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Genetic characterization of a novel astrovirus in Pekin ducks

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ABSTRACT

Three divergent groups of duck astroviruses (DAstVs), namely DAstV-1, DAstV-2 (formerly duck hepatitis virus type 3) and DAstV-3 (isolate CPH), and other avastroviruses are known to infect domestic ducks. To provide more data regarding the molecular epidemiology of astroviruses in domestic ducks, we examined the prevalence of astroviruses in 136 domestic duck samples collected from four different provinces of China. Nineteen goose samples were also included. Using an astrovirus-specific reverse transcription-PCR assay, two groups of astroviruses were detected from our samples. A group of astroviruses detected from Pekin ducks, Shaoxing ducks and Landes geese were highly similar to the newly discovered DAstV-3. More interestingly, a novel group of avastroviruses, which we named DAstV-4, was detected in Pekin ducks. Following full-length sequencing and sequence analysis, the variation between DAstV-4 and other avastroviruses in terms of lengths of genome and internal component was highlighted. Sequence identity and phylogenetic analyses based on the amino acid sequences of the three open reading frames (ORFs) clearly demonstrated that DAstV-4 was highly divergent from all other avastroviruses. Further analyses showed that DAstV-4 shared low levels of genome identities (50–58%) and high levels of mean amino acid genetic distances in the ORF2 sequences (0.520–0.801) with other avastroviruses, suggesting DAstV-4 may represent an additional avastrovirus species although the taxonomic relationship of DAstV-4 to DAstV-3 remains to be resolved. The present works contribute to the understanding of epidemiology, ecology and taxonomy of astroviruses in ducks.

1. Introduction

Astroviruses are small non-enveloped viruses with positive-sense, single-stranded, genomic RNA. In general, the astrovirus genomes have a common organization. From the 5′ end to the 3′ end, the genomes are arranged in a 5′ untranslated region (UTR), three open reading frames (ORFs 1a, 1b and 2), a 3′UTR and a poly (A) tail (Bosch et al., 2011). ORF1a and ORF1b encode the nonstructural proteins including several transmembrane (TM) helical motifs, a serine protease, a nuclear localization signal (NLS) and an RNA-dependent RNA polymerase (RdRp) (Jiang et al., 1993; Lewis et al., 1994; Willcocks et al., 1994). ORF2 encodes the structural proteins. All astroviruses share a rather conserved frameshift slippery structure between ORF1a and ORF1b, which directs the synthesis of an ORFla/lb fusion polyprotein (Jiang et al., 1993; Lewis et al., 1994; Lewis and Matsui, 1995, 1996, 1997; Marczinke et al., 1994).

The *Astroviridae* family is divided into two genera: *Mamastrovirus* and *Avastrovirus* (AAstV) that consist of astroviruses infecting mammalian and avian species, respectively (Bosch et al., 2011). Previously, classification within each genus was based only on the host of origin (Monroe et al., 2005). As such, the species do not correspond to genetic phylogenies. Currently, the astrovirus species is defined on the basis of genetic differences in the complete capsid sequence. According to the criteria, within the *Avastrovirus* genus, three species are created: *Avastrovirus 1*, including Turkey astrovirus 1 (TAstV-1); *Avastrovirus 2*, including Avian nephritis virus 1 (ANV-1) and 2 (ANV-2); and *Avastrovirus 3*, including Turkey astrovirus 2 (TAstV-2) and Duck astrovirus 1 (DAstV-1) (http://www.iahvirus.org/astroviridae/avastrovirus/avastrovirus.htm; http://www.ictvonline.org/virusTaxonomy.asp). Besides the three officially approved avastrovirus species, there are many other avastroviruses waiting to be classified, such as Chicken astroviruses (CAstVs) (Pantin-Jackwood et al., 2006; Todd et al., 2009; Kang et al., 2012), Duck hepatitis virus type 3 (DHV-3) (Todd et al., 2009), DHV-3-like astroviruses (Liu et al., 2014a), Duck astrovirus CPH (DAstV CPH) (Liu et al., 2014b), and astroviruses detected from wild birds (Chu et al., 2012).

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Based on sequence comparison of a partial ORF1b region, three divergent polymerase groups of DAstVs are recognized (Todd et al., 2009; Liu et al., 2014a,b). Besides the DAstV-1 group, which contains Duck hepatitis virus type 2 (DHV-2) M52 isolated in the UK and DHV-2-like astroviruses detected in China, duck astroviruses are found to form other two groups, termed DAstV-2 and DAstV-3 in this study. DAstV-2 contained DHV-3 X1222A Calnek isolated in the US and DHV-3-like astroviruses recently identified in China. While DAstV-3 refers to DAstV CPH identified recently in the newly hatched Pekin ducklings in China. To date, the complete sequences of DHV-2 and DHV-3 have not been determined. Phylogenetic analysis of the complete ORF1a, ORF1b and ORF2 sequences of the Chinese DAstV-1, DAstV-2 and DAstV-3 isolates further confirmed the existence of the same three genotypes defined by analysis of the partial ORF1b sequences. The three groups of DAstVs share mean amino acid genetic distance ranging from 0.577 to 0.612 in the ORF2 region, suggesting that they may represent three separate AAstV species. It should be noted that, if other avian astroviruses are employed in phylogenetic analysis, the three groups of DAstVs were shown to be closely related to different avastroviruses in different genomic regions. As suggested, this different phylogenetic clustering may have resulted from recombination events, making classification of AAstV species complicated (Liu et al., 2014a,b).

