Arch Virol (2012) 157:1143–1147
DOI 10.1007/s00705-012-1272-4

BRIEF REPORT

Astrovirus in wild boars (*Sus scrofa*) in Hungary

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Received: 13 January 2012 / Accepted: 25 January 2012 / Published online: 8 March 2012

Abstract The family *Astroviridae* consists of two genera, *Avastrovirus* and *Mamastrovirus* whose members are associated with gastroenteritis in avian and mammalian hosts, respectively. In this study, we report the first detection of astrovirus from fecal specimens of wild boars (*Sus scrofa*) using viral metagenomics and complete genome sequencing. The wild boar astrovirus (WBAstV-1/2011/HUN, JQ340310) genome is 6707 nucleotide long and had 76%, 95% and 56% amino acid (aa) identity in the ORF1a (852aa), ORF1b (522aa) and ORF2 (845aa) regions, respectively, to porcine astrovirus 4 (PAstV-4, JF713713), the closest match. This study indicates that wild boar could be a reservoir for astroviruses.

Keywords Astrovirus · *Mamastrovirus* · Wild boar · *Sus scrofa* · Feces · Species

Nucleotide sequence data reported is available in the GenBank database under accession number JQ340310.

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The family *Astroviridae* consists of small, non-enveloped viruses with a single-stranded positive-sense RNA genome that 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 1b, 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 [11].

The family *Astroviridae* consists of two genera, *Mamastastrovirus* and *Avastrovirus*, whose members are known to infect mammalian (humans, cheetahs, calves, pigs, sheep, deer, minks, dogs, kittens, mice, etc.) and avian (duck, chickens and turkeys) hosts, respectively. Astroviruses are reported to cause gastroenteritis in humans and some mammals; however, avian strains have been linked with both intestinal and extraintestinal manifestations [6, 11]. Historically, eight classical human astrovirus genotypes have been described in humans (HAstV1-8). However, a diverse group of novel astroviruses was identified recently in humans (HAstV-MLB and HMOAstV-A through C) whose members were phylogenetically separated by different lineages of animal astroviruses [3, 8]. Studies from the past three years have demonstrated that divergent astroviruses can infect the same animal species, including bats [2, 19], turkeys [4], domestic pigs [10, 13, 15], sea lions [9] and domestic sheep [14]. This means that astroviruses infecting one host species can be highly diverse, with different lineages likely reflecting independent origins. In this situation, continued characterization of astrovirus diversity in different host species will help our understanding of their origin and of their possible cross-species transmission. The presence of astrovirus in wild boars has not been investigated. This study describes the identification and complete genetic characterization of astrovirus in wild boars in Hungary.

Fecal samples (N = 10) from wild boar (*Sus scrofa*) piglets were collected from an animal park located in southwestern Hungary in April 2011. All animals were 6-8 weeks old and showed no signs of any clinical symptoms at the time of sample collection. The wild boars were in captive breeding and had no contact with domestic pigs.

Nucleic acid was extracted from specimens that had been diluted in 0.1 M phosphate-buffered saline (PBS), passed through a 0.45-µm sterile filter, and centrifuged at 6,000 × *g* for 5 min. The resulting pellet was treated with a mixture of nucleases to enrich for particle-protected nucleic acids [18]. 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 [18]. 454 pyrosequencing using 454 GS FLX technology was then performed as described previously [8, 18]. The pyrosequencing reads and assembled sequence contigs were compared to sequences in the GenBank nucleotide and protein databases using BLASTn and BLASTx, respectively.

Specific primer pairs were designed to determine the complete nucleotide sequence of the astrovirus by the genome-walking method using the sequence contigs from the pyrosequencing reads. The 5′ and the 3′ ends of the genome were determined using a 5′/3′ RACE PCR kit (Roche, Mannheim, Germany) as described previously [1]. Strain-specific astrovirus primers (WBAstV-ORF2-R, 5′-TGTATTACCCTGATTTGA-3′, at nt positions 4358-4375 and WBAstV-ORF1b-F, 5′-CATAATCATCCTGAC AGTGC-3′, at nt positions 3961-3980) were designed based upon the ORF1b/ORF2 junction of the genome for screening of wild boar astrovirus.

