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ORIGINAL ARTICLE

Molecular characterization of avian astroviruses

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Abstract Astroviruses are frequently associated with enteric diseases in poultry, being isolated from cases of runting-stunting syndrome (RSS) of broiler chickens, poult enteritis complex (PEC), and poult enteritis mortality syndrome (PEMS) of turkeys. Currently, five types of avian astrovirus have been identified: turkey astroviruses 1 and 2 (TAstV-1, TAstV-2), avian nephritis virus (ANV), chicken astrovirus (CAstV) and duck astrovirus (DAstV). The objective of this study was to molecularly characterize the different types of avian astroviruses circulating in commercial poultry. Sequence analysis of a region of ORF2, which encodes the capsid precursor protein associated with serotype and viral pathogenesis, revealed extensive variation in amino acid sequence within each subtype: TAstV-2 (81.5%-100%), ANV (69.9%-100%), and CAstV (85.3%-97.9%). However, this region was more conserved in TAstV-1's (96.2%-100%). Furthermore, a novel astrovirus was detected in chicken samples and found to be <64% similar to ANV and <30.6% similar to CAstV. The results of this study underline the great genetic variability of avian astroviruses and indicate that there are most likely multiple serotypes of each avian astrovirus circulating in commercial poultry.

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Introduction

Astroviruses have been associated with enteric disease in humans and young animals such as calves, lambs, pigs, deer, mice, dogs, cats, and minks [28]. In avian hosts, astroviruses have been linked to enteritis and increased mortality in young turkeys, chickens and guinea fowl [4, 5, 15, 18–20, 25, 34, 37, 40, 45, 46, 54, 60], to nephritis in chickens [17, 23], and to fatal hepatitis in ducklings [11, 13].

The family *Astroviridae* is divided into two genera: *Mamastrovirus* (mammal astroviruses) and *Avastrovirus* (avian astroviruses). Within each genus, numerous groups have been described based on serotype and genetic differences [1]. Astroviruses are small, non-enveloped, single-stranded, positive-sense RNA viruses of 28-30 nm diameter and have a star-like morphology [24]. Their genome is 6.4-7.7 kb in length and has three open reading frames (ORFs) and a poly-A tail [11, 28]. ORF1a and ORF1b encode the non-structural proteins, and ORF2 encodes a capsid precursor protein [28]. The greatest sequence variability in the astrovirus genome is found in ORF2; however, a high degree of conservation of the N-terminal 415 amino acids of ORF2 has been shown for HAstVs and other mammalian astroviruses. The avian astroviruses vary considerably from the mamastroviruses, although the basic character of the ORF2 amino terminus is

also conserved [28]. Downstream of the conserved 415 residues, considerable sequence variability is seen among astroviruses isolated from different species and even among different serotypes from the same animal species. Based on structural predictions, the conserved amino-terminal half of the astrovirus capsid protein could play a role in assembly of the capsid core, whereas the hyper-variable carboxy-terminal region could form the spikes of the virion and participate in the early interactions of the virus with the cellular receptors on the host cell [28].

Enteric diseases cause substantial economic losses of commercial turkeys and broilers due to reduced weight gain, increased morbidity and mortality, poor condition of the flock, and increased production costs from poor feed conversions and increased use of therapeutic anti-microbial treatments. Three enteric disease syndromes have been described: runting-stunting syndrome of broilers (RSS), poult enteritis complex (PEC), and poult enteritis mortality syndrome (PEMS). These diseases are considered to be polymicrobial, and numerous viruses have been associated with them; however, no definitive cause has been identified. Astroviruses are among the most common viruses found in cases of PEC and PEMS [2, 3, 19, 21, 35, 36, 38, 39, 60]. Astroviruses have also been isolated from recent cases of RSS in broilers and have been associated previously with poor weight gain, enteric disease and kidney disease in chickens [4, 12, 21, 41–43, 53]. To date, five different astroviruses have been identified in avian species based on the species of origin and viral genome characteristics: two turkey-origin astroviruses (TAstV-1 and TAstV-2); two chicken-origin astroviruses, avian nephritis virus (ANV) and chicken astrovirus (CAstV); and a duck-origin astrovirus (DAstV). ANV has also been detected in commercial turkey poults [30], and TAstV-2 in guinea fowl [5]. Molecular characterization of these viruses has been done mostly by sequencing ORFs 1a and 1b. However, with the exception of TAstV-2 viruses [31, 47], little or no characterization has been done of the ORF2 or capsid gene.

The study of the capsid protein genes is important for understanding the relatedness of these viruses. Antigenic characterization of avian astroviruses has been limited by the difficulty in isolating and growing these viruses. Currently, two serotypes of ANV and two of TAstV-2 have been described [17, 50], but sequence data suggest that there may be many more [47]. There also appears to be more than one serotype of CAstV [31, 55]. No serological characterization of TAstV-1 isolates has been reported.

