The Journal of Veterinary Medical Science

NOTE

Virology

A novel group of avian *Avastrovirus* in domestic geese, China

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J. Vet. Med. Sci.
80(5): 798–801, 2018
doi: 10.1292/jvms.18-0013

Received: 9 January 2018
Accepted: 2 March 2018
Published online in J-STAGE: 16 March 2018

ABSTRACT. Using an ORF1b-based astrovirus-specfic reverse transcription (RT)-PCR assay, a novel astrovirus-like was detected from domestic geese in China. Pairwise comparisons and phylogenetic analyzes suggested that a novel group of goose astrovirus, different with previously known astroviruses in the genus *Avastrovirus,* was found circulating in geese. This study has expanded our understanding about the role of domestic waterfowls as reservoirs for diverse astroviruses.

KEY WORDS: astrovirus, avian *Avastrovirus*, goose astrovirus

Astroviruses are non-enveloped viruses characterized by a positive sense, single-stranded RNA (ssRNA) viruses [4, 5]. The genomes of these viruses range in size from 6.1 kb to 7.9 kb, which are arranged in three open reading frames (ORF1a, ORF1b and ORF2), as well as a short 5′-untranslated region (UTR) and a 3′-UTR and a poly A tail. The ORF1a and ORF1b encode the non-structural proteins, including transmembrane helical motifs, serine protease, nuclear localization signal (NLS), and the RNA-dependent RNA polymerase (RdRp). The ORF2 encodes the viral capsid protein which is required for virion formation [2, 9–12, 22].

The family *Astroviridae* is divided into two genera: *Avastrovirus* and *Mamastrovirus* [5], which consist of astroviruses infecting mammalian [14, 15] and avian species, respectively (https://talk.ictvonline.org/taxonomy/). Previously, classification within each genus was based on the host of origin. But this classification method had the disadvantage that the species do not correspond to genetic phylogenies. Till now, three species (*Avastrovirus* 1, *Avastrovirus* 2 and *Avastrovirus* 3) were suggested in the genus *Avastrovirus*. The *Avastrovirus* 1 including Turkey astrovirus 1 (TAstV-1) [8]; the *Avastrovirus* 2 including two types of Avian nephritis virus (ANV-1 and ANV-2) [7]; and the *Avastrovirus* 3 including Duck astrovirus 1 (DAstV-1) [6] and Turkey astrovirus 2 (TAstV-2) [18] (https://talk.ictvonline.org/taxonomy/). To date, astroviruses were found from numerous avian species including turkeys (TAstV) [8, 18], ducks (DAstV) [9–12], chicken (CAstV) [19], guinea fowl (GFAstV) [2], pigeon (PiAstV) [23], geese [1, 9, 22], as well as wild aquatic and terrestrial birds including heron, doves, penguins [3, 5, 17]. These wide host range of astroviruses shared high genetic diversity, which have complicated attempts at a unified classification method [4, 5, 16]. Besides the three officially approved avastroviruses, there are numerous *Avastroviruses* waiting to be classified, particularly isolated from waterfowl (ducks and geese), which have not be approved as species by ICTV.

Recent works by Bipin *et al.* [1] have shown that goose embryo can be infected by avian nephritis virus (ANV), then a novel astrovirus in goose belong to the DAstV-3 cluster was identified by Liu *et al.* [12] and another goose astrovirus cluster identified by Zhang *et al*. [22]. In the present study, we report the detection of a goose astrovirus, different with previously known goose-origin astroviruses, which help us understanding not ducks but geese as reservoirs for various astroviruseses.

In January 2017, a disease occurred in two commercial *Shitou* geese flocks located in Southeast China, resulting in morbidity of about 35% and mortality rates nearly at 20% in 3–5 week-old domestic geese. Diseased geese showed inability to move and were unable to roost normally, with no typical necropsy and specific lesions were found.

Samples for each farm were collected including the intestine, spleen and liver were taken from the diseased geese for the purpose of diagnosis. The samples were homogenized in phosphate-buffered saline (20%, w/v) containing antibiotics (10,000 U/m*l* of Penicillin and 10 mg/m*l* of Streptomycin). The suspension was then clarified by centrifugation at 8,000 g at 4°C for 20 min, followed by filtration through a 0.22 μm-pore-size sterile filter (Millipore, Billerica, MA, U.S.A.). The filtrate was stored at-80°C until use.