PCR products were sequenced directly in both directions using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK) with the PCR primers and run on an automated sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, Stafford, USA). The study strains and the available astrovirus amino acid sequences obtained from the GenBank database were aligned, and similarity analysis was performed using GeneDoc 2.7 software [12]. Phylogenetic analysis of the astrovirus from wild boar with other astrovirus strains based on the deduced amino acid alignments of ORF1a, ORF1b and ORF2 were conducted using the neighbor-joining method of MEGA software (version 5.1) [16]. The evolutionary distances were computed using the Jones-Taylor-Thornton (JTT) method and are expressed in units of amino acid substitutions per site. Bootstrap values (based on 1000 replicates) for each node are given if >50%. The complete genome and amino acid sequences of the wild boar astrovirus (WBAstV1/2011/HUN) was submitted to GenBank under accession number JQ340310.

Astrovirus sequence contigs were identified in five (50%) of the 10 fecal samples by metagenomic analysis. One of the samples (WBAstV1) was selected, and the complete astrovirus genome sequence was determined. Seventeen astrovirus amino acid (aa) metagenomics-derived sequences were identified in this sample using BLASTx against the NR database of GenBank, covering eight regions of the astrovirus genome (Fig. 1). These sequences showed the closest identity to human MLB1 astrovirus (FJ227122) in the ORF1a region and porcine astrovirus (strain PAstV-2/2007/HUN, GU562296) in ORF1a and ORF2 regions at the time of the GenBank search.

The complete genome length of the wild boar astrovirus WBAstV1/2011/HUN is 6707 nucleotides (nt) excluding the poly(A) tail (Fig. 1). The 852-aa (2556 nt)-long ORF1a is preceded by an 5′ untranslated sequence of 103 nt. The ORF1b and the ORF2 regions are 522 aa (1566 nt) and 845 aa (2538 nt) long, respectively. The UTR at the 3′-end is 67 nt long. The heptanucleotide frameshift signal (AA AAAAC) at the ORF1a/1b junction and the highly conserved consensus ORF1ab/ORF2 junction and astrovirus promoter sequence UUUGGAGNGGNGGACCNAAN$_{4\text{-}11}$AUGNC initiating ORF2 (where the ORF2 AUG start codon is underlined; N stands for any of the four nucleotides) are present in wild boar astrovirus strain WBAstV1/2011/HUN. Just before the AUG start codon, this region includes 11 nucleotides (N_{11}) GCATAAGCCTA (complete sequence motif: UUUGGAGGGGCGGACCAAAN$_{11}$AUG GC) (Fig. 1). The characteristic YGDD aa RdRp motif is encoded by ORF1b. The conserved stem-loop-II-like (s2m) structure that is predicted at the 3′ end of the genomic RNA of several astroviruses [5] was not found in wild boar astrovirus strain WBAstV1/2011/HUN (data not shown). The nt and aa distances based on the complete ORF1a, ORF1b and ORF2 (capsid) regions between wild boar astrovirus strain WBAstV1/2011/HUN and the reference astroviruses are shown in Table 1. The highest aa identities, 76%

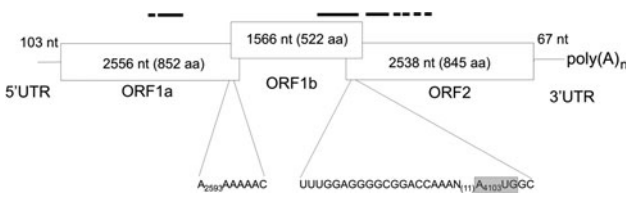

Fig. 1 Genome organization of wild boar astrovirus strain WBAstV-1/2011/HUN (JQ340310). Black bars represent the position of pyrosequencing contigs from metagenomic analysis. Nucleotide (nt) and amino acid (aa) sequence lengths are indicated in each genomic region. The nucleotide sequences represent highly conserved sequences present in the heptanucleotide frameshift signal (AAAA AAC) at ORF1a/ORF1b and just upstream of the transcription initiation site of the subgenomic RNA at the ORF1b/ORF2 junction

Table 1 Amino acid (aa) sequence identity in percentage or range of percentage (%) based on a comparison of the complete ORF1a, ORF1b and ORF2 (capsid) regions between wild boar astrovirus strain WBAstV-1/2011/HUN (JQ340310) (columns) and reference mamastroviruses (rows), including representations of the five known porcine astroviruses types (PAstV-1-5)