In addition to the three groups of DAstVs primarily found in domestic ducks, other avian astroviruses, including ANV, TAstV-1, TAstV-2 and CAstV, have also been identified in domestic ducks (Biđin et al., 2011, 2012a,b). The findings of different avastroviruses in domestic ducks have raised a concern about the role of domestic ducks as reservoirs for diverse astroviruses. Earlier work by Biđin et al. (2012b), which showed that ANV was detected from dead-in-shell goslings, suggests that it is necessary to pay attention to astrovirus infections in domestic geese.

In the present study, we report molecular detection of astroviruses in domestic ducks and geese. Using an ORF1b-based astrovirus-specific reverse transcription (RT)-PCR assay (Todd et al., 2009), two groups of astroviruses were found in our samples. A group of astroviruses detected from Pekin ducks, Shaoxing ducks and Landes geese in three different provinces in China were highly similar to DAstV-3. More interestingly, a novel group of astroviruses, which we named DAstV-4, was detected in Pekin ducks. Nucleotide sequence determination and sequence analysis of genome of one selected strain support the view that the virus will need be recognized as a novel avastrovirus.

2. Materials and methods

2.1. Sample collection

Feces and intestines were sampled from domestic ducks and geese from different regions in China from March to December 2011. Fecal samples were collected from Pekin ducks (*Anas platyrhynchos domestica*) in live-bird markets located in Guangdong and Hubei provinces, and Shaoxing ducks (*A. platyrhynchos domestica*) and Landes geese (*Anser anser Linn. var domestica*) in Jiangxi province. Intestinal samples were collected from dead-in-shell Pekin ducklings from a hatchery located in Shandong province (Table 1). There were no outbreaks of disease recorded among the egg-laying ducks and geese during sampling. The clinical status of the Pekin ducks before they were submitted to the live-bird markets was not known. The mortality of pre-hatching Pekin ducklings was about 10%.

2.2. Sample preparation

The samples were vigorously vortexed or homogenized in phosphate-buffered saline (20%, w/v) containing antibiotics (100 U/ml of Penicillin and 100 μg/ml of Streptomycin). The suspension was clarified by centrifugation at 13800×*g* for 10 min. The supernatant for each sample was filtered through a 0.22 μm-pore-size sterile filter (Millipore, Billerica, USA) to remove large particles. The filtrate was stored at −80 °C until use.

Table 1
Detection of astroviruses in samples collected from four different provinces of China.

Group and region	Bird species	Sample	No. of samples	No. (%) of astrovirus-positive samples
Live-bird market				
Guangdong	Pekin duck	Feces	61	8 (13.1)
Hubei	Pekin duck	Feces	29	0
Farm				
Jiangxi	Landes goose	Feces	19	7 (36.8)
	Shaoxing duck	Feces	18	2 (11.1)
Shandong	Pekin duck embryo	Intestine	28	3 (10.7)
Total			155	20 (12.9)

2.3. RT-PCR detection for astrovirus

Astroviruses in the samples were detected using a previously described degenerate primer-based RT-PCR assay, which was designed to amplify a fragment of approximately 430 bp from the ORF1b region of astroviruses (Todd et al., 2009). Total RNA was extracted from 200 μl of filtrate using an RNeasy Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The RNA was eluted in 50 μl RNase-free water.

2.4. Cloning of PCR product and sequencing

To confirm the presence of astroviruses in samples, all PCR amplicons with the expected product size were subjected to DNA sequencing. PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and then cloned into a pMD18-T vector (TaKaRa, Dalian, China), according to manufacturer's instructions. The vector was used to transform the competent *Escherichia coli* strain DH5a. After identification, positive transformants were submitted to a company (Taihe, Beijing, China) for nucleotide sequence determination. For each PCR product, three colonies were selected for sequencing.

2.5. Virus isolation

A fecal sample (designated YP2) from Guangdong province that was tested positive for astrovirus by RT-PCR was used for virus isolation. 0.2 ml of the filtrate was inoculated onto the chorioallantoic membrane (CAM) of five chicken embryos (9–10 days) and five duck embryos (9–10 days) respectively. Embryos were incubated at 37 °C and candled twice daily for 10 days. The CAMs, liver, kidneys, and intestines of embryos within 7–10 days postinoculation were harvested for further passages. The culture from each passage was tested by RT-PCR following the protocol described above.

2.6. Genome sequencing

The RNA extracted from the YP2 sample was subjected to full-length genome sequencing. Viral genome sequence was obtained using strategies that we had previously used for a novel picornavirus (Liao et al., 2014). Briefly, extracted RNA was randomly PCR amplified as described by Allander et al. (2005). The sequences from sequence-independent PCR amplification were analyzed and assembled into contiguous fragments (contigs) as described