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Mamastrovirus 5 detected in a crab-eating fox (*Cerdocyon thous*): Expanding wildlife host range of astroviruses

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ABSTRACT

Astroviruses are a common cause of gastroenteritis in children worldwide and can also cause infection in a range of domestic and wild animal species. Canine astrovirus (formally named as *Mamastrovirus 5,* MAstV5) has been reported worldwide, and its role as an enteric pathogen is still controversial. Herein, we describe the genomic characterization of a MAstV5 (strain crab-eating fox/2016/BRA) identified in a wild canid (*Cerdocyon thous*) diagnosed with canine distemper virus (CDV) as *causa mortis*. The nearly complete genome comprised 6579 nt in length and displayed the archetypal organization of astroviruses. The present report is the first evidence of MAstV5 infection in an animal species other than the dog and highlights a possible natural astrovirus spillover between domestic and wild canids. Moreover, these results show the first evidence of extra-intestinal MAstV5, suggesting a virus systemic spread. This work is expected to contribute to a better understanding of the astroviruses biology and their interactions with the wildlife health.

1. Introduction

Astroviruses (AstVs) are small, icosahedral, nonenveloped viruses, with a characteristic star-like surface structure. AstVs can infect humans and a variety of animals, and is transmitted via the fecal-oral route by ingestion or fomites [1]. The genome is single-stranded RNA with positive sense with 6.3–7.9 kb in length. It includes three open reading frames (ORFs) designated as ORF1a, ORF1b, and ORF2 [2]. ORF1 encodes a protease and an RNA-dependent RNA polymerase (RdRp) and has a frameshift structure between ORF1a and ORF1b [3]. ORF2 encodes the viral structural capsid protein that is expressed from a subgenomic mRNA [4]. Within each genus, AstVs are classified into genotype species, based on both genetic analyses of the ORF 2 encoded amino-acid sequence and the host species [5].

It has been reported that some AstVs species can cross the host species barrier [6]. This would be the case of some bat AstVs that can infect more than one bat species [7,8], and AstV species that can infect either cheetahs and cats [9]. Recently, neurotropic astrovirus associated with encephalitis was identified in a sheep. Interestingly, the similarity found among this strain and a astrovirus described in neurologically diseased cattle, indicates that astroviruses of the same genotype may cause encephalitis in different species [10]. So far, AstVs have been detected in over than 80 avian and mammalian host species [11]. Moreover, a phylogenetic analysis of the RdRp region suggests that the long-term evolution of AstVs is determined by cross-species transmission events, which occur among distinct ecological scenarios [6].

The crab-eating fox (*Cerdocyon thous*), also known as the Common Zorro, is a “false fox”, native to the South American pampas biome, which seems to be tolerant to human disturbance and is frequently seen

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Table 1
Oligonucleotide features used in the PCR for the detection of common enteric canine viruses.

Target	Primer	Sequence (5'-3')	Gene target	Product size (bp)	Reference
Astrovirus (pan-*Astroviridae*)	Astro F1	GARTTYGATTGGRCKCGKTAYGA	RdRp	422	Chu et al. [8]
	Astro F2	GGYTTKACCCACATNCCRAA			
	Astro R	CGKTAYGATGGKACKATHCC			
	Astro F3	AGGTAYGATGGKACKATHCC			
	Astro F4	GARTTYGATTGGRCKAGGTAYGA			
Mamastrovirus 5	46F	ATGTGTTCAGTGCCACCTTA	NSP1a	359	This study
	405R	CTTGTGTAAGCCTGTGCTGC			
Canine protoparvovirus 1	CPV 555F	CAGGAAGATATCCAGAAG	VP2	555	Buonavoglia et al. [51]
	CPV 555R	GGTGCTAGTTGATATGTA			
Canine adenovirus 1 and 2	HA1	CGCGCTGAACATTACTACCTTGTC	E3	1307	Linné [52]
	HA2	CCTAGAGCACTTCGTGTCCGCTT			
Canine coronavirus	CCoV 1F	TCCAGATATGTAATGTTCGG	M	450	Herrewegh et al. [53]
	CCoV 2R	TCTGTTGAGTAATCACCAGCT			
Canine rotavirus	BEG 9F	GGCTTTAAAAGAGAGAATTTCCGTCTGG	VP7	1062	Gouvea et al. [54]
	END 9R	GGTCACATCATACAATTCTAATCTAAG			
Canine distemper virus	CDV 1F	ACTGCTCCTGATACTGC	NC	480	Castilho et al. [55]
	CDV 2R	TTCAACACC**R**AC**Y**CCC			
	CDV 3F	ACAG**R**ATTGC**Y**GAGGACYT**R**T	NC	287	Frisk et al. [56]
	CDV 4R	CA**RR**ATAACCATGTA**Y**GGTGC			
Internal control	FC27	AGAGTTTGATCCTGGCTCAG	16S rRNA	530	Gontang et al. [17]
	R530	CCGCGGCTGCTGGCACGTA			

R = A/G; Y = C/T; K = G/T; H = A/C/T; N = A/C/G/T. Bold characters indicate modifications introduced to the original sequences published in the references.

in rural areas and close to urban regions [12]. The crab-eating fox's nocturnal scavenger habits bring it in close proximity with domestic animals. This is a negative factor for their conservation, since it increases the possibility of pathogen spillover from domestic dogs to wild canids [6]. It is known that the domestic dog (*Canis lupus familiaris*) may be a source and reservoir of virulent pathogens for wildlife, including the rabies virus, canine distemper virus (CDV), and canine parvovirus (CPV2) [13], also many wildlife species are reservoir of pathogens that threatens domestic animals [14,15].