Astrovirus reference strain(s)	Wild boar astrovirus strain WBAstV-1/2011/HUN (JQ340310)		
	ORF1a aa (%)	ORF1b aa (%)	ORF2 aa (%)
Porcine-PAstV-1 (Y15938)	Not available	Not available	22
Porcine-PAstV-2 HM756259	Not available	56	25
JF713712	30	56	26
Porcine-PAstV-3 (HM756261)	Not available	Not available	17
Porcine-PAstV-4 GU562296	Not available	76	48
JF713713	**76**	**95**	**56**
Porcine-PAstV-5 (JF713711)	18	40	19
Ovine-OAstV-1 (NC_002469)	16	42	16
Ovine-OAstV-2 (JN592482)	Not available	Not available	17
Bovine-BAstV (HQ916317)	29	52	25
Rat-RAstV (HM450381)	14	51	19
Deer-DAstV-1 (HM447045)	Not available	26	24
Human-HAstV 1-5 and 8 (NC_001943; L13745; AF141381; DQ070852; DQ028633; AF260508)	21	50	19–20
Human-HAstV-MLB1 (FJ222451)	22	48	20
Bat-BAstV-1 (EU847155)	Not available	43	17
Mink-MAstV (AY179509)	18	46	15

Boldface numbers indicate the highest level of amino acid identity. "Not available" means missing or only a partial sequence of the reference strain is available in the GenBank database for comparison

(ORF1a), 95% (ORF1b) and 56% (ORF2), were found to porcine astrovirus 4 (PAstV-4, JF713713), which was identified very recently in the USA. WBAstV1/2011/HUN has 48% aa identity in ORF2 to the prototype PAstV type 4 (GU562296) from Hungary. ORF2 is 20 aa longer in WBAstV1/2011/HUN than in PAstV-4 (JF713713).

Phylogenetic analysis confirmed that wild boar astrovirus strain WBAstV1/2011/HUN forms a common genetic lineage in the genus *Mamastrovirus* with porcine astrovirus type 4 (PAstV-4) in all ORF regions (Fig. 2).

Using the strain-specific astrovirus primers in a conventional RT-PCR assay, wild boar astrovirus were detected only in the same five fecal samples in which they were found by pyrosequencing, confirming the metagenomic results. The nt diversity was less than 3% between wild boar astroviruses in the partial ORF1b/ORF2 junction region.

Astroviruses have a wide range of host species. This report presents the first detection and complete genetic characterization of astrovirus in wild boars. Astroviruses were detected in half of the fecal samples collected from 6- to 8-week-old healthy wild boar piglets. This result indicates the endemic circulation of astrovirus strain WBAstV1/2011/HUN at that farm. Recently, up to five porcine astrovirus (PAstV-1-5) types were described in domestic pigs [10, 13, 15], confirming that, as is the case for humans, viruses of more than one astrovirus lineage/species may exist in the same host species. WBAstV1/2011/HUN was genetically most closely related, especially in ORF1b, to porcine astrovirus 4 (PAstV-4), which was identified from fecal samples of swine in Hungary [13] and the USA [15]. However, the maximum aa homology was only 56% in the ORF2 capsid region, indicating a novel astrovirus serotype. The lower level of homology to ORF2 relative to ORF1 is consistent with the results of other studies and is thought to be the result of strong positive selective pressure on the capsid-coding region from the host immune system [17].

Because of their genetic similarities, wild boar may be a reservoir for astroviruses infecting pigs and vice versa, and the PAstV-4 and WB lineages of astroviruses may have a single common origin. Further study and wild boar astrovirus sequences are needed to investigate more precisely the genetic and antigenic diversity (relationships and differences) of astroviruses in wild boars, the wildlife counterparts of domestic pigs, for confirmation of cross-species transmission of astroviruses between these and probably other hosts.

A highly conserved stem-loop-II-like motif (s2m) has been found in the ORF2/3′UTR of mamastrovirus and avastrovirus, equine rhinovirus, coronavirus and dog norovirus [2, 7]. The exact role of this motif is unclear, but