In this study, we sequenced new astrovirus isolates from chickens and turkeys and analyzed a region of the ORF2 (capsid proteins) of all available astroviruses detected in poultry.

Material and methods

Viruses

The viruses sequenced were obtained from the Southeast Poultry Research Laboratory (SEPRL) repository of intestinal contents collected from commercial turkeys and chickens throughout the United States between 2003 and 2007 [32, 33]. Specimens were from both healthy flocks and poorly performing flocks (Table 1). All samples were stored at -80°C until utilized for RNA extraction.

Viral RNA extraction and RT-PCR

Two-hundred microliters of intestinal contents was diluted in 1.2 ml of PBS, homogenized with sterile glass beads in a FastPrep homogenizer (Thermo-Savant, Inc., Waltham, MA) and centrifuged for 10 min at 800 × g. Total RNA was extracted directly from 250 µl of the supernatant using TRIzol LS reagent (Invitrogen Inc., Carlsbad, CA) according to the manufacturer's instructions. For the initial detection and characterization of the viruses, a segment of the polymerase gene (ORF1b) was amplified by standard RT-PCR using a QIAGEN OneStep RT-PCR Kit (QIAGEN Inc., Valencia, CA). The primers, which amplify all four avian astroviruses, have been reported previously [51]. Sequence data for the capsid gene (ORF2) were obtained by designing primers based on published sequence data from reference isolates of TAstV-1 (Y15936.2), TAstV-2 (AF206663.2), ANV-1(AB046864) and ANV-2(AB033 998.1). All primers were designed using the PrimerSelect program (DNASTAR, Madison, WI). Sequence data from the capsid gene was also obtained by RT-PCR using an oligo dT primer and specific primers directed toward the polymerase gene. The variation found in the capsid sequence among the astroviruses studied was so high that more than 96 sequencing primers had to be developed in order to obtain sequence data. Rarely could one set of primers be used on more than one astrovirus, even if it belonged to the same type. Amplifications were performed in a MJ Research DNA Thermal Cycler (Waltham, MA). The RT-PCR products were separated on an agarose gel by electrophoresis, and amplicons of the appropriate size were excised from the gel and extracted using a QIAquick Gel Extraction Kit (QIAGEN Inc.). Purified RT-PCR products were sequenced directly using a BigDye Terminator Kit (Applied Biosystems, Foster City, CA) run on an ABI 3730 sequencer (Applied Biosystems). Sequences were assembled and edited with LASERGENE 7.2 (DNASTAR) and then aligned to previously published astrovirus ORF2 sequences.

Table 1 Chicken and turkey astroviruses examined in this study

Isolate name	Flock condition	Age in days	Genotype	GenBank accession number
NC-TK-SEP T1-489-05*	Healthy	28	TAstV-1	HQ185551
NC-TK-SEP T1-552-05	Healthy	36	TAstV-1	HQ185552
NC-TK-SEP T1-703-05	Healthy	84	TAstV-1	HQ185553
MO-TK-SEP T1-822-05	Healthy	15	TAstV-1	HQ185554
NC-TK-SEP T1-928-06	Healthy	14	TAstV-1	HQ185555
VA-TK-SEP T2-33-03*	PEC	22	TAstV-2	HQ185556
NC-TK-SEP T2-222-03*	Unknown	57	TAstV-2	HQ185557
TX-TK-SEP T2-311-04*	Unknown	Unknown	TAstV-2	HQ185558
CA-TK-SEP T2-269-05*	Enteric disease	8	TAstV-2	HQ185559
GA-CK-SEP-CA-457-05*	RSS	6	CAstV	HQ185560
DE-CK-SEP CA-651-05*	Poor	7	CAstV	HQ185561
DE-CK-SEP CA-811-05	RSS	10	CAstV	HQ185562
GA-CK-SEP-CA-364-05*	RSS	7	CAstV	HQ185563
MO-CK-SEP-CA-883-06	RSS	15	CAstV	HQ185564
DE-CK-SEP ANV-651-05*	Poor	7	ANV-2	HQ188692
DE-CK-SEP ANV-811-05	RSS	10	ANV-2	HQ188693
GA-CK-SEP ANV-368-05*	RSS	10	ANV-2	HQ188694
NC-TK-SEP ANV-537-05*	Healthy	56	ANV-2	HQ188695
GA-CK-SEP ANV-451-05*	RSS	11	ANV-2	HQ188696
NC-TK-SEP-ANV-670-05*	Healthy	84	ANV-2	HQ188697
GA-CK-SEP- ANV-364-05*	RSS	7	ANV-2	HQ188698
GA-CK-SEP- ANV-458-05	Healthy	11	ANV-2	HQ188699
GA-CK-SEP-ANV-792-05	RSS	Unknown	ANV-like	HQ185565

