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Adenovirus surveillance in wild carnivores from Brazil

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ABSTRACT

Landscape transformation favors the spread of new pathogens that can be shared between domestic and wild animals. Certain adenoviruses (e.g., canine adenovirus 1 and 2, family *Adenoviridae*) can infect domestic and wild carnivores. In domestic canids, these viruses are associated with hepatic and respiratory diseases (among others). Nevertheless, information regarding adenovirus pathogenicity and molecular features in wild carnivores is still limited. Herein we surveyed adenovirus in free-ranging carnivores from Brazil. Total DNA was extracted from and subsequently tested by a nested panPCR in spleen and/or lung of 52 carnivores, representing species of the following families: Canidae ($n = 4$), Felidae ($n = 3$), Mustelidae ($n = 2$) and Procyonidae (n = 2). The obtained sequences were compared to others available at GenBank. Available tissue samples from the positive cases were evaluated histopathologically. One out of 52 (1.9%, CI 95%, 0.0–5.7%) carnivores was positive; a roadkilled ocelot (*Leopardus pardalis*). The obtained sequence presented a low deduced amino acid (78.1%) similarity with the closest adenovirus, identified in a pinniped from the United States of America. This fact and its detection in a novel host suggest it may be representative of a novel species and denominated ocelot adenovirus 1. None of the gross and microscopic findings of the positive case were associated with adenovirus. To the authors' knowledge, this is the first report of adenovirus in wild felids of South America and the second worldwide. Further studies are necessary to assess the epidemiology and potential pathogenicity of this agent in wild carnivores.

1. Introduction

The order Carnivora comprises over 290 species (https://www.catalogueoflife.org/data/taxon/VS). These animals play important ecological functions, such as the control of herbivore and smaller carnivore populations, seed dispersal and maintenance of local flora (Noss et al., 1996; Ripple et al., 2014; Roemer et al., 2009). Nevertheless, their dependence on large landscapes for shelter and reproduction, and stable prey populations makes carnivores vulnerable to habitat loss, modification, and fragmentation (Henle et al., 2004; Wilcox and Murphy, 1985). Landscape transformation also favors the spread of new pathogens (Sacristán et al., 2021), which can be potentially harmful, once free-ranging carnivores tend to be immunosuppressed (Daszak et al., 2000; Smith et al., 2009). Thus, carnivores are considered sensitive indicators of anthropogenic impact over the environment (Hunter and Barrett, 2018; Karanth and Chellam, 2009).

Adenoviruses are large nonenveloped double-stranded DNA viruses with high environmental resistance (Gerba, 2015; Harrach et al., 2011). These viruses tend to be species-specific (Harrach et al., 2019), are transmitted through direct or indirect (e.g., saliva, respiratory secretions, feces, urine) contact (Decaro et al., 2008) and able to establish persistent infections or reactivate under immunosuppressive conditions (Kosulin et al., 2016). Certain adenoviruses (i.e., canine adenovirus types 1 and 2 [CAdV-1 and CAdV-2] of the species *Canine mastadenovirus*

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A, genus *Mastadenovirus*, family *Adenoviridae*) are examples of pathogens shared between domestic and wild animals (García Marin et al., 2018).

CAdV-1 is the etiological agent of infectious canine hepatitis, a disease characterized by necrotizing hepatitis, vasculitis and disseminated intravascular coagulation, leading to encephalitis and glomerulonephritis (Walker et al., 2016). This disease has been commonly reported in dogs, and also diagnosed in brown bears (*Ursus arctos*), black bears (*Ursus americanus*), wolves (*Canis lupus*), red foxes (*Vulpes vulpes*), silver foxes (*Vulpes fulva*), a fennec fox (*Vulpes zerda*), a gray fox (*Urocyon cinereoargenteus*), and in a Eurasian river otter (*Lutra lutra*) (Dowgier et al., 2018; García Marín et al., 2018; Millán et al., 2016; Oleaga et al., 2021; Park et al., 2007). Moreover, unapparent CAdV-1 infections were described in red foxes (Walker et al., 2016). CAdV-2 is one of the etiological agents of the canine infectious respiratory disease, also known as "kennel cough", which is highly contagious in canine communities (Decaro et al., 2008; Reagan and Sykes, 2020). In wild carnivores, CAdV-2 has been detected in a wolf, a raccoon (*Procyon lotor*), and in red foxes (Balboni et al., 2013; Dowgier et al., 2018; Millán et al., 2016). Of note, fatal vaccine-induced CAdV-2 infection was described in captive maned wolves (*Chrysocyon brachyurus*) after administration of an attenuated vaccine (Swenson et al., 2012). In addition to CAdV-1 and 2, and human adenoviruses, some other species-specific mastadenoviruses have been described in carnivores; in polar bears (*Ursus maritimus*), a skunk (*Mephitis mephitis*), a pine marten (*Martes martes*), Eurasian river otters, sea otters (*Enhydra lutris*) and pinnipeds (Böszörményi et al., 2020; Chiappetta et al., 2017; Dayaram et al., 2018; Kozak et al., 2015; Siqueira et al., 2017; Walker et al., 2017). Moreover, a novel avian

