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Identification of a novel astrovirus in domestic sheep in Hungary

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Abstract

The family *Astroviridae* consists of two genera, *Avastrovirus* and *Mamastrovirus*, whose members are associated with gastroenteritis in avian and mammalian hosts, respectively. We serendipitously identified a novel ovine astrovirus in a fecal specimen from a domestic sheep (*Ovis aries*) in Hungary by viral metagenomic analysis. Sequencing of the fragment indicated that it was an ORF1b/ORF2/3′UTR sequence, and it has been submitted to the GenBank database as ovine astrovirus type 2 (OAstV-2/Hungary/2009) with accession number JN592482. The unique sequence characteristics and the phylogenetic position of OAstV-2 suggest that genetically divergent lineages of astroviruses exist in sheep.

Keywords

sheep astrovirus; domestic sheep; feces; species

The family *Astroviridae* consists of small, non-enveloped viruses with a single-stranded positive-sense RNA genome, which ranges in size from 6.4 to 7.3 kb. The astrovirus genome has three open reading frames (ORFs). ORF1a encodes the non-structural polyprotein 1a, while the longer ORF1b encodes polyprotein 1ab, including the RNA-dependent RNA polymerase (RdRp), which is expressed through a ribosomal frameshift at the ORF1a/1b junction. ORF2 encodes the viral capsid structural polyprotein [13].

The family *Astroviridae* consists of two genera, *Mamastrovirus* and *Avastrovirus*, which are known to infect mammalian (humans, cheetahs, calves, pigs, sheep, deer, minks, dogs, kittens, mice, bats) and avian (ducks, chickens and turkeys) hosts, respectively. Astroviruses are reported to cause gastroenteritis in humans and some mammals; however, avian strains have been linked to both intestinal and extra-intestinal manifestations [7, 13]. In humans, eight classical human astrovirus types (HAstV1-8), all members of the same species, are known; however, novel and diverse groups of human astroviruses have been found recently [3, 9]. Ovine astrovirus was first identified by electron microscopy in 1977 [18] and structurally described in 1981 [5]. The nucleotide sequences of the capsid region (ORF2) and, subsequently, the complete genome were determined in 2001–2003 [7, 8]. Until now, only this single astrovirus (Y15937) from Scotland has been detected in sheep [8]. In

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experimental infections, this prototype virus caused mild diarrhea in 2-day-old gnotobiotic lambs [17].

This study describes the identification and genetic characterization of a member of a candidate novel astrovirus species in domestic sheep in Hungary.

Fecal samples were collected from 8 and 9 young, healthy, domestic sheep (*Ovis aries*) aged less than 3 weeks from a farm located in central Hungary in March 2009 and April 2010, respectively. At this farm, merino ewes from Hungary were mated with blackhead meat rams from Germany. One fecal sample (TB3) collected in March 2009 was selected for viral metagenomic analysis. PBS-diluted specimens were passed through a 0.45-μm sterile filter and centrifuged at 6,000 × *g* for 5 min. The pellet was mixed with a mixture of nucleases to enrich for particle-protected nucleic acids [20]. Nucleic acids were extracted using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Viral RNA and DNA nucleic acid libraries were constructed by sequence-independent random RT-PCR amplification as described previously [20]. Pyrosequencing using 454 GS FLX technology was then performed as described previously [9, 20]. The pyrosequencing reads and assembled sequence contigs were compared to the GenBank nucleotide and protein databases using BLASTn and BLASTx, respectively. For the determination of the nucleotide sequence of the novel ovine astrovirus by RT-PCR, oligonucleotides covering conserved regions of human and animal astroviruses and the available ovine astrovirus nucleotide sequences were used to extend the ovine astrovirus sequence by the long-range PCR, 5′RACE and primer-walking methods. Fecal samples were also screened for novel ovine astrovirus using sequence-specific primers (TB3-AstV-F, 5′-AAGCACACTGACGCCACAC and TB3-AstV-R, 5′-CATGAACCACCAGCCACC) amplifying a 516-nt-long region of ORF2 at an annealing temperature of 55°C. PCR products were sequenced directly using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, UK) using the PCR primers and run on an automated sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, Stafford, USA). Phylogenetic analysis was conducted using MEGA version 5.0 [19]. The 2474-nucleotide-long ORF1b/ORF2/3′UTR sequence of ovine astrovirus type 2 (OAstV-2) strain Hungary/2009 was submitted to GenBank under accession number JN592482.