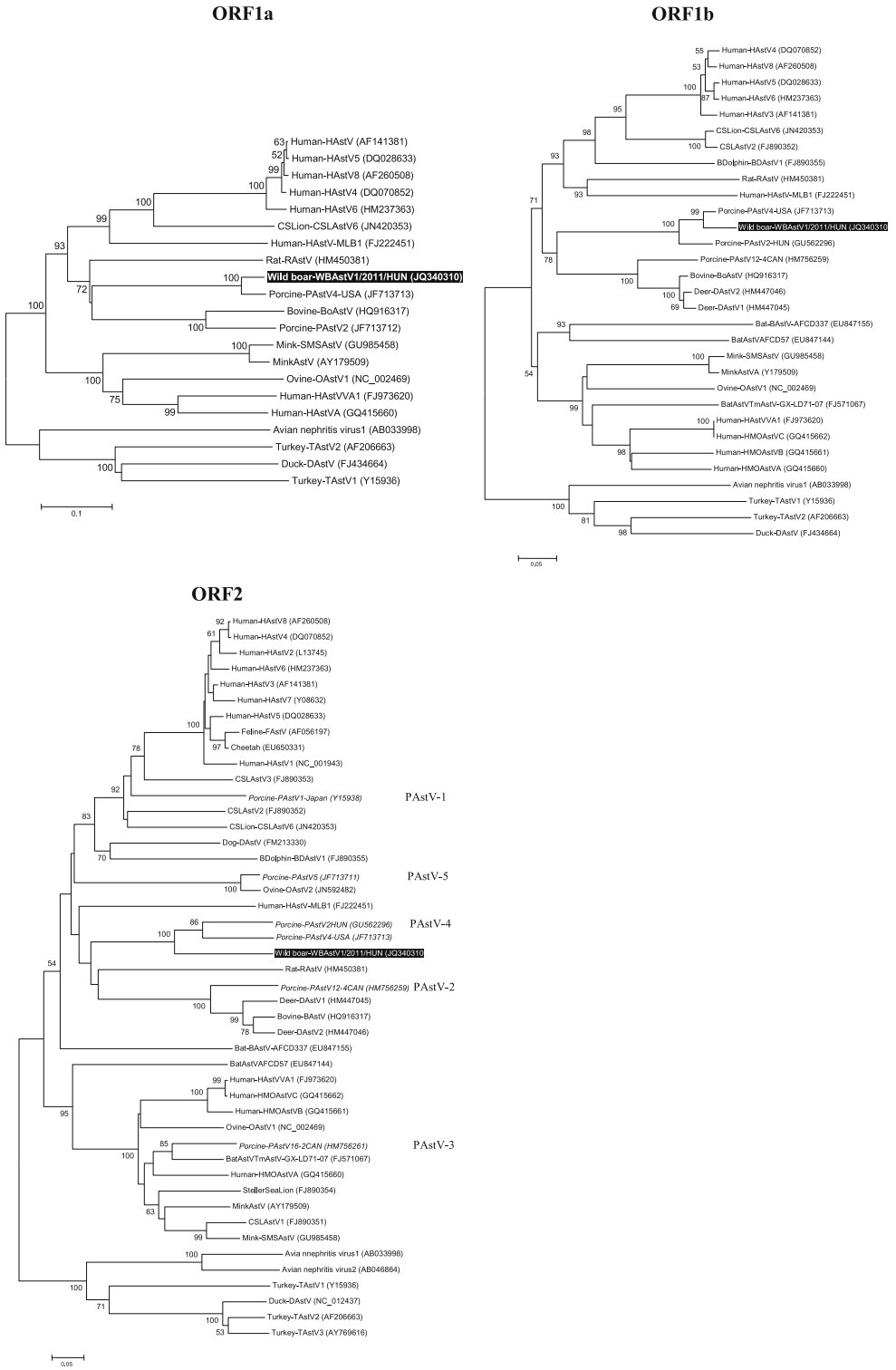

Fig. 2 Phylogenetic analysis of wild boar astrovirus (JQ340310) based upon of the complete amino acid sequences of the three ORF regions (ORF1a, ORF1b and ORF2). Porcine astroviruses (*italics*) representing the five porcine astrovirus types (PAstV-1-5) are indicated in the ORF2 tree

it is thought to be a universal feature of astroviruses [5]. Interestingly, this motif was not recognized in WBAstV1/2011/HUN, and it is also absent in the newly described astroviruses: porcine astrovirus type 2 [10], porcine astrovirus type 4 [13], turkey astrovirus 2, human astrovirus MLB1 [3] and bat astrovirus AFCD337 [2].

It is clear that astroviruses have a broader spectrum of host species as well as higher genetic and antigenic diversity than previously thought. In addition, members of multiple astrovirus species can exist in the same host species. As (re)emerging infectious diseases pose a continuous health threat to wild and domestic animals as well as to humans, continued characterization of astrovirus diversity in different host species will help our understanding of their origin and of their possible cross-species transmission.

Acknowledgments This work was supported by a grant from the Hungarian Scientific Research Fund (OTKA, K83013). G.R. was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences; B.K. was supported by a fellowship from the Rosztoczy Foundation and E.D. by NHLBI grant E01HL083254.

Conflict of interest The authors declare that they have no conflict of interest.

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