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Genome analysis of canine astroviruses reveals genetic heterogeneity and suggests possible inter-species transmission

Eszter Mihalov-Kovács[a], Vito Martella[b], Gianvito Lanave[b], Livia Bodnar[b], Enikő Fehér[a], Szilvia Marton[a], Gábor Kemenesi[c], Ferenc Jakab[c], Krisztián Bányai[a,*]

[a] Institute for Veterinary Medical Research, Centre for Agricultural Research, Hungarian Academy of Sciences, Hungária krt. 21, Budapest, H-1143, Hungary
[b] Dipartimento di Medicina Veterinaria, Universitá Aldo Moro di Bari, S.p. per Casamassima km 3, 70010 Valenzano, Italy
[c] Virological Research Group, Faculty of Sciences, Institute of Biology, University of Pécs, Ifjúság útja 20, H-7624 Pécs, Hungary

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ABSTRACT

Canine astrovirus RNA was detected in the stools of 17/63 (26.9%) samples, using either a broadly reactive consensus RT-PCR for astroviruses or random RT-PCR coupled with massive deep sequencing. The complete or nearly complete genome sequence of five canine astroviruses was reconstructed that allowed mapping the genome organization and to investigate the genetic diversity of these viruses. The genome was about 6.6 kb in length and contained three open reading frames (ORFs) flanked by a 5′ UTR, and a 3′ UTR plus a poly-A tail. ORF1a and ORF1b overlapped by 43 nucleotides while the ORF2 overlapped by 8 nucleotides with the 3′ end of ORF1b. Upon genome comparison, four strains (HUN/2012/2, HUN/2012/6, HUN/2012/115, and HUN/2012/135) were more related genetically to each other and to UK canine astroviruses (88–96% nt identity), whilst strain HUN/2012/126 was more divergent (75–76% nt identity). In the ORF1b and ORF2, strains HUN/2012/2, HUN/2012/6, and HUN/2012/135 were related genetically to other canine astroviruses identified formerly in Europe and China, whereas strain HUN/2012/126 was related genetically to a divergent canine astrovirus strain, ITA/2010/Zoid. For one canine astrovirus, HUN/2012/8, only a 3.2 kb portion of the genome, at the 3′ end, could be determined. Interestingly, this strain possessed unique genetic signatures (including a longer ORF1b/ORF2 overlap and a longer 3′UTR) and it was divergent in both ORF1b and ORF2 from all other canine astroviruses, with the highest nucleotide sequence identity (68% and 63%, respectively) to a mink astrovirus, thus suggesting a possible event of interspecies transmission. The genetic heterogeneity of canine astroviruses may pose a challenge for the diagnostics and for future prophylaxis strategies.

1. Introduction

Astroviruses (AstV), family *Astroviridae,* are non-enveloped viruses with a diameter of 28–30 nm and with a typical star-like shape. The genome is a single strand of positive sense, RNA of 6.4-7.3 kb in size, containing three overlapping ORFs (ORF1a, ORF1b and ORF2) with a 3′ poly(A) tail (Mendez and Arias, 2007). ORF1a encodes a serine 3C type of viral protease. ORF1b is separated from ORF1a by a heptameric frame shift signal (AAAAAAC) and encodes the viral RNA polymerase. ORF2 encodes an 87-kDa polypeptide, which functions as the capsid precursor. AstV infection is associated with gastroenteritis in many animal species and humans, and they are also associated with extra-intestinal diseases, such as nephritis in chickens, hepatitis in ducks and shaking syndrome in minks (Imada et al., 2000; Fu et al., 2009; Blomström et al., 2010). The evolution of AstVs is driven by mechanisms of genetic drift, recombination and, possibly, inter-species transmission (Finkbeiner et al., 2008; De Benedictis et al., 2011; Martella et al., 2014).

So far, AstVs are classified into two distinct genera, *Mamastrovirus* (MAstV) and *Avastrovirus* (AvAstV) with 19 MAstV (mammalian) species and 3 AvAstV (avian) species listed officially by the International Committee on Taxonomy of Viruses (ICTV) (Bosch et al., 2010). However, taking advantage of broad-range PCR primers for AstVs and metagenomic protocols, several novel AstVs have been identified in a number of mammalian and avian species (Chu et al., 2008; Finkbeiner et al., 2008; Bosch et al., 2010; De Benedictis et al., 2011). Using the classification criteria adopted

* Corresponding author at: Krisztián Bányai, Institute for Veterinary Medical Research, Hungária krt. 21, Budapest, H−1143, Hungary.
E-mail address: bkrota@hotmail.com (K. Bányai).

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in the 9th ICTV report that is based on genetic analysis of the full-length ORF2, an additional 14 MAstV and 4 AvAstV candidate species have been defined recently (Schultz-Cherry, 2013).

AstV-like particles have been detected only occasionally in dogs by EM, either alone or in co-infection with other enteric viruses (Williams, 1980; Marshall et al., 1984; Vieler and Herbst, 1995; Toffan et al., 2009). More recently, AstVs have been identified in dogs with enteric signs and characterized molecularly, suggesting that the detected viruses may represent a distinct AstV species (Toffan et al., 2009). Also, a canine AstV, strain ITA/08/Bari, was successfully adapted to replicate *in vitro* on MDCK cells, and AstV-specific antibodies were detected in convalescent canine sera (Martella et al., 2011a,b, 2012). The prevalence of AstV infection seems higher in dogs with enteric disease than in asymptomatic animals (Martella et al., 2011a,b; Zhu et al., 2011; Caddy and Goodfellow, 2015; Takano et al., 2015). Also, monitoring of natural infection by AstV in dogs has revealed that the acute phase of gastroenteritis overlaps with peaks of viral shedding (Martella et al., 2012). At present, limited and partial information on canine AstV genomes is available in GenBank. This limited amount of information seems to suggest that canine AstVs are genetically heterogeneous (Martella et al., 2011a,b; Caddy and Goodfellow, 2015), thus posing a challenge for the diagnostic and for the understanding of the genetic and biological properties of these viruses in dogs.