In this study, we report for the first time, the infection of a wild canid with *Mamastrovirus* 5 (canine astrovirus) in the context of a concurrent infection with canine distemper virus. Additionally, this astrovirus genome has been nearly fully sequenced, characterized, and a discussion of the possible spillover of this virus among wild Canidae species is presented.

2. Material and methods

2.1. Clinical history and pathological features

Veterinarians sighted an adult crab-eating fox (*Cerdocyon thous*) showing signs of motor incoordination in the peri-urban area of Porto Alegre city, Southern Brazil. The animal was then referred to the veterinary hospital by the regional official service (Secretaria do Meio Ambiente e Desenvolvimento Sustentável do Rio Grande do Sul, SEMA-RS) presenting clinical signs resembling central nervous system disease, which was suggestive of a canine distemper virus infection. Upon clinical examination, the crab-eating fox presented apathy, pale mucous membranes, mild dehydration (< 8%), mild eye discharge and multiple neurological signs, which were characterized by ataxia and motor incoordination, evolving to lateral decumbency and severe myoclonia. After 72 h of supportive treatment and no clinical improvement, the animal was euthanized and submitted for necropsy.

At necropsy, the brain, spinal cord, lungs, liver, spleen, lymph nodes, stomach, small and large intestines, kidneys, skeletal muscle, heart, large intestine, adrenal, esophagus and pancreas were collected, fixed in 10% neutral buffered formalin for 24–48 h, trimmed and processed routinely for histopathology. Tissues were then embedded in paraffin, cut at 3 μm and these sections were stained with hematoxylin and eosin (H&E).

Immunohistochemistry for canine distemper virus (CDV) antigens was performed on sections of the cerebellum, hippocampus, thalamus and telencephalic cortex. CDV antigens were detected employing the monoclonal antibody anti-CDV nucleoprotein (VMRD, Pullman, WA, USA) at a 1:400 dilution in phosphate buffered saline (PBS, pH 7.4), and revealed with a 3-amino-9-ethylcarbazole (AEC, Dako North America, Carpinteria, CA, USA) as chromogen.

2.2. Sample collection, nucleic acids isolation and cDNA synthesis

The cerebral cortex, lungs, small intestine, mesenteric lymph nodes, feces, urine and serum were collected at the time of necropsy and stored at −80 °C. Samples were diluted to 20% (w/v) in PBS (pH 7.4). DNA was isolated using NewGene Preamp (Simbios Biotecnologia, Cachoeirinha, RS, Brazil) based on guanidine isothiocyanate and silica [16]. RNA was isolated using TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized with GoScript™ Reverse Transcription System (Promega, Madison, WI, USA) using random primers (0.5 μg/reaction) in a final volume of 20 μL, following the manufacturer's recommendations.

2.3. Detection of common canine enteric viruses

Viruses that infect domestic dogs were screened by specific cDNA/DNA amplifications from feces, urine, serum, and pooled organs. PCR were conducted using primer pairs that were already reported in literature for the detection of astrovirus (AstV), canine distemper virus (CDV), carnivore protoparvovirus 1 (CPV2), canine coronavirus (CCoV), canine rotavirus (CRV), and canine adenovirus 1 (CAdV1) and CAdV2. In addition, the 16S rRNA gene was amplified using the primer pair FC27 and R530 as an endogenous internal control in the feces sample [17]. In order to discriminate in which organs the AstVs would be present, a pair of primers was selected for SYBR-based real-time PCR. The conditions of this qPCR were in accordance with the manufacturer's recommendations (GoTaq® qPCR Master Mix, Promega, Madison, WI, USA). The data about the oligonucleotide features and references are shown in Table 1.

2.4. Illumina genome sequencing and sequence analysis

RNA virome sequencing was performed as previously described [18]. Briefly, the brain, lungs, lymph nodes, intestines, urine and feces, which were collected from the crab-eating fox, were pooled, macerated, centrifuged at a low speed, filtered through a 0.45 μm filter to remove

small debris, and subjected to ultracentrifugation under a 25% sucrose cushion (~150,000 × *g* for 4 h). The resulting viral pellet was mixed with nucleases to eliminate non-capsid-protected nucleic acids. After the nucleases treatment, RNA was isolated with TRIzol® LS Reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions and subsequently enriched using a whole transcriptome amplification kit (WTA2, Sigma-Aldrich, Saint Louis, MO, USA). Subsequent to the amplification, the viral nucleic acids were purified using PureLink® PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). Their quality and quantity were assessed using a spectrophotometer and a fluorometer, respectively.