Using viral metagenomic analysis, two amino acid sequence hits were identified by GenBank BLASTx in sample TB3 for astrovirus: a partial 141-nt/47-aa-long ORF1b sequence contig (LSMLILSHNDPPDSPLRCYVRRCVDILRARVGSDLPVFSERILSYLW), which showed 38% amino acid (aa) identity to bat astrovirus strain Tm/Guangxi/LD77/2007 (FJ571066) and a partial, 128-nt/42-aa-long astrovirus ORF2 capsid sequence contig (GARKHTDATPGRRVEFRLGRRDLQGPRDGWWYTDTSNGVQS), which showed 57% aa identity to human astrovirus 1 strain GGH-2004 (AY590262). The entire 2474-nt-long continuous sequence of the partial ORF1b (185 nt) and the complete ORF2/3′UTR (2305 nt) of the ovine astrovirus strain Hungary/2009 was characterized. The putative ORF2 consisted of 2,214 nt (737 aa). The nt and aa distances based on the N-terminal half and complete capsid (ORF2) regions between ovine astrovirus strain Hungary/2009 and the reference astroviruses are shown in Table 1. The highly conserved consensus ORF1ab/ORF2 junction and promoter astrovirus sequence UUUGGAGNGGNGGACCNAAN$_{4\text{-}11}$AUGNC initiating ORF2 (where the ORF2 AUG start codon is underlined; N stands for any of the four nucleotides) is present in ovine astrovirus strain Hungary/2009. Just before the AUG start codon, this region includes eight nucleotides (N_8) AAGAGATG (compete sequence motif: UUUGGGGGGGAGGACCAAAN$_8$AUGGC). The N-terminal half (384 aa) of ORF2 with multiple basic Arg (R) residues – 9 (47%) of the first 19 aa – was found to be related (35–

36% in aa) to human astroviruses, with the highest identity to HAstV-6 (HM237363); however, the C-terminal half is highly different. In the complete ORF2, the highest aa identities (22–23%) were found with deer astroviruses (HM447045 and HM447046). The ORF2 protein of ovine astrovirus strain Hungary/2009 is shorter by 25 aa than the known ovine astrovirus (Y15937), and the aa identity between them in the capsid region is 18%. A highly conserved stem-loop-II-like (s2m) nucleotide sequence is present in ovine astrovirus strain Hungary/2009 at the boundary of ORF2/3′UTR (Fig. 1A). However, the s2m amino acid motif SRGHAE – which is thought to be a characteristic protein sequence at the carboxy-terminal end of the ORF2 (capsid) region of astroviruses – is in a different frame (frame 3) in sheep astrovirus. The predicted stop codon (TGA) is located at the beginning of the stem-loop-II-like motif (Fig. 1B). The 3′UTR is 91 nt long.

Phylogenetic analysis confirmed that ovine astrovirus strain Hungary/2009 forms a genetic lineage that is distinct from the previously reported ovine astrovirus capsid sequence (Fig. 2). Ovine astrovirus strain Hungary/2009 is phylogenetically basal to astrovirus strains identified in human (strain MLB-1), rat, porcine and deer (Fig. 2). Maintaining the continuity of the current nomenclature, ovine astrovirus strain Hungary/2009 was provisionally named ovine astrovirus type 2 (OAstV-2) (Fig. 2). OAstV-2 was not detected by RT-PCR using a specific OAstV-2 astrovirus primer pair from the other 16 fecal samples collected from domestic sheep less than 3 weeks old at the same farm.