In this study, the complete or nearly complete genome sequence of five canine AstVs and the partial genome sequence of one canine AstV were determined and analysed, providing information on the genome organization and genetic diversity of these viruses.

2. Materials and methods

2.1. Samples

During 2012 samples were collected from diarrheic and non-diarrheic dogs from a Hungarian shelter. There was no age restriction. A total of 63 samples obtained from 50 animals were tested for AstV by using a pan-astrovirus specific primer set (Chu et al., 2008) as described elsewhere (Mihalov-Kovács et al., 2014) and 37 (from 33 dogs) were randomly selected for viral metagenomics.

Fecal samples were diluted 1: 10 in PBS (phosphate buffered saline) and homogenized. The homogenates were centrifuged at 10000*g* for 5 min and the supernatant was collected for extraction of viral RNA (Zymo DirectZol viral RNA extraction kit, Zymo Research).

2.2. Semiconductor sequencing

Templates for deep sequencing were prepared as described previously (Mihalov-Kovács et al., 2015). In brief, viral RNA samples were denatured at 97 °C for 5 min in the presence of 10 μM random hexamer tailed by a common PCR primer sequence (Djikeng et al., 2008). Reverse transcription was performed with 1 U AMV reverse transcriptase (Promega, Madison, WI, USA), 400 μM dNTP mixture, and 1 × AMV RT buffer at 42 °C for 45 min following a 5 min incubation at room temperature. Then, 5 μL cDNA was added to 45 μL PCR mixture to obtain a final volume of 50 μL and a concentration of 500 μM for the PCR primer, 200 μM for dNTP mixture, 1.5 mM for MgCl2, 1 × Taq DNA polymerase buffer, and 0.5 U for Taq DNA polymerase (Thermo Scientific, Vilnius, Lithuania). The reaction conditions consisted of an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of amplification (95 °C for 30 s, 48 °C for 30 s, 72 °C for 2 min) and terminated at 72 °C for 8 min.

We subjected 0.1 μg of random PCR product to enzymatic fragmentation and adaptor ligation (NEBNext Fast DNA Fragmentation & Library Prep Set for Ion Torrent kit, New England Biolabs, Ipswich, MA, USA). The barcoded adaptors were retrieved from the Ion Xpress Barcode Adapters (Life Technologies, Carlsbad, CA, USA). The resulting cDNA libraries were measured on an Qubit 2.0 device using the Qubit dsDNA BR Assay kit (Invitrogen, Eugene, OR, USA). The emulsion PCR that produced clonally amplified libraries was carried out according to the manufacturer's protocol using the Ion PGM Template kit on an OneTouch v2 instrument. Enrichment of the templated beads (on an Ion One Touch ES machine) and further steps of presequencing setup were performed according to the Page 3 of 11 200-bp protocol of the manufacturer. The sequencing protocol recommended for Ion PGM Sequencing Kit on an 316 chip was strictly followed.

2.3. 5′ rapid amplification of cDNA ends (5′ RACE)

To determine the actual 5′ end genomic sequence of two AstV strains, a 5′ RACE protocol was used, following the manufacturer's instructions (Invitrogen Ltd). The amplicons were visualized on 1.5% agarose gel, and cleaned up with Qiagen Qiaquick Gel Extraction Kit, according to the manufacturer's instructions. The amplicons were cloned using TOPO XL PCR Cloning (Invitrogen Ltd) and the clones were subjected to sequencing in both directions using Big Dye v3.1 chemistry on a 3730xl instrument from Applied Biosystems (Foster, CA). For sequencing accuracy, a minimum of three independent clones for each fragment type were selected for sequencing in both directions using the universal M13F/R primers.

2.4. 3′ rapid amplification of cDNA ends (3′ RACE)

The First Strand III kit (Invitrogen Ltd) was used to generate cDNA from the poly-A tailed RNA target, as described previously (Martella et al., 2011a,b). Forward primers were designed about 1 kb upstream of the 3′ terminus of the genome of the various AstV strains and used with a reverse poly-T oligonucleotide with a 5′-anchored tail. The 3′ RACE products were sequenced on Ion Torrent PGM.

2.5. Sequence and phylogenetic analysis

Sequence data generated by the Ion Torrent PGM were trimmed and analysed by the CLC Bio software (www.clcbio.com). The same software package was utilized to map sequence reads to reference eucaryotic viral sequences retrieved from GenBank. Sequence editing, annotation and analysis were carried out using Geneious software v9.1.4 (Biomatters LTD, New Zealand). Open reading frames (ORF) were predicted with the ORF finder. Multiple alignments were prepared and manually adjusted, whereas phylogenetic analysis was performed by using the MEGA6 software (Tamura et al., 2013) with a neighbor-joining method, Jukes Cantor genetic distance model and bootstrapping over 1000 replicates. Mean amino acid genetic distances were computed using the p-distance method of MEGA6. The protein sequence analysis and classification were prepared with the aid of EMBL-EBI website (http://www.ebi.ac.uk/interpro/). Transmembrane domains were determined using Phobius web server (Käll et al., 2007). Nuclear localization signals (NLS) were predicted by http://www.moseslab.csb.utoronto.ca/NLStradamus/.

2.6. GenBank accession numbers

AstV genomic sequences were deposited in the GenBank with the following accession numbers: HUN/2012/2, KX599349; HUN/2012/6, KX599350; HUN/2012/115, KX599351;