DNA fragment libraries were prepared with one ng of DNA from WTA using a Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. Illumina sequencing was performed in an Illumina® MiSeq System with a MiSeq Reagent Kit V2 (2 × 150 cycles). The reads quality were evaluated with FastQC, trimmed in Geneious software (version 9) and were *de novo* assembled into contigs using SPAdes (3.6 version) [19]. The contigs were compared to known sequences in the GenBank nucleotide and protein databases using BLASTn/BLASTx [20]. Geneious software was used for an open reading frame (ORF) prediction and genome annotations. The ORF1a disorder prediction was performed with Fold Index software program [21] (Appendix A in Supplementary material).

2.5. Phylogenetic inferences

For phylogenetic inferences, multiple nucleotide sequence alignments were produced with the aid of the ClustalW software. The phylogenetic tree whole genome and capsid protein were reconstructed using the Maximum Likelihood (ML) inference and the protocol to generate these phylogenetic trees was calculated using the "find best DNA/protein model" tool from MEGA6 [22]. Phylogenetics analysis of ORF1a and ORF1b were performed with neighbor-joining method, Junkes Cantor genetic distance model. Bootstrap values were determined by 1000 replicates to assess the confidence level of each branch pattern. The complete genomic sequence of the MAstV5 strain of the crab-eating fox/2016/BRA was deposited in GenBank under the accession number KY765684.

3. Results and discussion

3.1. Histopathological and immunohistochemistry data

At necropsy, the animal presented severe cachexia, pale mucosae and severe tick infestation (*Amblyomma aureolatum*). The brain leptomeningeal blood vessels were severely distended (hyperemia). Also, there was moderate splenomegaly and consolidation areas in diaphragmatic lung lobes.

On histopathology, the cerebellum contained a diffuse and severe white matter demyelination (Fig. 1A) that is associated to large amounts of Gitter cells and gemistocytic astrocytes, with occasional intranuclear and intracytoplasmic eosinophilic inclusion bodies and mild perivascular lymphoplasmacytic cuffs (Fig. 1B). The thalamus showed a focal area of white matter demyelination, which was observed multifocally on the spinal cord in addition to mild perivascular lymphoplasmacytic cuffs. The hippocampus and telencephalic cortex did not show any abnormalities. The lung lesions consisted of parasitic granulomatous pneumonia (*Angiostrongylus spp.*), which is characterized by a focally extensive granulomatous inflammatory infiltrate arranged concentrically around larval structures and embryonated eggs located inside blood vessels and sometimes in alveolar spaces.

Upon immunohistochemistry examination for the CDV antigen, the cerebellum showed a marked intranuclear and intracytoplasmic staining, mainly in astrocytes of the white matter (Fig. 1C and D). Mild immunostaining was observed on sections of the hippocampus and thalamus, while no immunostaining was noted on the telencephalic cortex sections. In this assay, the presence of multifocal intracytoplasmic and intranuclear immunostaining was an important microscopic finding for canine distemper diagnosis. The cerebellum was an adequate organ for the detection of the CDV antigen, being a good auxiliary method in the post mortem and definitive diagnosis of the *causa mortis*.

3.2. Detection of common canine enteric viruses

The crab-eating fox samples were submitted for molecular screening of the common canine enteric viruses by PCR. The internal control resulted positive in all molecular detection assays from fecal samples, which confirmed the nucleic acid quality. The detection assays were negative for CDV, CPV2, CCoV, CRV and CAdV-1/2 in all tested samples. MAstV5 was detected in cDNA derived from the pooled organs using a pan-AstV RT-PCR protocol [8] followed by sequencing. In order to discriminate MAstV5 in each organ, the MAsTV5 was detected in cerebral cortex, small intestine, mesenteric lymph nodes and feces (Table 2) using a specific MAstV5-RT-PCR protocol with primers 46F and 405R (Table 1).

The data presented herein shows two important findings: (i) it is the crab-eating fox/BRA/2016 strain was likely derived from the canine host, and (ii) the extra intestinal MAstV5 presence. It has been reported that some AstVs infections can cross the species barrier [6]. This would be the case of some bat AstVs that infect related bat species [7,8], and AstV species that can infect either cheetahs and cats [9].

Beyond to the gastrointestinal tract infection, some human astroviruses (HAstVs) such as VA1/HMO-C, MLB and the classical HAstV genotypes have already been identified causing encephalitis and meningitis in immunocompromised patients. The proximity to animals, the intravenous treatment of immunoglobulins and the stem cell graft were some of the suggestions from sources of transmission origin, but it has not been confirmed [23].

A spillover from the natural reservoir requires more than the availability of pathogen from the natural host. It also requires that the natural host be brought into physical proximity with a second species, and that this second host be susceptible to infection. These findings are likely to provide new insights into the ecology of astroviruses and transmission among species; especially in peri-urban areas, where factors such as deforestation and human expansion may endanger wildlife populations.