The supernatants were used for DNA/RNA extraction using the EasyPure Viral DNA/RNA Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Classical endemic and emerging viruses once outbreak in geese, such as goose

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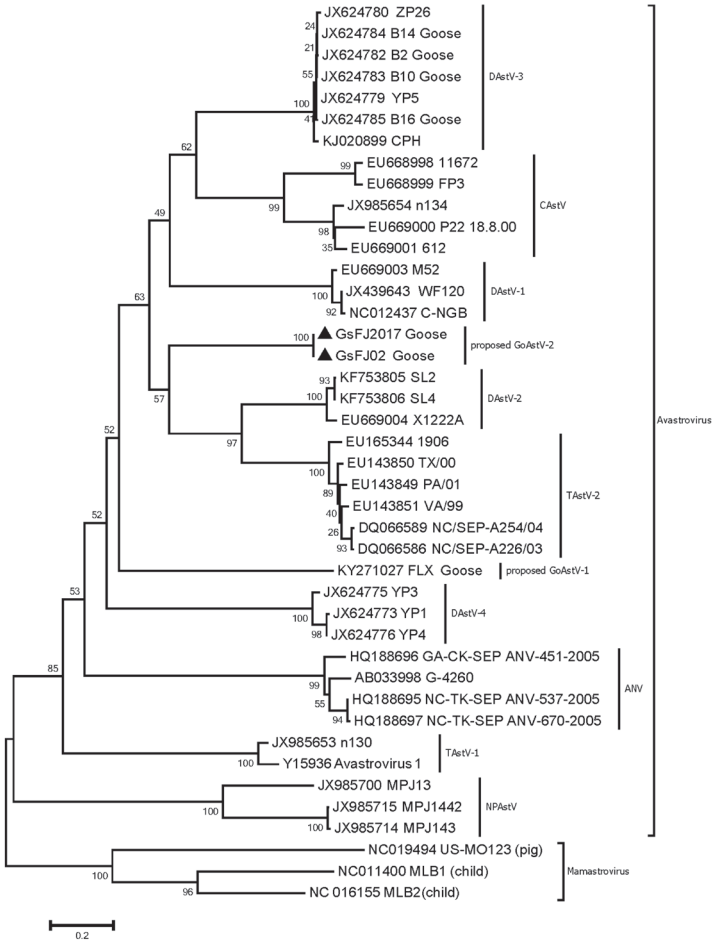

Fig. 1. Phylogenetic relationship of astroviruses detected in this study to other astroviruses, based on alignment of nucleotide sequences from an approximately 391-bp RdRp region. The tree was generated by MEGA 6.0 software, using Neighbor-Joining method (bootstrap=1,000). The scale bar represents the number of nucleotide substitutions per site. The goose origin astroviruses (GsFJ2017 and GsFJ02, proposed as GoAstV-2) in this study were indicated with black triangle (▲). Reference sequences obtained from GenBank are indicated by accession number and strain name. Goose origin astroviruses are indicated with goose followed the accession number and strain name. Of the astroviruses detected in this study, 37 avian astroviruses (belong to the genus *Avastroviruses*) were included in the analysis, including three classic *Avastrovirus* clades (TAstV-1, ANV and DAstV-1), six unclassified avian astroviruses (CAstV-1, DAstV-2, DAstV-3, DAstV-4, TAstV-2 and NpAstV) and newly identified goose astrovirus (GenBank No. KY271027, proposed as GoAstV-1). Three *Mamastroviruses* (GenBank No. NC019494, NC011400 and NC016155) were used as out-group. Clades were indicated on the right side of the trees.

parvovirus, avian influenza virus, avian Tembusu virus, avian paramyxovirus type 1, goose circovirus, *Escherichia coli*, *Riemerella anatipestifer* and *Salmonella anatum* spp. were excluded as the causative agent by PCR (RT-PCR) method [13, 20, 21].

As part of etiological studies, the extracted RNA was screened for astrovirus using a previously described degenerate primer based RT-PCR with primers, which was designed to amplify a fragment of *RdRp* gene [3, 12, 19]. Using the RT-PCR method, DNA fragments of approximately 430-bp were obtained from the diseased *Shitou* geese. After cloning and sequencing of the amplicons, two goose origin astroviruses (designated as GsFJ2017 and GsFJ02) were obtained and submitted to the GenBank under the accession numbers MF576430 and MG696113, 391-bp sequences were used for further analyzes.

