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Molecular characterization of a duck hepatitis virus 3-like astrovirus

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ABSTRACT

Using an ORF1b-based astrovirus-specific reverse transcription (RT)-PCR assay, a duck hepatitis virus type 3 (DHV-3)-like astrovirus was detected from four intestinal samples collected from diseased ducks in China. Complete genome sequencing and comparative sequence analysis showed that the four duck astrovirus (DAstV) isolates were closely related and possessed a typical astrovirus genome organization. Genetic analysis of the complete ORF2 region revealed that mean amino acid genetic distances between the DHV-3-like isolates and previously known avastrovirus species were between 0.579 and 0.721, suggesting that the DHV-3-like isolates could be classified as an additional avastrovirus species. In the ORF1a and ORF1b regions, however, mean amino acid genetic distances between the DHV-3-like viruses and the turkey astrovirus 2 (TAstV-2)-like isolates were substantially less than those between TAstV-2-like isolates and DAstV/C-NGB-like astroviruses belonging to the same species. Pairwise comparisons and phylogenetic analyses demonstrated that the DHV-3-like isolates were most closely related to TAstV-2-like viruses in ORF1a and ORF1b, while showed highest similarity with the chicken astrovirus (CAstV) 612-like viruses in ORF2. These findings provide evidence that recombination events may have occurred during evolution of the avastroviruses and support the view that genomic analysis is required for classification of the avastroviruses.

1. Introduction

Astroviruses have been demonstrated to infect both mammalian and avian hosts, mostly associated with gastroenteritis in young individuals (Bosch et al., 2011). Unlike other species, astroviruses in ducks have been associated with duck viral hepatitis (DVH), a highly contagious and fatal disease in young ducklings (Woolcock, 2003). To date, two different astroviruses have been described in ducks, historically known as duck hepatitis virus types 2 (DHV-2) and 3 (DHV-3) (Asplin, 1965; Toth, 1969; Haider and Calnek, 1979; Gough et al., 1985; Todd et al., 2009). Recent work by Todd et al. (2009) showed that the two duck astroviruses (DAstVs) shared low nucleotide (nt) and amino acid (aa) identity values in a 391 nt open reading frame (ORF) 1b region, suggesting that they may represent different astrovirus species. Astrovirus infection has been documented in ducks in China. Based on sequence comparisons of the partial ORF1b region, the Chinese DAstV isolate (designated C-NGB) is probably isolate of DHV-2 (Fu et al., 2009). The DHV-3-associated DVH was last observed in 1975 on a flock of White Pekin ducks in Long Island, New York, USA

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(Haider and Calnek, 1979) and there have been no published reports of the disease occurring since then.

Astroviruses are non-enveloped, single-stranded, positive-sense RNA viruses. The genomes of these viruses are arranged in three ORFs: ORF1a and ORF1b at the 5′ end encoding the viral protease and the RNA-dependent RNA polymerase (RdRp), and ORF2 at the 3′ end encoding the precursor capsid protein (Bosch et al., 2011). A frame-shifting mechanism located between ORF1a and ORF1b is employed to translate the RdRp (Jiang et al., 1993; Lewis et al., 1994; Marczinke et al., 1994). Earlier work in our laboratory revealed that the DHV-2-like astrovirus strain C-NGB possesses a typical astrovirus genome organization (Fu et al., 2009). Although DHV-3 was first isolated 44 years ago (Toth, 1969), little was known regarding its molecular biology.

The *Astroviridae* is currently divided into two genera: *Mamastrovirus* and *Avastrovirus*. Based on genetic analysis of the complete capsid region at the amino acid level, three species were established within the genus *Avastrovirus*, *Avastrovirus* GI.A, including Turkey astrovirus 1 (TAstV-1); *Avastrovirus* GI. B, including Avian nephritis virus types 1 (ANV-1) and 2 (ANV-2); and *Avastrovirus* GII.A, including Turkey astrovirus 2 (TAstV-2) and DAstV/C-NGB (Bosch et al., 2011). Most recently, the three species were renamed *Avastrovirus 1*, *Avastrovirus 2*, and *Avastrovirus* 3 respectively (http://www.iah-virus.org/astroviridae/). Due to the lack of data relating to the molecular characteristics of DHV-3, the virus is now regarded as an unassigned species in the genus *Avastrovirus* (Bosch et al., 2011).