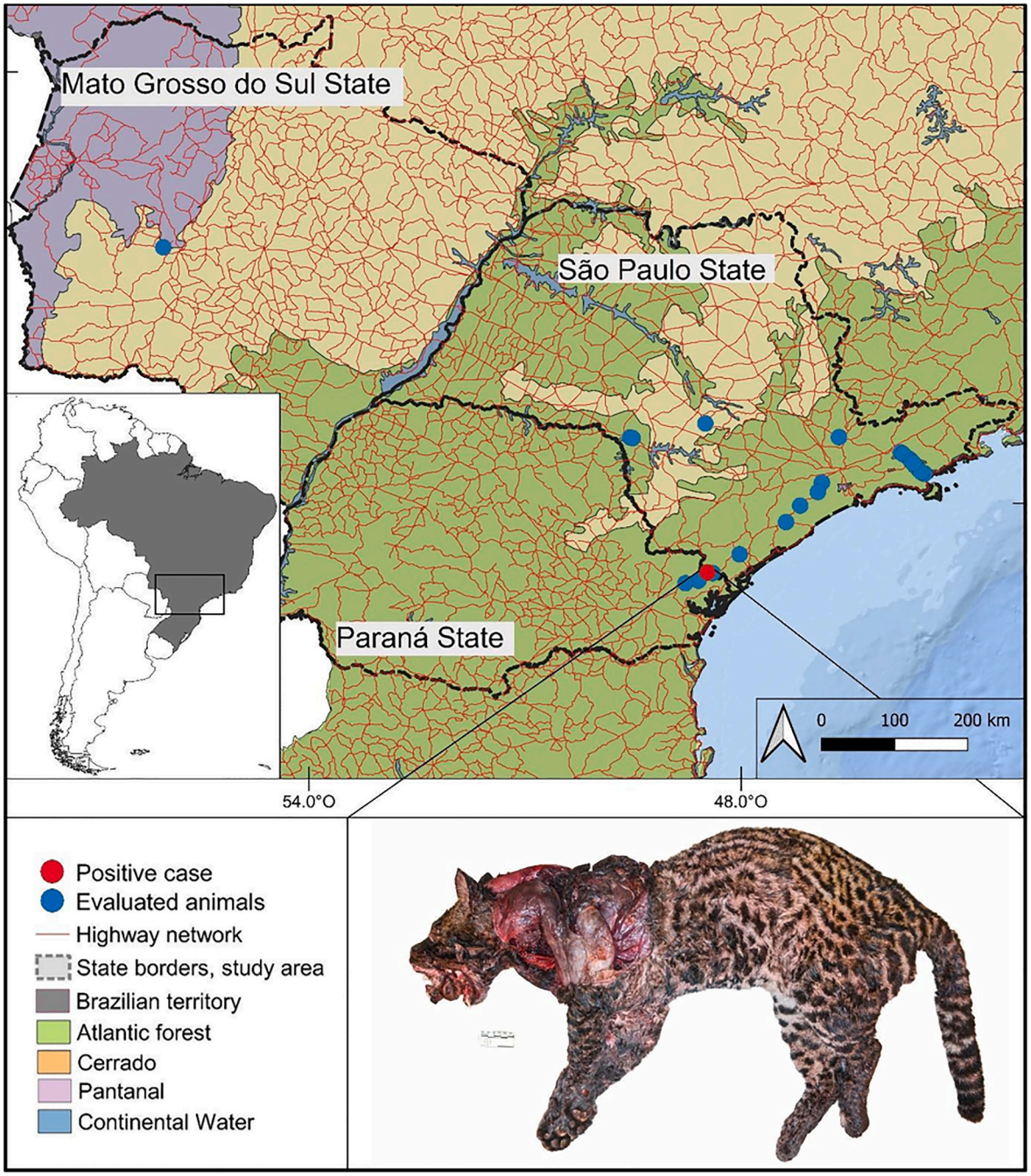

Fig. 1. Location of the 52 carnivores included in the study. Bottom left: lateral view of the adenovirus-positive ocelot (*Leopardus pardalis*).

adenovirus has been detected in fecal and liver samples of pine martens (Walker et al., 2017). In spite of that, the confirmed reports of adenoviral infection in free-ranging terrestrial South American carnivores are scarce, limited to the detection of human and canine adenoviruses in pampa (*Lycalopex gymnocercus*) and crab-eating foxes (*Cerdocyon thous*) from Brazil (Monteiro et al., 2015).