Recent study showed a 391-bp RdRp region (partial of the 430-bp), which agreed with those obtained astroviruses sequences identity analyzes, can be used for different avian species astroviruses classification [3, 12, 19]. Here, 391-bp RdRp sequences from previously known 37 avian astroviruseses and 3 mammalian astroviruses retrieved from GenBank were used. Nucleotides identities were analyzed using Lasergene software v10.0 (DNAStar, Madison, WI, U.S.A.) by ClustalW method. Phylogenetic tree of the 391-bp RdRp sequences were constructed using the neighbor-joining implemented in MEGA 6. Bootstrap analysis was performed with 1,000 replications.

To investigate further the relationship of the two goose origin astroviruses detected in the study with other avastroviruses, phlylogenetic analysis was performed based on the alignments of RdRp sequences. The tree demonstrated that 37 astrovirus isolates from birds were clustered into nine distinct clades (ANV, CAstV, DAstV-1, DAstV-2, DAstV-3, DAstV-4, TAstV-1, TAstV-2 and NpAstV). The GsFJ2017 and GsFJ02 isolates in this study were grouped into a distinct clade (proposed GoAstV-2 candidates), different with newly identified goose astrovirus (GenBank No. KY271027, proposed GoAstV-1), under genus *Avastroviruses* (Fig. 1).

Table 1. Nucleotides comparison of astroviruses detected in this study to other astroviruses, based on alignment of nucleotide sequences from an approximately 391-bp RdRp region

Viruses	TAstV-1	ANV	DAstV-1	CAstV-1	DAstV-2	DAstV-3		DAstV-4	GoAstV-1	TAstV-2	NpAstV
						Duck origin	Goose origin				
Identities (%)	58.8–64.2	55.9–60.3	62.9–63.7	55.9–63.6	66.0–67.3	66.2–67.3	66.5–67.3	63.1–64.1	62.8–63.0	67.3–67.8	53.7–54.2

In terms of sequence identity, the two goose origin astroviruses (GsFJ2017 and GsFJ02) shared the highest identity values at 99.7%, significantly higher than those with newly identified goose astrovirus (GenBank No. KY271027) (62.8–63.0%), the DAstV-3 cluster (66.2–67.3% with duck origin and 66.5–67.3% with goose origin) and the ANV cluster (55.9–60.3%), respectively. Goose had been tested with astrovirus positive previously from the viruses with belong to the GoAstV-1 (proposed), DAstV-3 cluster [12, 22] and ANV cluster [1, 7]. The lower identities (53.7–67.8%) shared by the GsFJ2017 and GsFJ02 isolates and other selected avastroviruses were shown in Table 1.

Moreover, positive samples were submitted to viral isolation by inoculation of clarified homogenate onto the chorioallantoic membrane (CAM) of 9-day-old embryonated SPF chicken eggs and 10-day-old embryonated goose eggs for three passages. Embryos were incubated at 37°C and candled twice daily for 10 days. No deaths and pathological changes were observed in embryos after three passages, with no positive signal observed at every passage by the RT-PCR technology. These data means attempts to isolate the virus were unsuccessful. Experimental infections of goslings with unpropagated viruses that are present in a clarified intestinal sample could not reproduce the disease observed in the field. Thus, although the novel goose astroviruses were detected in diseased ducks, and was possibly associated with clinic pathological changes, it is not concluded that the astrovirus described here must be the causative agent of the disease. Further studies, including propagation assays and pathogenicity tests of the novel goose astrovirus and other possible pathogens, are needed to elucidate the causative agent of the disease.

To conclude, this study demonstrates the occurrence of a novel astrovirus in geese, distinguished with all previously reported avian astroviruses. The findings of different avastroviruses in domestic ducks and geese have raised a concern about the role of domestic waterfowls as reservoirs for diverse astroviruses. More extensive surveillance for avian astroviruses in different ducks and geese may enhance our understanding of the molecular epidemiology and ecology of *Avastroviruses*.

CONFLICTS OF INTEREST. All authors have declared no conflict of interest.

ACKNOWLEDGMENTS. This work was found by Natural Science Foundation of China (31602068), China Agriculture Research System (CARS-42), Fujian Academy of Agriculture Science Innovative Research Team (STIT2017-3-10) and Young Talent Program (YC2015-12), and the Fujian Public Welfare Projects (2018R1023-5).

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