Recent studies have characterized novel astroviruses in humans [3, 9], bats [2], California and Steller sea lions [15], bottlenose dolphins [15], deer [16] and rats [1], indicating that astroviruses have a wide range of host species. Interestingly, multiple lineages of astroviruses have been identified in humans (classical HAstV, HAstV-MLB and HMOAstV), and these are phylogenetically separated by different lineages of animal astroviruses [3, 10]. This means that human astroviruses are highly diverse genetically, and each lineage is likely to have an independent origin [3, 10]. Divergent astrovirus lineages can also infect the same animal species, for example, bats [2, 21], turkeys [4], domestic pigs [12, 14] and sea lions [11]. Only one ovine astrovirus sequence from sheep has been published [8]. In this study, we detected a novel ovine astrovirus (OAstV-2), indicating that genetically different lineages of astroviruses also exist in domestic sheep.

A highly conserved stem-loop-II-like (s2m) motif has been found at the boundary of ORF2/3′UTR of mamastroviruses and avastroviruses, equine rhinoviruses, coronaviruses and dog noroviruses [2, 10]. s2m is thought to be an universal feature of astroviruses [6]. In addition, the stop codon of the HAstV capsid protein precursor gene is thought to be located at the top of the s2m structure. Interestingly, this motif was not recognized in recently identified astroviruses, including human astrovirus MLB1 [3], bat astrovirus AFCD337 [2] and porcine astrovirus type 2 [14]. Ovine astrovirus strain Hungary/2009 has an s2m motif; however, the predicted stop codon is located at the beginning of the structure as in turkey astrovirus 1 (Y15936). These data indicate that s2m is an important but not universal feature of astroviruses and that the stop codon can be in different positions in s2m in different astroviruses.

The genetic diversity and clinical impacts of astroviruses – especially in humans – is being gradually determined. However, results from the previous three years indicate that our knowledge is far from complete. This is true of both the diversity of the astroviruses –in both animal and human hosts - and the clinical spectrum of astrovirus infection. It is clear that astroviruses have a broader spectrum of host species as well as higher genetic and antigenic diversity that previously thought. In addition, members of multiple astrovirus species can exist in the same host species. Continued characterization of astrovirus diversity

in different host species will help our understanding of their origin and of their possible cross-species transmission.

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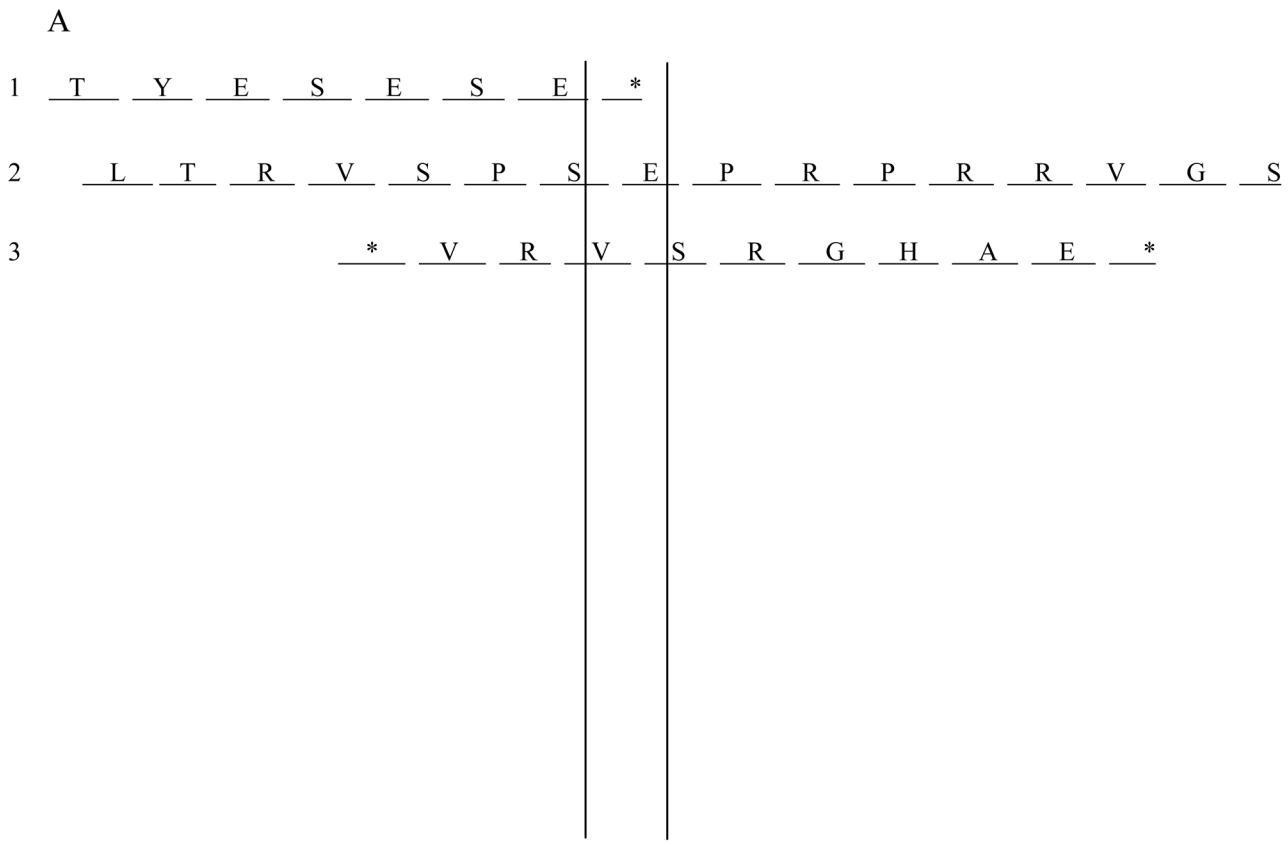