*Partial ORF1b reported previously [30, 31]

Sequence analysis

An 861-894-nucleotide region of ORF2 was used to evaluate phylogenetic relationships among avian astroviruses. This region corresponded to the following regions of the reference viruses: TAstV-1, nucleotides (nt) 5329-6204; TAstV-2, nt 5474-6340; ANV-1, nt 5054-5945; DAstV, nt 5844-6710. There is no full-genome sequence published for CAstV. The following capsid sequences from avian astrovirus published in GenBank were included in the phylogenetic analysis: TAstV-1 (Y15936.2); TAstV-2 (AF206663.2), AK/98, CA/00, CO/01, MN/01, MO/01, PA/01, MI/01, TX/00, VA/99 (EU143843.1 to EU143851.1), TAstV-1987 (AY769616.1), TAstV-2001 (AY769615.1); ANV-1 (AB046864); ANV-2 (AB033998.1); DAstV (FJ434664.1); HAstV-1 (L123513); HAstV-2 (Q82446.1); HAstV-3 (AAD17224.1); HAstV-4 (BAA93446.1); HAstV-5 (AAA56750.1); HAstV-6 (CAA86616); HAstV-7 (CAA 69922); HAstV-8 (AAF85964); Dog AstV (CAR82569.1); porcine AstV (CAB95000); sea lion TAstV (ACR54274.1); Bat AstV (EU847155); feline AstV (AAC13556.1); ovine AstV (Y15937); bottlenose dolphin AstV (ACR54280.1); mink AstV (AY179509). Also included was the sequence of CAstV-3 (US Patent 209009263) and GA-UGA_Ast1-Oct2006 (CAstV-UGA-2006, Patent WO/2010/059899), a CAstV isolated at the University of Georgia, Athens, GA, from a flock of chickens with RSS (GenBank accession number HQ185566).

The sequence information was compiled using the Seqman progam (Lasergene V. 6.01, DNASTAR, Madison, WI), and multiple alignments of nucleotide and amino acid sequences were constructed with the Megalign application of the same software package using the Clustal W alignment algorithm (Slow/Accurate, Gonnet). Pairwise sequence alignments were also performed with the Megalign program to determine nucleotide and amino acid sequence identity.

In addition, a 2.5-kb region of the ANV-2's corresponding to nt 3710 to 6569 of ANV-1, spanning part of ORF1b and ORF2, was analyzed using SimPlot software version 3.5.1 [22]. The polymerase-capsid region was aligned using Clustal W of the MEGA 4 software [49], and ANV-1 and GA-CK-SEP- ANV-364-05 were used as the query sequences for similarity analysis. Similarity was calculated in each window of 200 bp using the Kimura two-parameter method.

Phylogenetic trees

Sequences were aligned with Clustal W (Lasergene, V. 8.0.2 DNAStar, Madison WI). Nucleotide trees were

constructed with merged duplicate runs of BEAST v. 1.4.8 [9] using HKY substitution, empirical base frequency, gamma heterogeneity, codon 2 partitions, relaxed lognormal clock, Yule process tree prior with default operators with unweighted pair group mean with arithmetic average starting tree, and a Markov chain Monte Carlo length of 10^6. Protein trees were constructed with the same program and parameters, except the Blosum 62 substitution matrix and the gamma + invariant site heterogeneity model were used.

Sequence accession numbers

The assigned GenBank accession numbers for the sequences generated from the capsid region of TAStV-1, TAstV-2, CAstV, and ANV viruses are HQ185551-65, and the ANV sequences encompassing ORF1b and capsid are HQ1888692-99 (Table 1).

Results and discussion

The capsid precursor protein gene is the most variable astrovirus gene, containing sequence differences that certainly reflect variation in antigenicity and possibly also in pathogenicity [45]. In this study, we compared the nucleotide and predicted amino acid sequences of a section of the hypervariable region of the capsid (ORF2) of 19 avian astroviruses from chickens and turkeys, along with capsid gene sequences of 22 other avian astroviruses retrieved from the database. Pairwise comparisons of the nucleotide and amino acid sequences of the ORF2 region of the viruses analyzed in this study showed great variability among all groups of avian astroviruses, with the exception of TAstV-1, which had between 96.2% and 100% amino acid identity (Table 2). TAstV-2 had between 81.5% and 100% amino acid identity, and CAstV had between 85.3% and 97.9% amino acid identity. ANV strains were most closely related to the reference ANV-2 strain, with 85.8%-100% amino acid identity among them, and 69.9% to 74.0% amino acid identity with ANV-1. One astrovirus detected in chickens (GA-CK-SEP ANV-792-05) did not group with any of the known avian astroviruses but was most closely related to ANV-1, with which it had 64% amino acid identity. DAstV was closest to TAstV-2 in amino acid sequence similarity, with 72.8%-75.7% identity. This is interesting, considering that these two viruses differ in their genome organization [11].