To date, MAstV5 was reported only in samples derived from the gastrointestinal tract of dogs [24,25,34,26–33], and the recovery of MAstV5 in the crab-eating fox's CNS is an interesting and unexpected finding. However, the detection of other AstV species in the central nervous system (CNS) of mink [35], cattle [36,37], human [38], pigs [39] and sheep [40] has been the focus of differential diagnosis of non-suppurative encephalitis.

It was not possible to detect CDV by RT-PCR in the cerebral cortex, or in any other sample available to us, but the CDV antigen was detected in the cerebellum by IHC (Fig. 1). Unfortunately, the samples that were tested by IHQ were not available to be tested by RT-PCR. The recognized effects of CDV on nervous tissue include acute, subacute to chronic forms of encephalopathy, and rare distinct chronic variant encephalomyelitis of mature dogs, termed old dog encephalitis (ODE) [41]. The clinicopathologic features of progressive cortical neurologic signs along with multifocal severe perivascular and parenchymal lymphoplasmacytic encephalitis involving mainly the cerebrum and brain stem are characteristic lesions of ODE that was confirmed at the histopathology description [42]. A number of different hypotheses have been postulated to explain the occurrence and pathogenesis of ODE [43,44]. For instance, ODE may represent the cumulative effects of end stage chronic subclinical CDV encephalitis. In dogs, ODE has almost exclusive predilection for seropositive adults, often with complete vaccination histories. External reinfection of immune dogs by wild-type CDV with subsequent rapid immune-mediated suppression of the

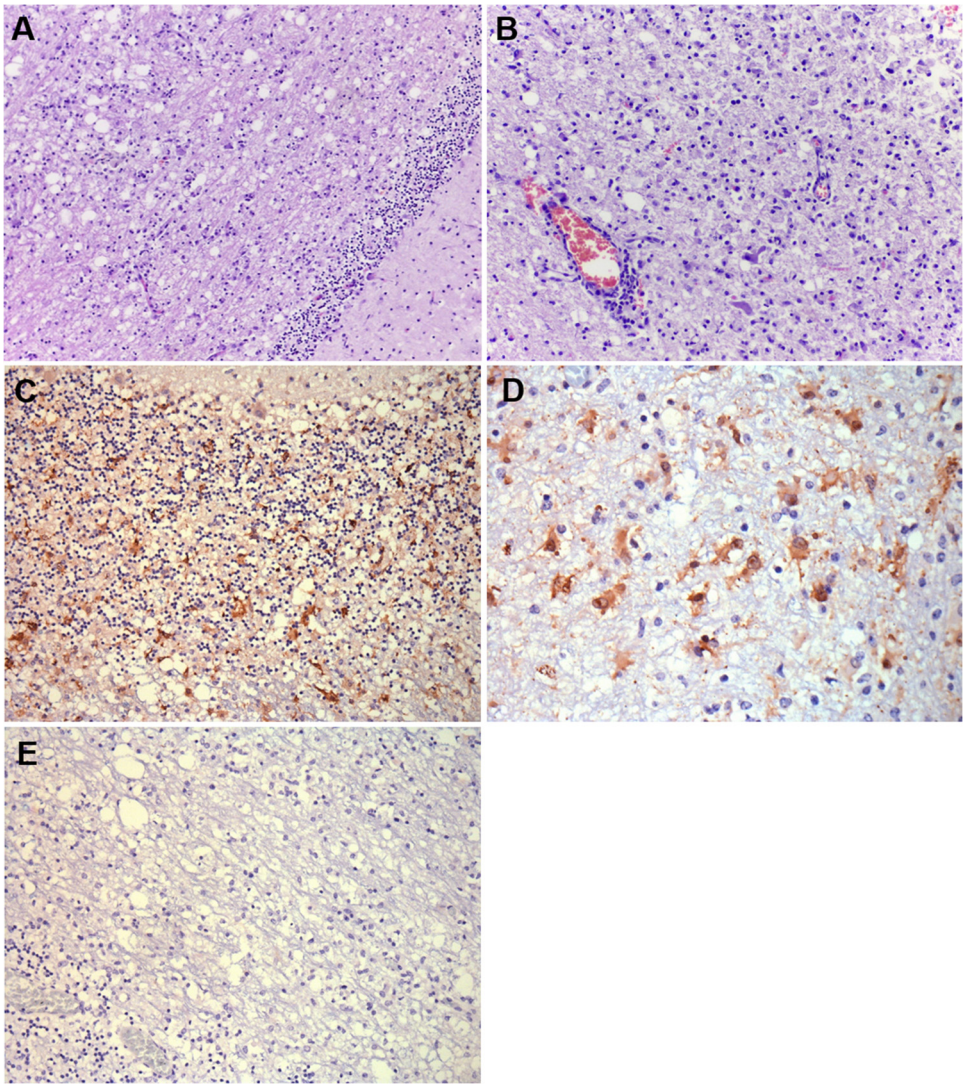

Fig. 1. Pathological findings in *Cerdocyon thous* brain.
A. Cerebellum with a diffuse and severe white matter demyelination (H&E, obj. 10X). B. Cerebellum showing focally extensive areas with large amounts of Gitter cells and gemistocytic astrocytes with mild perivascular lymphoplasmacytic cuffs (H&E, obj. 20X). C–D. Astrocytes at cerebellum white matter showing marked and multifocal immunostaining to CDV in the cytoplasm (obj. 40X). E. Negative control.

