments of ORF1a performed and visualized using ESPript. Abbreviation: ORF, open reading frame.

virus evolution. Considering that the range of virus hosts is still expanding, the recombination of strains from Henan, Hubei, and Hebei provinces should alert local farmers to undertake strategies to prevent and control the vertical transmission of AstVs in geese.

In the present study, most of the mutations occurred in the ORF1a and ORF2 regions, with the JS01 strain especially showing high amino acid variations in ORF1a at loci 357–400 (Supplementary Tables 2–4 and Figure 4). ORF1a not only encodes nonstructural proteins but also plays a crucial role in virus replication, regulation, and immunity (Patel et al., 2017). The abundant mutations were presumed to influence virus pathogenicity. Accordingly, the continuous mutations in the ORF1a gene of the JS01 strain suggested the changes in the virus pathogenicity. In addition, fewer amino acid mutations were observed generated in the ORF1b protein which seemed to occur in a highly conserved region;

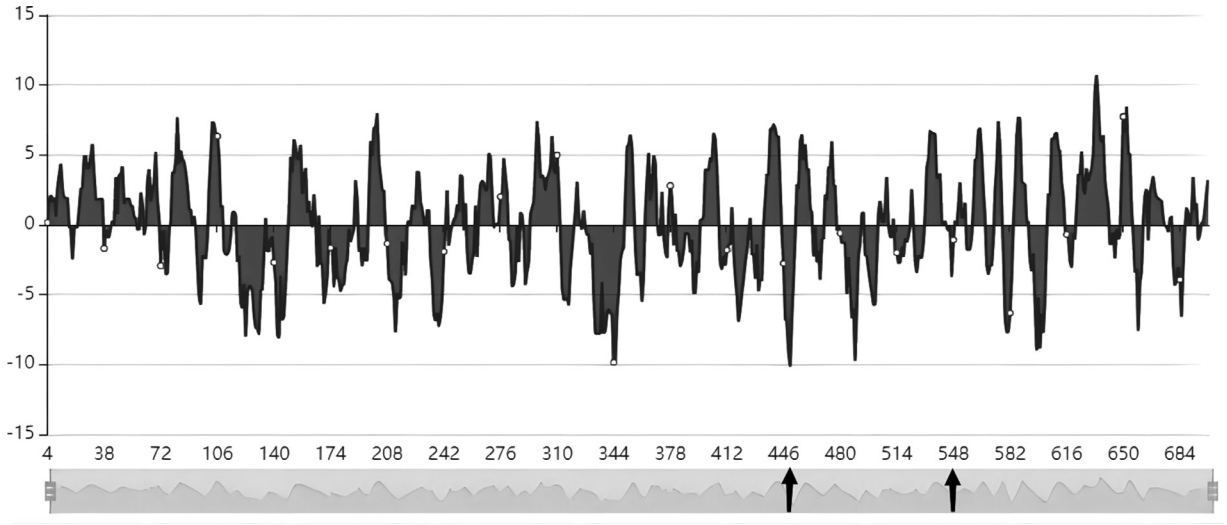

Figure 5. Antigenic epitope of the ORF2 protein of GoAstV-JS01. "↑" represents mutation sites at loci 446 and 540. Abbreviation: ORF, open reading frame.

Table 1. ORF2 epitope location and epitope sequence.

Epitope	Loci	Epitope sequence
1	134−153	AGRANILGSVVFLDIEQEAN
2	208−227	PAINMWTYLRTVNALSTRAQ
3	311−330	VGEVFWAVSTEVVETVASAL
4	354−373	SGSTYLIYSSVSDAQIDSRI
5	441−460	TTESCSFLVFGIPQADSRSR
6	535−542	TSTGGQIT

ORF1b exhibited a stable structure, indicating that it was an essential factor in virus replication (Liao et al., 2015). The ORF2 protein of astrovirus mainly determined the cell tropism, and stimulated the host cell to respond and participate in virus particle assembly, nucleic acid packaging, and virus particle maturation (Ren et al., 2020). In addition, ORF2 harbored a large number of amino acid mutations; among the 14 isolated strains, loci 446 and 540 showed an especially high frequency of mutations. Additionally, as shown by Table 1, we founded that loci 446 and 540 were located in the center of the antigen epitope. Virus evolution under antibody pressure may cause these mutations. The following loci were predicted using SVMTriP: 1) 134−153; 2) 208−227; 3) 311−330; 4) 354−373; 5) 441−460; and 6) 535−542. These results provide important data to develop vaccines against GoAstV.

This research reports the recent characteristics and antigenic mutations of GoAstV, enriching the understanding of this virus and providing a foundation for its effective prevention and control as well as for the manufacture of relevant vaccines.

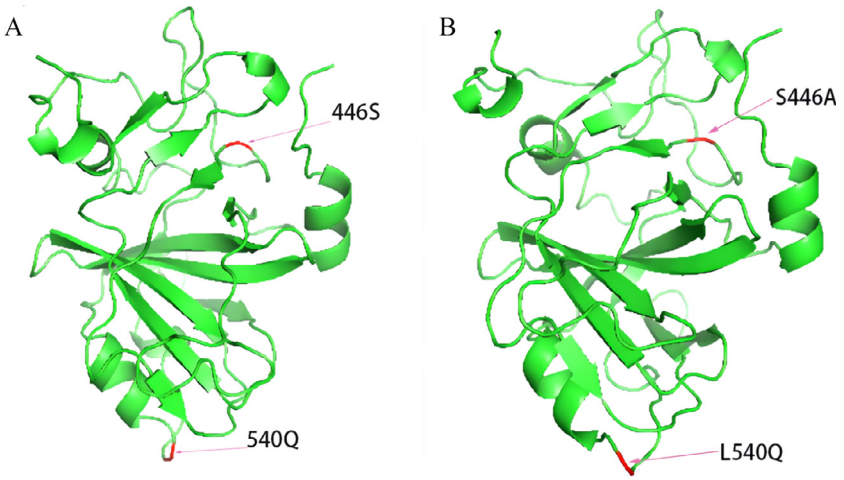

Figure 6. Cartoon scheme of the ORF2 protein structure. (A) Conserved domain of the ORF2 protein of GoAstV-AHAU2; (B) mutant structure of the ORF2 protein of GoAstV-HN01. Fourteen strains mutated at loci 446 and 540. Abbreviations: GoAstVs, gosling *astroviruses*; ORF, open reading frame.

Figure 7. Recombination events in GoAstVs. Abbreviation: GoAstVs, gosling *astroviruses*.

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Data availability: The data generated or analyzed during this study are available within the article and its supporting information.

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Ethics statement: The autopsy and sampling protocols for dead birds were approved by the South China Agricultural University Committee for Animal Experiments (approval ID: SYXK-2014-0136).

DISCLOSURES

The authors have nothing to declare.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.101888.

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