The amount of variation observed in most of the avian astroviruses groups studied suggests that there is antigenic variation among them, since for HAstV, Walter *et al.* [56] found that when two strains had less than 95% nucleotide homology, they could be distinguished serologically. TAstV2001 and TAstV1987 have been reported to represent distinct serotypes, sharing only 82.8% amino acid sequence identity for the complete capsid region [52]. This level of sequence conservation is close to that of HAStV capsid genes from different serotypes (<80% nt similarity). These sequence distances suggest that many of the astroviruses studied may represent different serotypes in each group. However, in order to corroborate this, serological cross-reactivity analysis of the viruses still needs to be conducted. Similar remarkably high genetic diversity has also been observed within bat astroviruses [6, 61].

The degree of similarity at the amino acid level to mamastroviruses in the region examined was 20.7%-29.3% for TAstV-2, 21.1%-26.7% for TAstV-1, 20.3-28.9 for CAstV, and 22.4%-27.9% for ANV. The mamastroviruses analyzed were 31.4% to 61.2% similar among themselves,

Table 2 Range of percent amino acid and nucleotide (*italics*) identity among avian astrovirus isolates analyzed in this study. One ANV-1, nine ANV-2, seven CAstV, six TAstV-1, fifteen TAstV-2, one DAstV, and isolate ANV792 viruses were compared

	ANV-1	ANV-2	CAstV	TAstV-1	TAstV-2	DAstV	ANV792
ANV-1	-	69.9-74.0	31.6-33.7	30.4-38.1	28.6-31.7	28.1	64.0
	-	*66.0-67.5*	*42.9-44.8*	*45.6-46.0*	*42.6-45.2*	*42.0*	*65.1*
ANV-2		85.8-100	29.4-33.7	28.6-39.2	29.4-33.7	26.1-29.9	61.5-64.4
		85.4-98.2	*42.7-47.2*	*45.8-48.0*	*42.7-47.2*	*42.8-44.9*	*61.9-63.6*
CAstV			85.3-97.9	38.7-42.9	39.0-42.0	41.3-42.4	29.5-30.6
			74.0-91.8	*49.2-51.0*	*48.2-53.2*	*48.5-50.0*	*40.7-43.5*
TAstV-1				96.2-100	45.0-48.2	45.3-45.9	29.3-37.2
				95.5-100	*52.1-54.6*	*51.2-51.6*	*45.5-46.4*
TAstV-2					81.5-100	72.8-75.7	28.2-45.2
					81.4-100	*65.2-70.1*	*43.7-45.2*
DAstV						-	29.2
						-	*42.5*

with the exception of human and feline astroviruses, which were > 72.6% similar (data not shown).

Phylogenetic analysis based on the partial ORF2 amino acid sequences shows that the avian astrovirus form four groups (Fig. 1): TAstV-1-like, TAstV-2-like, CAstV-like and ANV-1-like viruses, similar to previous reports analyzing ORF1b sequences [30]. The phylogenetic relationships of the nt sequences were similar to those of the aa sequences. The ANV group contained both chicken-origin and turkey-origin isolates. The TAstV-1, TAstV-2 and CAstV groups contained only turkey and chicken-origin viruses, respectively. The TAstV-2 group was distant from TAstV-1 and was closely related in this region to DAstV. A phylogenetic tree based on amino acid sequences of the ORF2 region of the avian and selected mammalian astroviruses shows that the avian astroviruses form distinct groups consistent with the nucleotide tree and are separate from the mammalian isolates (Fig. 2). The mammalian isolates vary widely in their similarity to one another. The HAstV isolates clustered together with the feline astroviruses on a branch that was distant from other astrovirus groups. Several other isolates formed species-specific branches, including the porcine, canine, bottlenose dolphin and sea lion astroviruses. Conversely, some disparate isolates including mink, ovine and bat astroviruses clustered together.