In the present study, we report the detection and complete genome characterization of a novel duck astrovirus, which is probably an isolate of DHV-3, from four intestinal samples collected from diseased ducks in China. The molecular features of the newly identified astrovirus and its phylogenetic relationships to previously sequenced avastroviruses were investigated.

2. Materials and methods

2.1. Clinical samples

In September 2012, a disease occurred in a commercial Cherry Valley Pekin duck flock located in south China, resulting in a morbidity of about 40% in 4–5-week-old ducks. Diseased ducks showed inability to move and were unable to roost normally, with most of effected ducks lay supine. On necropsy, no specific lesions were found. In some cases, hemorrhage or grey-white annular bands were observed on the surface of intestinal mucosa. Twenty-five samples including intestine ($n = 16$), liver ($n = 6$) and heart ($n = 3$) were taken from the diseased ducks for the purpose of diagnosis. The samples were homogenized in phosphate-buffered saline (20%, w/v) containing antibiotics (10,000 U/ml of Penicillin and 10 mg/ml of Streptomycin). The suspension was then clarified by centrifugation at 12,000 × *g* at 4 °C for 15 min, followed by filtration through a 0.22 μm-pore-size sterile filter (Millipore, Billerica, USA). The filtrate was stored at −80 °C until use.

2.2. RNA extraction

RNA was extracted from 250 μl of filtrate using a TRIzol® Reagent (Life Techologies™, Carlsbad, USA), following the manufacturer's instructions.

2.3. Detection of astrovirus in the samples

As part of etiological studies, the extracted RNA was screened for astrovirus using a previously described degenerate primer-based reverse transcription (RT)-PCR assay targeting the RdRp gene (Todd et al., 2009). All PCR amplicons with the expected product size (~430 bp) were subjected to DNA sequencing for confirmation.

2.4. Amplification of the astrovirus genome

Most of the full-length sequences of duck-origin astroviruses were determined using RT-PCR with primers (Supplementary Table S1) designed based on the conserved regions in genomes of DAstV/C-NGB (Fu et al., 2009) and TAstV-2 isolates (Koci et al., 2000; Strain et al., 2008). For cDNA synthesis, 5 μl of extracted RNA was mixed with 20 pmol of reverse primer, incubated at 70 °C for 5 min and then at 4 °C for 5 min. A reaction mixture of 19 μl containing 5 μl of M-MLV RT 5 × Buffer, 5 μl of dNTP mixture, 1 μl of 200 U/μl M-MLV reverse transcriptase (Promega, Madison, USA) and 1 μl of 40 U/μl Recombinant Ribonuclease Inhibitor (TaKaRa, Dalian, China) was added. The reaction mixture was incubated at 42 °C for 60 min, followed by an enzyme inactivation step at 94 °C for 5 min. 5 μl of the RT product was then used as a template in a subsequent 25 μl PCR mixture containing 20 pmol of each of the upstream and downstream primers and 12.5 μl of 2 × PCR SuperMix (Vigorous, Beijing, China). PCR was performed at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 47–60 °C for 30 s and 72 °C for 30–100 s (depending on the length of genes amplified) and a final extension at 72 °C for 10 min.

The 5′ and 3′ ends of the genome were obtained by 5′ and 3′ rapid amplification of cDNA ends (RACE) strategies. The reactions and conditions used for reverse transcription and amplification were similar to those as described above. To obtain the 3′ end of viral genome, 5 μl of extracted RNA was reverse transcribed by M-MLV reverse transcriptase with primer 3Rr. A semi-nested PCR was performed using 3Rr as antisense primer and 5729f and 6008f as first- and second-round PCR sense primers, respectively. To amplify the 5′ end of the DAstV genome, 5 μl of extracted RNA was reverse transcribed by M-MLV reverse transcriptase with primer 387r. The cDNA was purified using a TIANquick Mini Purification kit (TIANGEN Biotech, Beijing, China), and a poly (T) tail was added to the 3′ end of cDNA using Terminal Deoxynucleotidyl Transferase and dTTP (TaKaRa, Dalian, China), following the manufacturer's instructions. Subsequently, a semi-nested PCR was carried out employing 5Rf as sense primer and 292r and 250r as first- and second-round PCR antisense primers, respectively.

The initial genome sequence was confirmed by determination of 10 overlapping DNA fragments amplified using RT-PCR with additional primers.