Duck astroviruses (DAstVs) are known to cause duck viral hepatitis; however, little is known regarding their molecular biology. Here, we report the complete sequence of a DAstV associated with a recent outbreak of fatal hepatitis in ducklings in China. Sequence analyses indicated that the genome of DAstV possessed a typical astrovirus organization and also exhibited two unique features. The polyadenylated genome comprised 7722 nt, which is the largest among astroviruses sequenced to date. The ORF2 of DAstV was not in the same reading frame as either ORF1a or ORF1b, which was distinct from all other astroviruses. Sequence comparisons and phylogenetic analyses revealed that DAstV was more closely related to turkey astrovirus (TAstV) type 2, TAstV-3 and TAstV/MN/01 (a possible new TAstV serotype) than to TAstV-1 or other astroviruses. These findings suggest that astroviruses may transmit across ducks and turkeys.

Astroviruses are non-enveloped, single-stranded, positive-sense RNA viruses, which have been demonstrated to infect both mammalian and avian hosts (Monroe et al., 2005). The genomes of these viruses range in size from 6.1 to 7.3 kb, consisting of three ORFs (1a, 1b, and 2) (Finkbeiner et al., 2008). All astroviruses share a rather conserved frameshift slippery sequence in the overlap region between ORF1a and ORF1b, which directs the synthesis of an ORF1a/lb fusion polyprotein (Jiang et al., 1993; Lewis et al., 1994; Lewis & Matsui, 1996; Marczinke et al., 1994). ORF1a and ORF1b encode the non-structural proteins, which include several transmembrane helical motifs, a serine protease, a nuclear localization signal (NLS), and an RNA-dependent RNA polymerase (RdRp) motif (Jiang et al., 1993; Lewis et al., 1994; Willcocks et al., 1994). ORF2 encodes the capsid protein that is required for virion formation.

Astroviruses in ducks have been associated with a fatal hepatitis which occurred in the UK, historically known as duck hepatitis virus type 2 (DHV-2) (Asplin, 1965; Gough et al., 1984, 1985). Originally thought to be a picornavirus, DHV-2 was later characterized as an astrovirus by morphology and renamed duck astrovirus 1 (DAstV-1) (Gough et al., 1984, 1985; Monroe et al., 2005). DHV-1 and a later described serotype 3 (DHV-3), isolated in the USA (Haider & Calnek, 1979), are still classified as picornviruses (Stanway et al., 2005). Recently, DHV-1 was confirmed as a picornavirus belonging to a novel genus (Ding & Zhang, 2007; Kim et al., 2006; Tseng et al., 2007). It has also been proposed that DHV-1 be renamed duck hepatitis A virus (DHAV) genotype A (DHAV-A) and that two other newly discovered DHV serotypes (Kim et al., 2007; Tseng & Tsai, 2007) be designated DHAV-B and DHAV-C (Fu et al., 2008; Wang et al., 2008). More recently, a 391 nt RdRp sequence has been determined for DAsTV-1 and DHV-3 and comparisons indicate that they are both astroviruses (Todd et al., 2009). In this study, we report the complete genomic sequence of a DAsTV strain associated with a fatal hepatitis which occurred in China in 2008. The genomic features of this virus and its relationship to other astroviruses were investigated.

In July 2008, a severe outbreak of duck viral hepatitis occurred in a commercial Cherry Valley duck flock in China, resulting in a mortality of about 50% in 1- to 2-week-old ducklings. A liver sample, designated C-NGB, was obtained from a 2-week-old dead duckling displaying the typical haemorrhagic lesions of duck viral hepatitis. Total RNAs were extracted from the sample using a BioSpin RNA Simply P Purification kit (Bioeer Technology) according to the manufacturer’s instructions. The sample was found to be negative for DHAV using DHAV-specific RT-PCR as recently described (Fu et al., 2008). An RT-PCR, designed to amplify avian astrovirus RdRp sequences of approximately 430 nt (Todd et al., 2009), was performed and an amplicon of the expected size...
was obtained. The sequence generated from this amplicon was identified by BLASTP analysis in GenBank as encoding an astrovirus-like RdRp.

To determine further the full-length sequence of DAstV, CLUSTAL W 1.83 (Thompson et al., 1994) was used to align nucleotide and amino acid sequences of the three ORFs of astroviruses, including human astrovirus 1 (HAstV-1) (Lewis et al., 1994), mink astrovirus (MAstV) (Mittelholzer et al., 2003), ovine astrovirus (OAstV), turkey astrovirus (TAstV) 1 (Jonassen et al., 2003), TAstV-2 (Koci et al., 2000), and avian nephritis virus 1 (ANV-1) (Imada et al., 2000). New primers were designed based on conserved motifs in astroviruses and DAstV-specific sequences obtained, using Primer Premier 5.0 (Premier Biosoft International; Supplementary Table S1). The strategy for amplification of the DAstV genome is illustrated in Fig. 1 (RT-PCR conditions is shown in supplementary methods).