A 2.5-kb nucleotide sequence spanning a region of the polymerase and capsid genes of eight ANV was also analyzed by comparison to the ANV-1 reference strain. The SimPlot computer program [22] was used to analyze the alignment of the eight ANV using a window size of 200 nucleotides that was moved along in 20-nucleotide steps. The percent identity was calculated for each window and plotted in a line chart. Figure 3 shows that the ANV-1 reference strain shared a lower level of sequence identity in the capsid region with the ANV analyzed, whereas, based on the phylogenetic analysis, these viruses were more closely related to ANV-2, a different ANV serotype. Because there are no sequence data available for the ORF1b of the ANV-1 virus, it was not included in the analysis. Alignment of the ORF1b-ORF2 region sequences of eight ANV2 strains showed that they shared high levels of sequence identity in the conserved domain (N-terminal 415 amino acids), whereas the carboxy-terminus of ORF2 showed higher levels of variation (Figure 4). This is consistent with results reported previously for HAstV [14, 27, 57]. Recent work by Strain *et al.* involving the analysis of nine complete turkey astrovirus genomes provided evidence for recombination between astrovirus genomes and

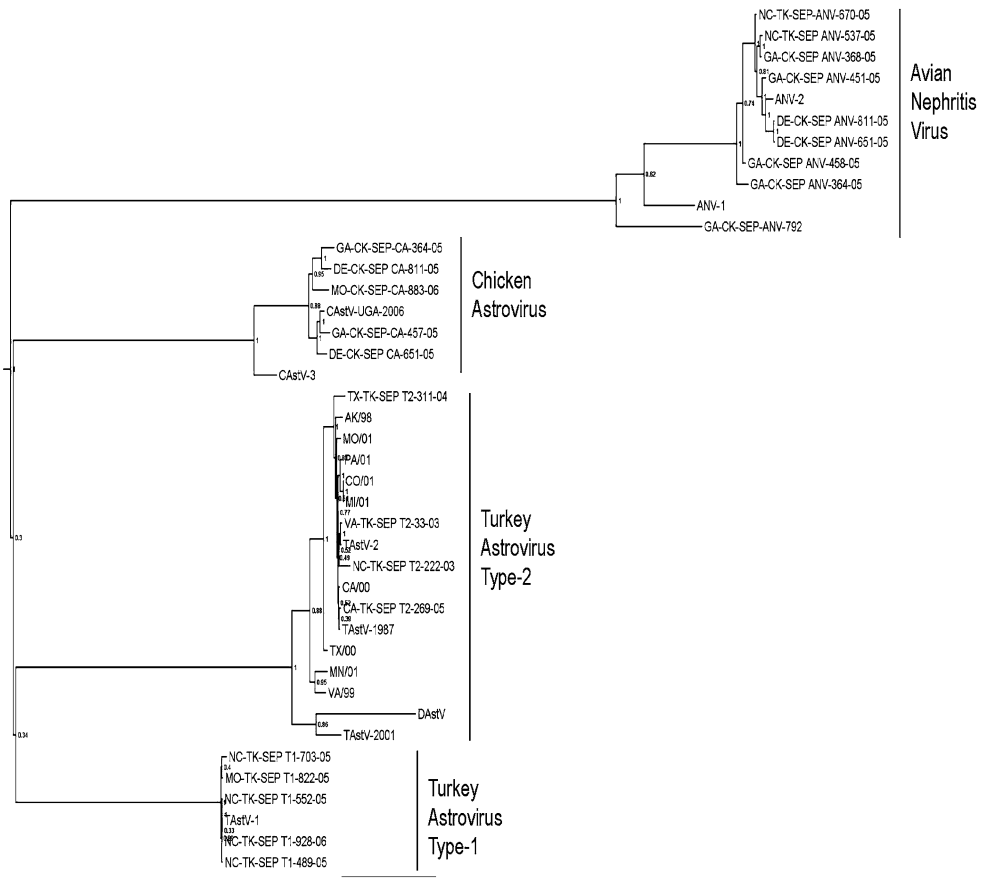

Fig.1 Phylogenetic tree of the nucleotide sequences of avian astrovirus capsid genes. Node posterior values are given