Table 2
Summary of PCR screening of most common canine enteric viruses in the *Cerdocyon thous* sample.

Virus target	Cerebral cortex	Lungs	Small intestine	Mesenteric lymph nodes	Feces	Urine	Serum
MAstV5	+	–	+	+	+	–	–
Canine protoparvovirus 1	–	–	–	–	–	–	–
Canine adenovirus 1 and 2	–	–	–	–	–	–	–
Canine coronavirus	–	–	–	–	–	–	–
Canine rotavirus	–	–	–	–	–	–	–
Canine distemper virus	–	–	–	–	–	–	–

(+) positive. (-) negative.

extracellular virus production within the CNS could explain the development of ODE [42]. Our results sustains the hypothesis that the Pampa Fox was infected by CDV, possibly when juvenile, it was able to clear the infection and survive, but the virus persisted in the CNS, leading to a late onset of neurological symptoms compatible to what is seen in ODE cases [45]. This hypothesis could explain why the virus was only detected in CNS tissues. In addition to the CDV infection, the parasitic granulomatous pneumonia contributed to the immune depression and made it possible to MAsTV5 strain crab-eating fox/2016/BRA spread to the extra-intestinal tissues.

It is important to highlight that it is not possible to affirm that MAstV5 strain crab-eating fox/2016/BRA was associated with CNS lesions with the assays applied. Whether MAstV5 might be associated to any pathology remains to be investigated in the future. Regardless of the involvement of MAstV5 in disease, this work is expected to contribute to a better understanding of the biology of astroviruses, and its interactions and possible spillover with the wild hosts.

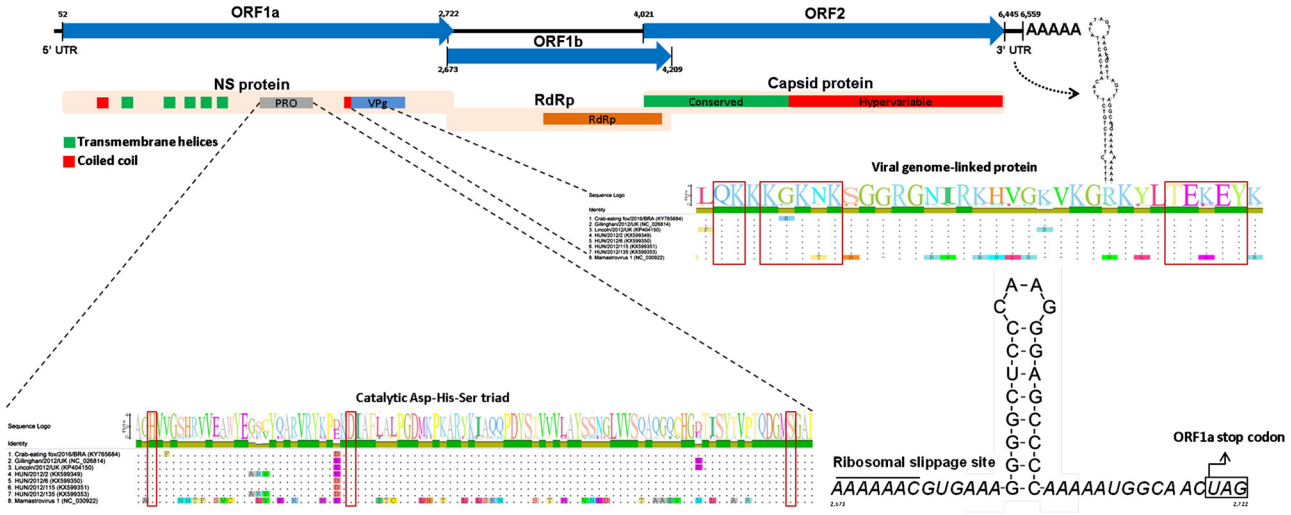

Fig. 2. Organization of MAstV5 strain crab-eating fox/2016/BRA genomic RNA.
The ORF1 is predicted to encode a serine protease (PRO) and VPg protein. The PRO and VPg amino acid sequences are highlighted and compared with other seven AstV sequences. The motifs present in PRO and VPg are marked in boxes in the alignment.