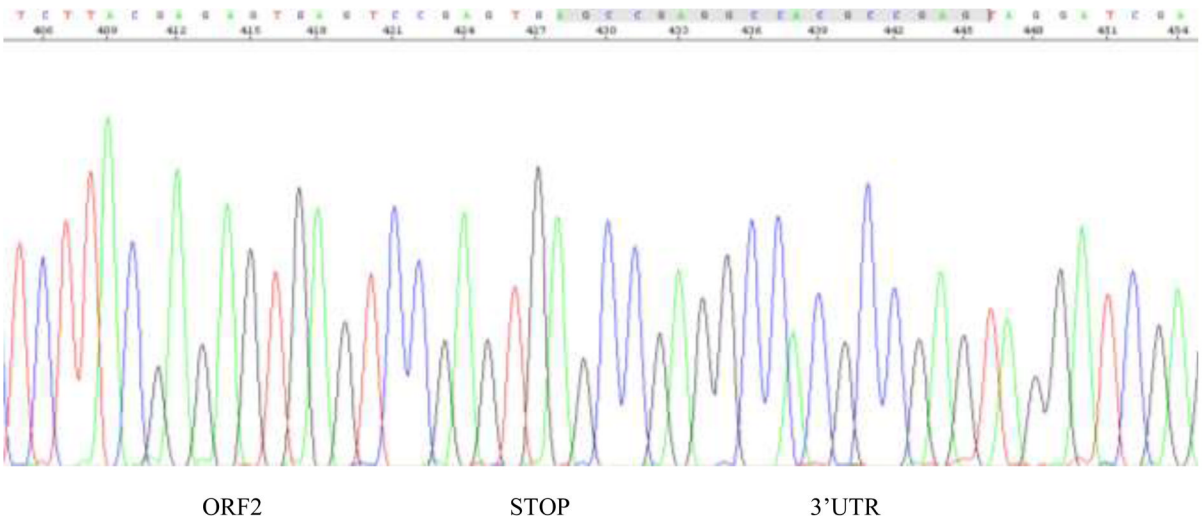

B

Fig. 1.

(A) Translation of the highly conserved stem-loop-II-like nucleotide sequence (in shaded box) region of sheep astrovirus in three frames (1, 2 and 3) at the boundary of ORF2/3′UTR. The amino acid motif SRGHAE, which is thought to be a characteristic amino acid sequence at the carboxy-terminal end of the ORF2 (capsid) region of astroviruses, is translated in a different reading frame (frame 3) in sheep astrovirus. The predicted coding sequence is represented in frame 1. (B) Predicted RNA secondary structure of the stem-loop-II-like nucleotide sequence of sheep astrovirus as determined using the Mfold program. The position of the predicted stop codon (TGA) is indicated by shaded boxes.