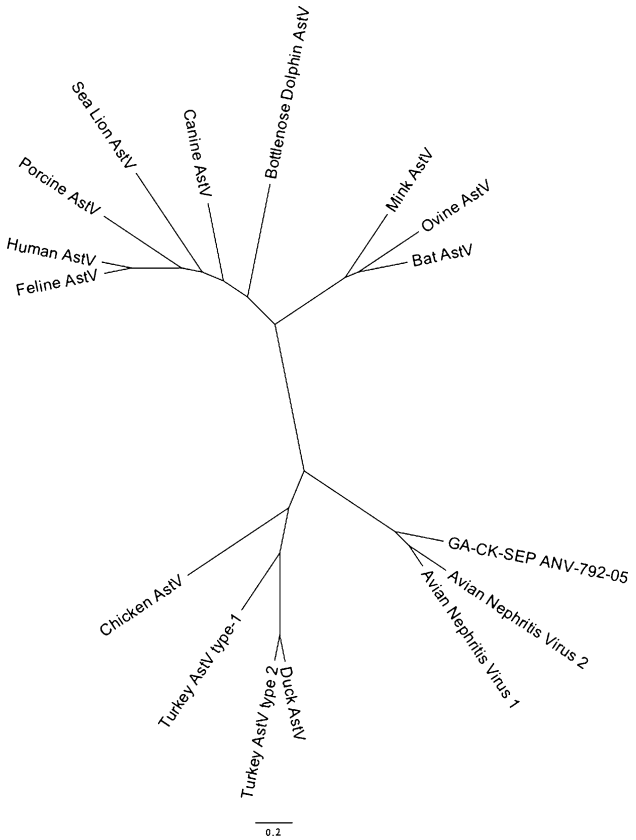

Fig. 2 Unrooted phylogenetic tree of the amino acid sequences of phylogenetically representative isolates from different species groups

supported the view that sequences from different regions of the astrovirus genome should be considered for phylogenetic analysis on which taxonomic subdivisions might be based [47]. With recombination, it is possible that different ANV-1 ORF1b sequences could be contiguous with ANV-1 capsid protein sequences, or conversely, they could have different ORF2 sequences, as was observed with the ANV-2 isolates analyzed. These data suggest that potential recombination could occur within ORF2, resulting in the emergence of new viruses.

Astroviruses appear to be widely disseminated and endemic in poultry in the US, and concomitant infection of flocks with two or more enteric viruses is common [19, 30, 32, 33]. The high incidence of astroviruses in turkeys affected with enteric disease has been documented previously [37]. Astroviruses have also been found in turkeys with poult enteritis mortality syndrome (PEMS), a disease that has caused severe economic losses to the turkey industry [2, 3, 35, 59, 60]. Although TAstV-2 is frequently detected in turkey poults, it is not clear how many serotypes exist. Phylogenetic analysis of TAstV-2 isolates revealed a high level of genetic variation, particularly in the capsid gene, suggesting the possibility of several serotypes [31, 47, 52]. Tang and Saif antigenically characterized two TAstV-2 isolates, TAstV1987 and TAstV2001, showing that they belonged to different serotypes [50], and in our analysis, these viruses are clearly of different genotypes. Until more serology is done to determine how many TAstV-2 serotypes exist, genetic characterization of the virus serves as a useful alternative for type assignment of avian astroviruses and permits the characterization of the astroviruses circulating in poultry. TAstV-1 strains, on the other hand, appear to be more closely related to each other and are less commonly detected on turkey farms. The reason for this is not clear, but it could be due to sample bias or real biological features of the virus.

Avian nephritis virus (ANV), previously classified as a member of the genus *Enterovirus*, family *Picornaviridae*, has been isolated from chickens and was re-classified as an astrovirus following the complete sequencing of the viral genome [17]. This virus was first isolated in 1976 and is associated with growth depression and kidney lesions in young chickens [17]. Avian nephritis virus has been shown to be widely distributed in chicken flocks in Japan [16], some European countries [7, 8, 29], and even in specific-pathogen-free flocks [26]. Under field conditions, clinical signs associated with this virus infection in broiler chickens have varied from subclinical to outbreaks of RSS and baby chick nephropathy [10, 16, 23, 42, 44, 48, 58]. At least two serotypes of ANV and different pathotypes have been reported [43, 54]. Based on sequence comparisons of part of the non-structural protease gene (ORF 1a), ANV could be grouped into three genotypes [23]. ANV has also been reported in turkeys [30], and antibodies to ANV have been detected in turkeys in Northern Ireland and England, but nothing has been reported about clinical signs of infection [7, 29]. The ANVs analyzed in this study, although closely related to ANV-1 in ORF1b, were mostly ANV-2-like based on ORF2, indicating the possibility of recombination. It is not clear if the GA-CK-SEP ANV-792-05 is in fact an ANV. Further genetic analysis of these viruses is required.