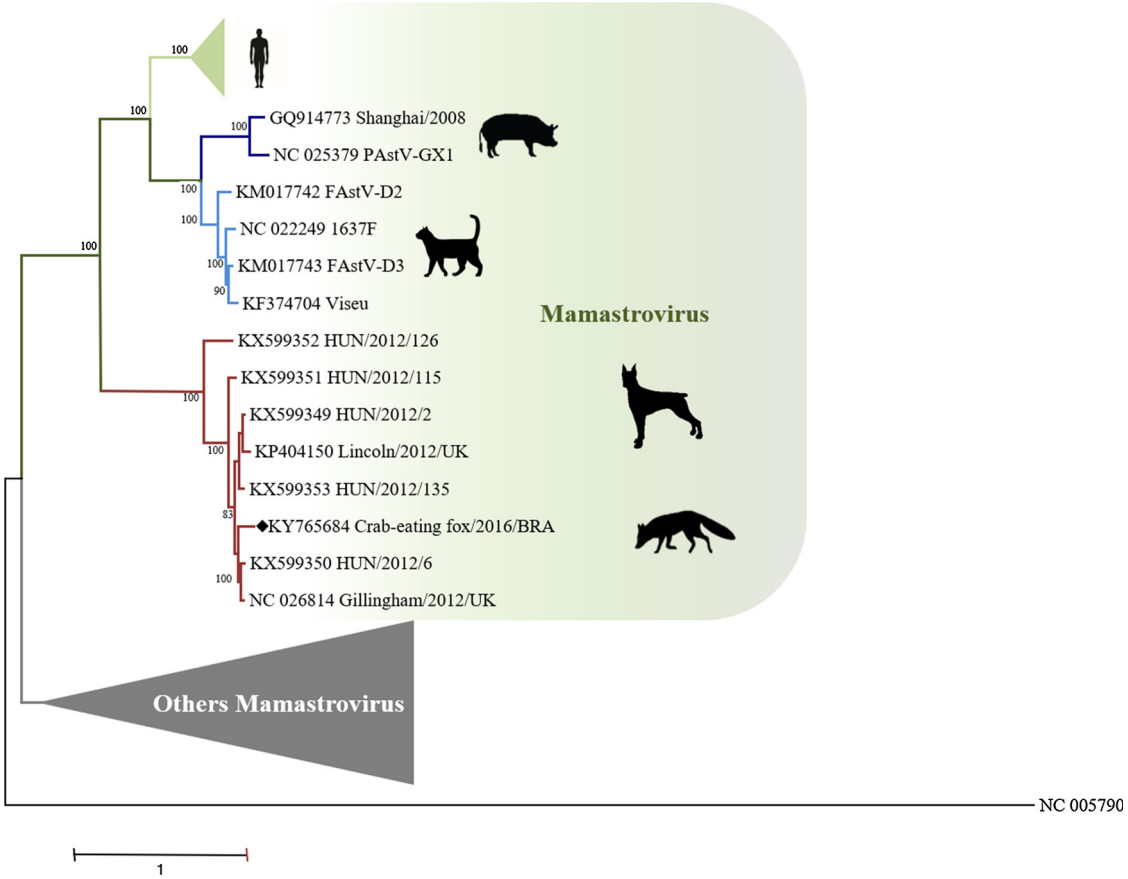

Fig. 3. The whole genome phylogenetic tree.
Nucleotide phylogenetic tree (A) was reconstructed using General Time Reversible (GTR) model. Gamma distribution with invariant site (G + I) were applied to both inferences. The percentage of replicate trees in which the associated taxa are grouped in the bootstrap test (1000 replicates) is shown next to the branches. GenBank accession numbers are listed for all sequences analyzed in the tree. The crab-eating fox/2016/BRA sequence is labeled with a black diamond (◆).

3.3. MAstV5 genome sequencing and genomic analysis

The Illumina MiSeq sequencing generated a total of 71,746 high quality paired-end reads with an average length of 112.5 bp. One contig with ~6.6 kb was *de novo* assembled and showed high genomic identity with canine astroviruses (MAstV5). This contig was obtained with 48,901 reads (coverage ~ 885X). The MAstV5 strain crab-eating fox/2016/BRA nearly full genome is 6559 nt (excluding the poly-A tail) with a GC content of 44.8%. The genome displays typical AstV organization that includes a 5' untranslated region (5'UTR), followed by three ORFs (ORF1a, ORF1b and ORF2), 3' untranslated region (3'UTR) and poly-A tail (Fig. 2).