The 5' and 3' ends of the genome were obtained by 5' and 3' rapid amplification of cDNA ends (RACE) strategies (Sambrook & Russell, 2001). To obtain the 5' end sequence, extracted RNAs were reverse transcribed by SuperScript III reverse transcriptase (Invitrogen) with primer F1, followed by purification of cDNA using TIANquick Mini Purification kit (TIANGEN Biotech), and ligation of dTTP to the 3' end of cDNA using Terminal Deoxynucleotidyl Transferase (TaKaRa), following the manufacturer’s instructions. Subsequently, a semi-nested PCR was carried out employing F4 as sense primer and F2 and F3 as first- and second-round PCR antisense primers, respectively.

In all cases, PCR products were purified by TIANgel Midi Purification kit (TIANGEN Biotech) according to the manufacturer’s instructions, cloned into pGM-T vector (TIANGEN Biotech), and sequenced (AuGCT Biotechnology). Sequence data for all clones were edited and translated into amino acid sequences with DNAMAN 5.2.2 (Lynnon). Sequence similarity was evaluated using BLASTP. The complete genomic sequence of DAstV C-NGB was obtained and confirmed by the process of primer design and primer walking.

Sequence analyses using DNAMAN 5.2.2 (Lynnon) revealed that the complete genome of DAstV C-NGB was 7722 nt, excluding the poly(A) tail, making it the largest among astroviruses so far sequenced. The polyadenylated genome was organized into three overlapping ORFs of 3723 (ORF1a), 1551 (ORF1b) and 2196 nt (ORF2), a short 5’ UTR of 22 nt and a 3’ UTR of 217 nt (Fig. 1a). The ORFs were identified as astrovirus-like by BLASTP analysis in GenBank.

By analogy to other astroviruses, a ribosomal frameshift signal (Jiang et al., 1993; Lewis et al., 1994; Lewis & Matsui, 1996; Marczinke et al., 1994) was observed in the 43 nt overlap region between ORF1a and ORF1b of DAstV, consisting of the heptameric AAAAAAC sequence from nt 3736 to 3742, followed by a stem–loop sequence from nt 3750 to 3772. However, DAstV appeared to deviate from all other astroviruses in the genomic structure. The ORF2 of DAstV was not in the same reading frame as either ORF1b or ORF1a, because the start codon of ORF2 was 23 nt downstream of the stop codon of ORF1b and 1564 nt downstream of the stop codon of ORF1a. In contrast, the ORF2 of ANV-1 and most mammalian astroviruses (except MAstV) is in the same reading frame as ORF1a, whereas the ORF2 of MAstV and TAstVs is in the same reading frame as ORF1b (Chu et al., 2008; Finkbeiner et al., 2008; Mittelholzer et al., 2003; reviewed by Koci & Schultz-Cherry, 2002; Strain et al., 2008).

Pairwise comparisons based on the amino acid sequences of the three ORFs were undertaken to determine the

![Fig. 1.](http://vir.sgmjournals.org)
relationship of DAscV with the other completely sequenced astroviruses, including HAstV types 1–5 and 8 (Jiang et al., 1993; Lewis et al., 1994; Méndez-Toss et al., 2000; Oh & Schreier, 2001; Silva et al., 2006) as well as astrovirus MLB1 (AstV-MLB1) (Finkbeiner et al., 2008), MAscV (Mittelholzer et al., 2003), OAscV, TAscV-1 (Jonassen et al., 2003), TAscV-2 (Koci et al., 2000), TAscVMN/01 (a possible new TAscV serotype) (Strain et al., 2008), and ANV-1 (Imada et al., 2000). Pairwise comparisons were performed using CLUSTAL W 1.83 (Thompson et al., 1994; http://align.genome.jp/), with the Gonnet matrix as the comparison scoring table. We found that DAscV was more closely related to TAscVs than to other astroviruses.

DAscV appeared to be more closely related to TAscV-2 and TAscVMN/01 rather than to TAscV-1. The amino acid identity between DAscV and TAscV-2 was around 44, 69 and 68 % in ORF1a, ORF1b and ORF2, respectively. A similar result was also obtained from amino acid identity between DAscV and TAscVMN/01, which was about 44, 69 and 69 % in the three ORFs, respectively. In contrast, the identities were lower between DAscV and TAscV-1 in terms of the amino acid sequences in the three ORFs, and were approximately 39, 55 and 36 %, respectively. Similarly, the amino acid identities between TAscV-1 and the other two TAscV serotypes were approximately 33, 56–57 and 35–37 % in the three ORFs, respectively.

When the amino acid sequence of DAscV ORF2 was compared with those of the partially sequenced astroviruses, including HAscV-6 (Lee & Kurtz, 1994), HAscV-7, feline astrovirus (FAstV), porcine astrovirus (PAstV) (Jonassen et al., 2001), bat astrovirus (BatAscV) (Chu et al., 2008), ANV-2 (Imada et al., 2000), and TAscV-3 (Tang et al., 2005), it was found that DAscV was more closely related to TAscV-3 than to other astroviruses. The amino acid identity shared by DAscV and TAscV-3 was 71 %, which was slightly lower than that among TAscV-2, TAscV-3 and TAscVMN/01 (81–85 %), but much higher than that between TAscV-1 and TAscV-3 (34 %).