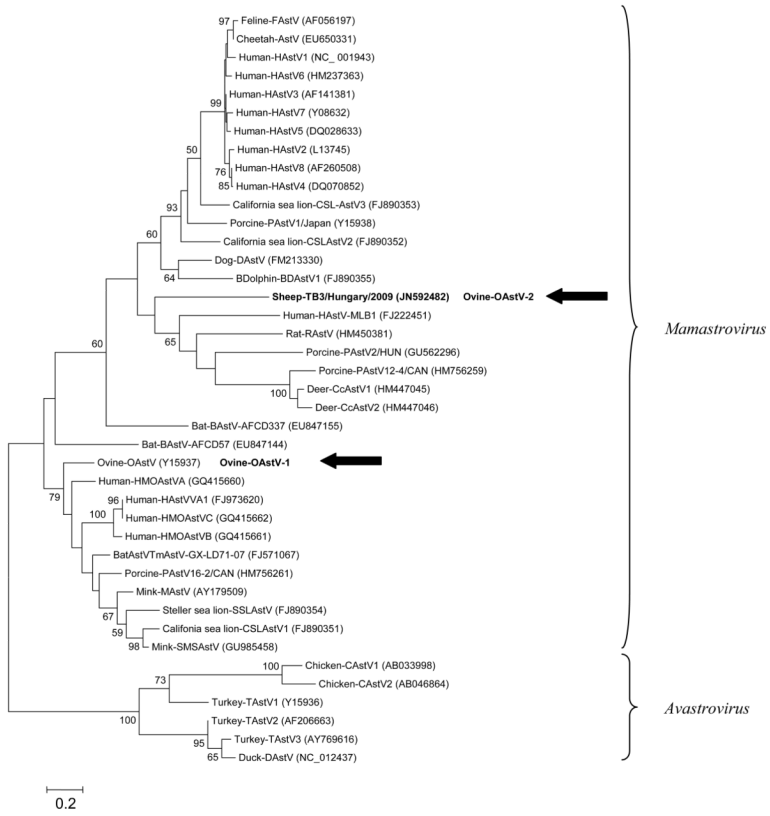

Fig. 2.

Phylogenetic analysis of the complete amino acid sequence of ORF2 of ovine astrovirus type 2 (OAstV-2) strain Hungary/2009 (JN592482). The ORF2 region of a representative of each species belonging to the mammalian and avian astrovirus genera *Mamastrovirus* and *Avastrovirus*, respectively, was obtained from GenBank and used as a reference. Any astroviruses detected in sheep are indicated by black arrows. The tree was constructed using the neighbour-joining method implemented in MEGA4. The novel ovine astrovirus is indicated by bold letters. Bootstrap values >50% are shown at the branches.

Table 1

Nucleotide (nt) and amino acid (aa) sequence identity in percentage or range of percentage (%) based upon the N-terminal (1152-nt/384-aa-long) and complete capsid (ORF2) regions of ovine astrovirus type 2 (OAstV-2) strain Hungary/2009 (JN592482) (columns) and reference astroviruses (rows). Boldface numbers indicate the highest level of nucleotide and amino acid identity.

Astrovirus reference strain(s)	**Ovine astrovirus strain Hungary/2009 (JN592482)**	
	ORF2 (N-terminal) nt/aa (%)	**ORF2 (complete) nt/aa (%)**
Ovine-OAstV (NC_002469)	42/31	32/18
Porcine-PAstV (GU562296; HM756259; HM756261)	39–41/29–31	27–31/18–22
Human-HAstV 1–5 and 8 (NC_001943; L13745; AF141381; DQ070852; DQ028633; AF260508)	42–**43**/35–**36**	31–**33**/19–21
Human-HMOAstV (GQ415660)	40/31	31/19
Human-HAstV-MLB1 (FJ222451)	38/29	31/20
Bat-BAstV-1 (EU847155)	42/33	30/18
Deer-DAstV (HM447045; HM447046)	41–42/32	32/22–**23**
Rat-RAstV (HM450381)	41/32	30/20
Mink-MAstV (NC_004579)	40/30	31/18
Avian-AAstV (CAstV: NC_003790); DAstV: NC_012437; TAstV-1: Y15936)	35–37/21–24	29–31/14–17