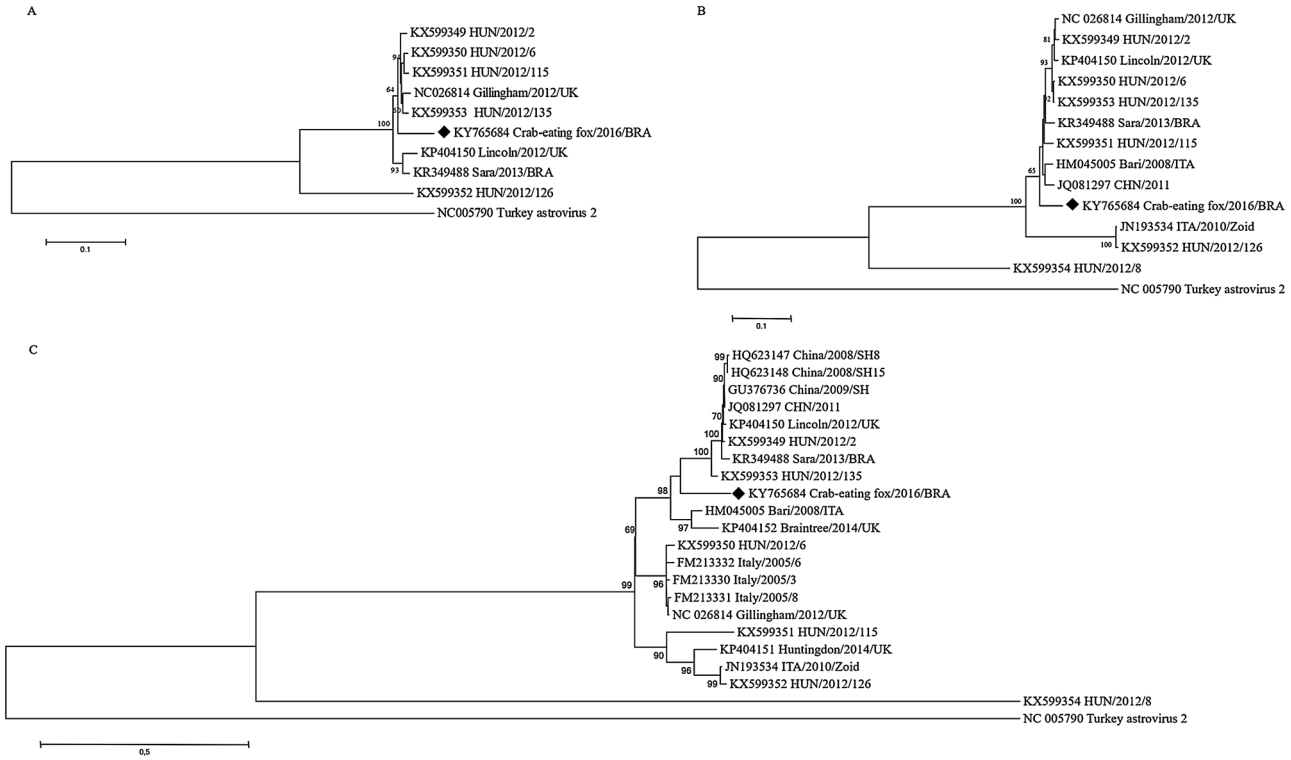

Fig. 4. The ORFs phylogenetics trees.
(A–B) Phylogenetics analysis of nucleotide sequences of ORF1a and ORF1b were performed with neighbor-joining method, Junkes Cantor genetic distance model. (C) While the capsid phylogenetic tree was reconstructed using amino acid sequences with Jones-Taylor-Thornton (JTT) model Gamma distribution with invariant site (G + I) were applied to both inferences. The percentage of replicate trees in which the associated taxa are grouped in the bootstrap test (1000 replicates) is shown next to the branches. GenBank accession numbers are listed for all sequences analyzed in the tree. The crab-eating fox/2016/BRA sequence is labeled with a black diamond (◆).

The ORF1a sequence of the crab-eating fox/2016/BRA strain presents 890 amino acids length in agreement with other MAstV species in which ORF1a range 787–950 amino acids [1,46]. The presence of the putative catalytic triad in the ORF1a that represent the serine protease motif was observed (Fig. 2).

The sequence for the ribosomal frameshift site between ORF1a and ORF1b, which is conserved in the *Astrovirudae* family members [38], is present in the crab-eating fox/2016/BRA nearly full genome (Fig. 2). This translational frameshift is started by a ribosomal slippage site (RSS) that possesses the heptamer sequence 5′-AAAAAAC-3′ at position 2,673, followed by a GC-rich stretch which forms a stem loop structure. The 3′ end of ORF1a overlaps with ORF1b by 49 nucleotides.

As expected, the most conserved region of the MAstV5 strain crab-eating fox/2016/BRA nearly full genome is the RNA-dependent RNA polymerase (RdRp). The analysis of the putative 511 residues of RdRp reveals high sequence identity, when compared to those of other MAstV5 RdRp sequences. These identities range from 78.4% (with the KX599352 sequence) to 94.2% (KP404150 sequence), both recovered from dogs in Hungary (Appendix A in Supplementary material).

The highest identity of the ORF1b of the crab-eating fox/2016/BRA, relative to sequences from other AstV species, was 73% with the partial sequence of the California Sea Lion AstV (AEM37630). The same similarity between the RdRp belonging to the two AstV species can also be verified in previous studies. The identity of the putative RdRp from the crab-eating fox/2016/BRA compared with other MAstV species is described in the Appendix A in Supplementary material.

The ORF2 of the crab-eating fox/2016/BRA contains 2454 nucleotides in length corresponding to 817 putative residues. In general, it ranges from 672 to 851 amino acids among the *Astroviridae* family members [1]. This ORF encodes the putative capsid protein [47]. It was also observed an overlapping reading frame in the C-terminal portion of the polymerase and the N-terminal portion of the capsid precursor of 188 nucleotides (Fig. 2). This observation is in agreement with recently reported MAstV5 genomes [24,48].

3.4. Phylogenetic inferences

In order to reconstruct the evolutionary history of the crab-eating fox/2016/BRA, this sequence was compared to reference sequences of each MAstV species available in GenBank. A phylogenetic tree was reconstructed with the complete genome sequences of viruses belonging to the genus *Mamastrovirus* (Fig. 3).