Based on the alignments above, a number of characteristic amino acid motifs were identified in DAscV. In ORF1a, a serine protease domain was predicted, containing a catalytic triad formed by histidine, aspartate, and serine (aa 616, 648 and 713). An NLS motif was also identified at aa positions 913–952. Further analysis of the amino acid sequence using the TMHmm program (http://www.cbs.dtu.dk/services/TMHHMM) suggested five possible transmembrane domains (aa 229–251, 398–415, 422–444, 459–481 and 486–508) in the N-terminal half of ORF1a. The 3723 nt sequence of ORF1a was the largest among the known astroviruses, which range from 2364 nt in AstV-MLB1 (Finkbeiner et al., 2008) to 3381 nt in TAscVMN/01 (Strain et al., 2008). The increased length of DAscV ORF1a compared with that of TAscVMN/01 was largely attributed to three insertions that contained a total of 100 aa located between the protease and NLS regions. The characteristic YGDD motif found in the RdRps (Jiang et al., 1993; Lewis et al., 1994) was encoded by DAscV ORF1b, beginning at nt 4864.

The ORF2 of DAscV was predicted to encode the structural proteins with a total length of 731 aa. The N-terminal half of the ORF2 protein has been previously shown to be more highly conserved among astroviruses than the C-terminal half, and has been proposed as the core assembly domain of the viral capsid (Krishna, 2005); this was also the case in DAscV. A highly conserved basic stretch in the N-terminal part of the capsid precursor of HAscV, FAscV, PAscV and TAscV-2 (Jonassen et al., 2001) was also present in the corresponding region of DAscV. The sequence of the basic region of DAscV was the most closely related to those of TAscV-2, TAscV-3 and TAscVMN/01. All four viruses contained an SR dipeptide (Jonassen et al., 2001), which was repeated six times in TAscV-2 and TAscV-3 and TAscVMN/01, but seven times in DAscV.

The sequence analyses of 5′-end in astrovirus genomes, as described previously by Jonassen et al. (2003), demonstrated that the highly conserved CCGAA motif in avian astroviruses was also conserved in DAscV. This motif was also found in the 23 nt space between the ORF1b stop codon and the ORF2 start codon of DAscV. Like TAscV-2, AstV-MLB1 and BatAscV, DAscV lacked a common RNA motif found at the 3′ end of the genomes of some other astroviruses (Chu et al., 2008; Finkbeiner et al., 2008; Jonassen et al., 1998).

To gain further insight into the evolutionary relationship of DAscV with other astroviruses for which corresponding sequences were available, phylogenetic trees were constructed using MEGA4.0 (Tamura et al., 2007) based on amino acid sequences from the three ORFs (Fig. 2). All the three trees (Fig. 2a–c) indicated that DAscV was the most closely related to some turkey astroviruses. Interestingly, DAscV clustered with TAscV-2, TAscV-3 and TAscVMN/01, whereas TAscV-1 formed another clade with ANV-1 and ANV-2 in the ORF2 tree (Fig. 2c). In particular, the distances between the capsid sequence of DAscV and those of TAscV-2, TAscV-3 and TAscVMN/01 were comparable to the distances between the eight HAscV types. In the ORF1a and ORF1b trees (Fig. 2a and b), the branching patterns of TAscV-1 showed minor differences from those observed in the ORF2 tree. However, DAscV always clustered together with TAscV-2 and TAscVMN/01, with TAscV-1 being an outgroup.

Comparison of the recently published partial polymerase sequences of DAscV-1 (DHV-2; GenBank accession no. EU669003) and DHV-3 (EU669004) (Todd et al., 2009) with the same region of DAscV C-NGB showed relationships of 93 and 64 % nt identity and 95 and 69 % aa identity, respectively, whereas DAscV-1 (DHV-2) and DHV-3 are only 64 and 69 % identical in their nucleotide and amino acid sequences. These observations suggest that DAscV C-NGB is probably an isolate of DAscV-1, although comparisons of their capsid sequences would be needed for conclusive proof.
Taken together, we found that the overall genome organization of DAstV was identical to that of known astroviruses. In addition, there were two unique features about the DAstV genome in comparison with other astroviruses. One feature was that the genome of DAstV was the largest among the astroviruses, and the other was that the ORF2 of DAstV was not in the same reading frame as ORF1a or ORF1b, as commonly observed in other astroviruses. Furthermore, the sequence comparisons and phylogenetic analyses revealed that DAstV was more closely related to TAstV-2, TAstV-3 and TAstV/MN/01 than to TAstV-1 or other astroviruses. These findings suggest that astroviruses may transmit across ducks and turkeys, which may be of help to the diagnosis and control of viral hepatitis in ducks.

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