A second type of chicken astrovirus was shown to be antigenically and genetically distinct from ANV [4]. Although the sequence of this virus is still not available, we will refer to it as the reference CAstV (it has been patented as CAstV-2). CAstVs sharing high levels of nucleotide sequence identity with this first characterized CAstV have been detected in the United States in broiler chickens affected with RSS [30]. Recently, molecular and antigenic characterization of entero-like viruses (ELVs) demonstrated that they were CAstV, some of them closely related to the reference CAstV, and some of them more distantly related [54]. CAstV was detected by serology in broiler flocks in the United Kingdom, the Netherlands, Spain, Australia, and the United States [4]. Serological evidence also indicated that CAstV infections were common in

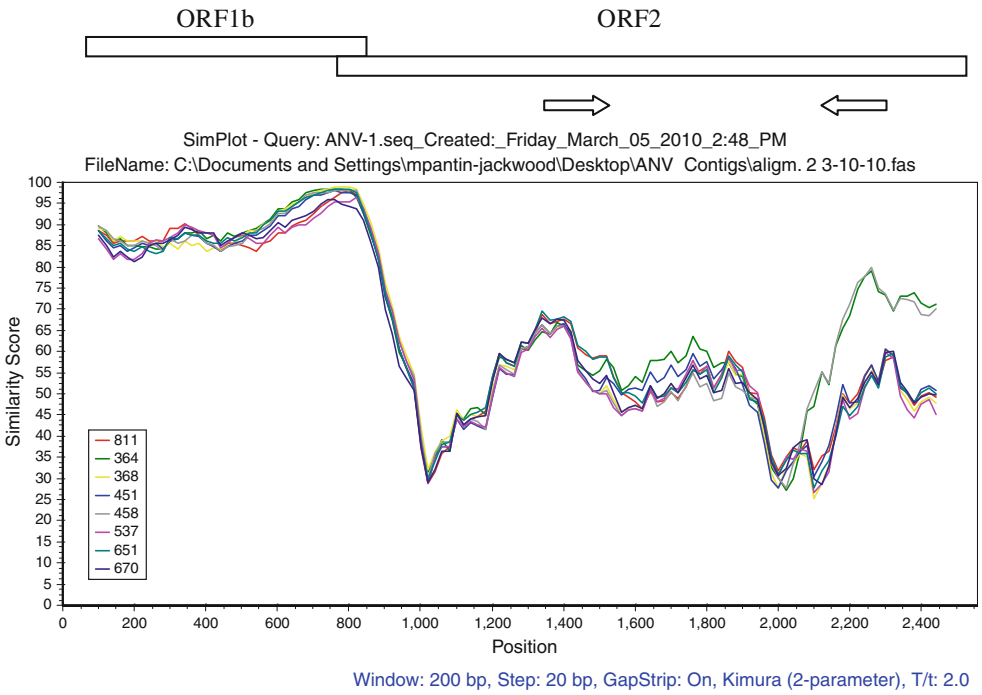

Fig. 3 Similarity analysis of ANV based on partial sequences of ORF1b and ORF2. Nucleotide identity plot of the 2.5 kb region comprising the amplified polymerase and capsid regions of the following ANVs compared with ANV-1: DE-CK-SEP ANV-651-05 (651), DE-CK-SEP ANV-811-05 (811), GA-CK-SEP ANV-368-05 (365), NC-TK-SEP ANV-537-05 (537), GA-CK-SEP ANV-451-05 (451), NC-TK-SEP-ANV-670-05 (670), and GA-CK-SEP-ANV-364-05 (364). The bar above the plot represents the regions corresponding to ORF-1b (polymerase) and ORF-2 (capsid). The arrows indicate the region amplified from the capsid gene and used in the phylogenetic analysis