The crab-eating fox/2016/BRA nearly full genome, as expected, grouped in the MAstV5 cluster with all other characterized canine astroviruses. The crab-eating fox/2016/BRA nearly full genome sequence clustered in the same terminal node as Gillinham/2012/UK and HUN/2012/6 strain (GenBank accession numbers NC_026814 and KX599350).

In addition, phylogenetic trees were constructed comparing the alignments of ORF1a, ORF 1b and ORF2 belonging *Mamastrovirus 5* species (Fig. 4A–C). The ORF1a and ORF1b alignments were based on the full-length ORF nucleotide sequences, also included selected sequences above 1167 and 714 nucleotides, respectively. The ORF2 alignment only included full-length capsid protein sequences, both in nucleotide-based and in amino acid-based analysis.

The ORF1a tree formed two distinct branches with the crab-eating fox/2016/BRA strain being closely related to the Lincoln/2012/UK and Gillingham/2012/UK strains, ranging from 93.4% to 93.3% of nucleotide identity, respectively (Table 3). The upper branch that crab-eating fox/2016/BRA has been included was composed by Hungarian strains (GenBank accession number KX599349, KX599350, KX599351, KX599353), United Kingdom strains (GenBank accession number

Table 3
Sequence comparison among Canine AstV and crab-eating fox/2016/BRA strain.

Strains	GenBank accession number	crab-eating fox/2016/BRA (KY765684)			
		Genome nt	ORF1a nt	ORF1b nt	ORF2 aa
Bari/2008/ITA	HM045005	–	–	94.0	83.0
ITA/2010/Zoid	JN193534	–	–	80.9	72.5
Italy/2005/3	FM213330	–	–	–	78.8
Italy/2005/6	FM213332	–	–	–	77.7
Italy/2005/8	FM213331	–	–	–	78.4
China/2008/SH15	HQ623148	–	–	–	82.0
China/2008/SH8	HQ623147	–	–	–	81.8
China/2009/SH	GU376736	–	–	–	82.3
CHN/2011	JQ081297	–	–	93.4	82.3
Braintree/2014/UK	KP404152	–	–	–	80.2
Gillingham/2012/UK	KP404149	87.6	93.3	94.1	78.1
Huntingdon/2014/UK	KP404151	–	–	–	75.8
Lincoln/2012/UK	KP404150	87.8	93.4	94.2	82.2
HUN/2012/115	KX599351	85.1	92.1	92.8	73.0
HUN/2012/126	KX599352	73.2	74.4	78.4	71.5
HUN/2012/135	KX599353	87.4	93.0	93.9	83.1
HUN/2012/2	KX599349	87.1	92.9	93.7	82.0
HUN/2012/6	KX599350	86.7	92.6	94.2	77.6
HUN/2012/8	KX599354	–	–	56.7	23.7
Sara/2013/BRA	KR349488	–	92.9	93.4	81.6

KP404149 and KP404150) and Brazilian strain (GenBank accession number KR349488). More distant related to then, HUN/2012/126 form the second branch of the OFR1a tree (Fig. 4A).

The same pattern of topology was found in the ORF1b, despite the inclusion of new sequences (GenBank accession number HM045005, JQ081297, JN193534 and KX599354). The crab-eating fox/2016/BRA strain being in the middle of the two first branches, even nucleotide similarity of upper branch does not vary above from 1.4%. Therefore, we consider that crab-eating fox/2016/BRA strain still belonging to the upper branch. The below branch was composed by the highly virulent strain ITA/Zoid/2010 and the HUN/2012/126 strain sharing 80.9% and 78.4% of nucleotide identity with crab-eating fox/2016/BRA, respectively (Fig. 4B and Table 3). As expected, the strain HUN/2012/8 form another isolated branch distantly related to all others *Mamastrovirus 5* as was reported [48].

According to the taxonomic guidelines of the *Astroviridae* family [49], species classification is performed not only on the basis of the host, but also on the phylogenetic differences based on the analysis of the complete ORF2 amino acid sequence. Moreover, the mean genetic distance of the amino acid sequences (p-distance), relative to the sequence of the crab-eating fox/2016/BRA strain, remained within the established parameters for the *Mamastrovirus 5* species (Fig. 4C). There were clearly visible sub-cluster patterns within the MAstV5 cluster that exhibited the same patterns already reported [48]. Therefore, this result support our found that the crab-eating fox/2016/BRA formed a distinct subset based on the lower amino acid identity between the other strains (Table 3). As the Astroviridae family, the MAstV5 constitute a remarkably genetically diverse species each was nomenclature and taxonomy must be discussed and update regularly [50].

4. Concluding remarks

This report shows a canine-like astrovirus identified in a wild Canidae (*Cerdocyon thous*). This is also the first detection of MAstV5 presence in an extra-intestinal tissue, together with canine distemper virus. The findings presented here are expected to help understand how viral infections of domesticated dogs may impact the wild canid population's health, and its potential as sources of viruses, which may potentially infect other animal species.

Competing interests

None of the authors have any potential financial conflict of interest related to this manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2018.08.002.

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