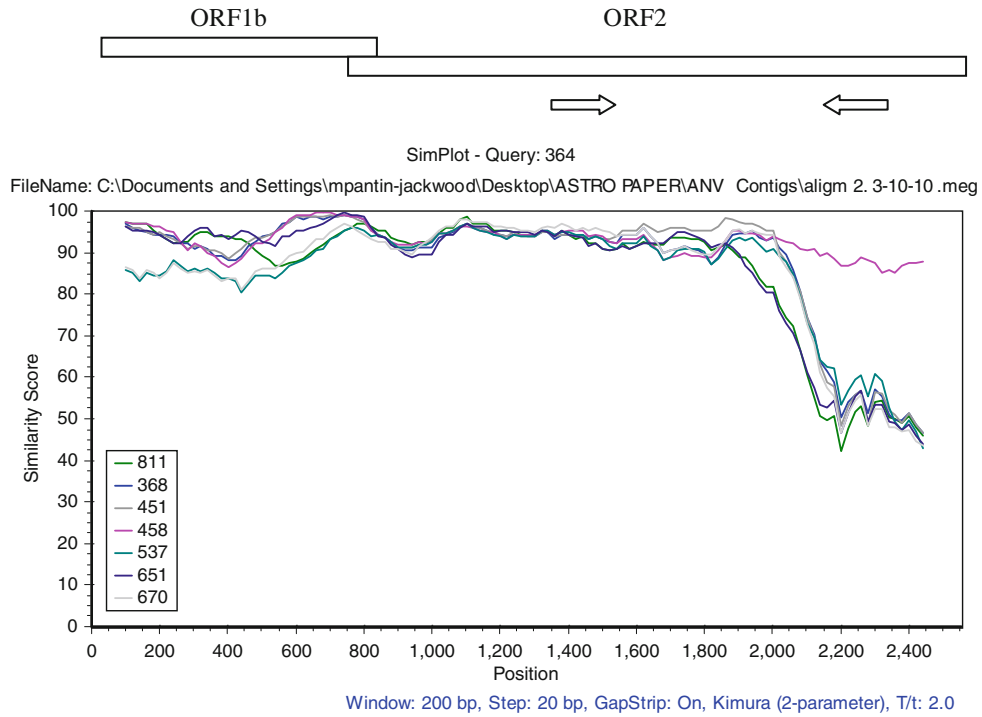

Fig. 4 Similarity analysis of ANV based on partial sequences of ORF1b and ORF2. Nucleotide identity plot of the 2.5-kb region comprising the amplified polymerase and capsid regions of the following ANVs: DE-CK-SEP ANV-651-05 (651), DE-CK-SEP ANV-811-05 (811), GA-CK-SEP ANV-368-05 (365), NC-TK-SEP ANV-537-05 (537), GA-CK-SEP ANV-451-05 (451), NC-TK-SEP-ANV-670-05 (670), and GA-CK-SEP-ANV-364-05 (364). The query isolate used was isolate 364. The bar above the plot represents the region corresponding to ORF-1b (polymerase) and ORF-2 (capsid). The arrows indicate the region amplified from the capsid gene and used in the phylogenetic analysis

broiler parent flocks within the United Kingdom and also appeared to be widespread in European breeder flocks [55]. CAstV was commonly detected by RT-PCR in broiler flocks with growth problems or ones that were affected by RSS [30, 45, 55]. Phylogenetic analysis based on a region of the ORF1b demonstrated the existence of two distinct clades [30, 45]. In this study, the CAstV isolates grouped separately from CAstV-3 and also formed two different groups (Fig.1). The complete genome of CAstV has not been published, so many question related to the genome structure and how they relate to other avian astroviruses remain to be answered.

A virus producing hepatitis in ducks (DHV-2) was identified to have astrovirus morphology and was renamed

duck astrovirus 1 (DAstV-1) [1, 13]. Sequence analysis of the ORF 1b region of this virus showed that it was closely related to CAstV. A second virus, DHV-3, was also identified as an astrovirus, but based on the amino acid identities in the ORF 1b region of this virus with that of DHV-2 (69%) it appears that they might be of different genotypes, and DHV-3 is more closely related to TAstV-2 [54]. The complete sequence of a duck astrovirus (DAstV) associated with an outbreak of fatal hepatitis in ducklings in China was published recently, and phylogenetic analysis revealed that DAstV was most closely related to TAstV-2 [11]. Our results also corroborate this.

A new taxonomy proposal for avian astroviruses has been submitted to the International Committee on Taxonomy of Viruses (ICTV) for approval (http://talk.ictvonline.org/files/proposals/taxonomy_proposals_vertebrate1/m/vert01/2358.aspx). This proposal is the result of the work done in agreement by all members of the *Astroviridae* Study Group during the preparation of the 9th ICTV Report. Based on new data, the *Astroviridae* Study Group states that a classification based on genetic criteria is more appropriate than classifying the viruses into species within the genus *Avastrovirus* based only on the host of origin. A phylogenetic analysis of avastroviruses based on the amino acid sequence of the full-length ORF2 results in three groups (genotype species). Mean amino acid genetic distances (p-dist) range between 0.576-0.742 and 0.204-0.284 between and within groups, respectively. This new classification establishes three new species within the genus: *Avastrovirus* GI.A, including turkey astrovirus 1; *Avastrovirus* GI.B, including avian nephritis virus 1 and 2; and *Avastrovirus* GII.A, including turkey astrovirus 2 and duck astrovirus. Some chicken astroviruses for which only partial sequences are available (chicken astrovirus 2, DQ324850), or no sequences at all, are deposited in GenBank (chicken astrovirus 3) and would be listed as "related viruses that might be members of the genus *Avastrovirus* but have not been assigned to a species".

As more sequence data become available, a clearer picture will emerge with regard to avian astroviruses. This will have to be complemented by serological and biological characterization of these viruses.

In conclusion, comparative analysis of avian astrovirus capsid genes revealed extensive genetic variation and the presence of distinct genotypes of TAstV-2, ANV and CAstV circulating in poultry. Changes in the capsid protein induced by mutations or recombination most likely have an effect on the pathogenicity and antigenicity of the viruses, and consequently, practical implications for virus detection methods, epidemiological studies and development of potential vaccines against astrovirus infections.

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