Landscape transformation could promote the spread of CAdV-1 and 2 from domestic to wild carnivores. Additionally, due to the coevolution of most adenoviruses with their host, one may expect a high number of adenoviral species. Nevertheless, the current knowledge on adenoviruses in wild carnivores is still limited, particularly in South America. The goals of the study were to: (1) identify the presence of adenovirus (es) in wild carnivores from Brazil, and (2) characterize potential histopathologic adenovirus-associated lesions.

2. Materials and methods

2.1. Samples

Necropsies were conducted in 52 carnivores from three Brazilian regions: São Paulo (southearstern), Paraná (southern) and Mato Grosso do Sul states (central west) (Fig. 1). Evaluated animals comprised 11 carnivore species, of the families Felidae (ocelot [*Leopardus pardalis*, $n = 10$], southern tiger cat [*Leopardus guttulus*, $n = 2$], jaguarundi [*Puma yagouaroundi*, $n = 5$] and puma [*Puma concolor*, $n = 4$]), Canidae (crab-eating fox, n = 10, maned wolf, n = 5, and bush dog [*Speothos venaticus*, n = 1]), Procyonidae (crab-eating raccoons [*Procyon cancrivorus*, $n = 9$] and South American coati [*Nasua nasua*, n = 2]), and Mustelidae (lesser grison [*Galictis cuja*, $n = 3$] and neotropical river otter [*Lontra longicaudis*, n = 1]). Samples from 50 of these carnivores were collected as part of an ongoing study to assess the health status of roadkill wild mammals in Brazil, between 2014 and 2019 (Table 1). Additionally, the samples from two other animals - a maned wolf from Serra da Canastra National Park (Minas Gerais state) necropsied in January 2020 and a crab-eating fox from Ibitinga (São Paulo state) necropsied in September 2020 - were provided by CENAP (Centro Nacional de Pesquisa e Conservação de Mamíferos Carnívoros), and by the Universidade Estadual Paulista Júlio de Mesquita Filho, respectively (Table 1). Representative tissue samples of all these animals were kept at −20 °C until tested. An additional set of samples was fixed in formalin and subsequently embedded in paraffin. Spleen and/or lung samples from all animals were selected for initial adenovirus screening. Subsequently, all remaining available tissues from adenovirus-positive animals were extracted and tested for adenovirus.

2.2. Molecular techniques

After manual or automatic homogenization and enzymatic digestion, total DNA was extracted from lung ($n = 38$) and spleen ($n = 46$) using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. A nested endpoint panPCR targeting the DNA-dependent DNA polymerase gene of adenovirus was performed (Li et al., 2010), amplifying a fragment of approximately 260 base pairs. A CAdV-2 vaccine (Vanguard HTLP 5/CV-L, Zoetis, Lincoln, NE, USA) was used as a positive control, while diethyl pyrocarbonate treated water was selected as no template control. The amplicons of expected size were purified using ExoSap-IT (USB Corporation, OH, USA) and directly sequenced using an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The consensus sequence was

Table 1
General biological and ecological data of the 52 wild carnivores included in this study.

Species	Conservation	Sex		Age		Body condition	Season			Biome	
	ICMBio/ IUCN*	Female	Male	Adult	Juvenile	Good	Regular	Dry	Rain	Atlantic forest	Cerrado
Canidae											
Cerdocyon thous (n = 10)	LC/LC	40% (4/10)	50% (5/10)	90%(9/10)	10% (1/10)	100% (10/10)	0% (0/10)	60% (6/10)	30% (3/10)	90% (9/10)	10% (1/10)
Chrysocyon brachyurus (n = 5)	VU/NT	40% (2/5)	40% (2/5)	80% (4/5)	20% (1/5)	80% (4/5)	20% (1/5)	20% (1/5)	60% (3/5)	100% (5/5)	0% (0/5)
Spheotos venaticus (n = 1)	VU/NT	0% (0/1)	100% (1/1)	100% (1/1)	0% (0/1)	100% (1/1)	0% (0/1)	100% (1/1)	0% (0/1)	0% (0/1)	100% (1/1)
Felidae											
Leopardus guttulus (n = 2)	VU/VU	50% (1/2)	50% (1/2)	50% (1/2)	50% (1/2)	100% (2/2)	0% (0/2)	0% (0/2)	100% (2/2)	100% (2/2)	0% (0/2)
Leopardus pardalis (n = 10)	LC/LC	20% (2/10)	80% (8/10)	80% (8/10)	20% (2/10)	90% (9/10)	10% (1/10)	50% (5/10)	50% (5/10)	100% (10/10)	0% (0/10)
Puma concolor (n = 4)	VU/LC	50% (2/4)	50% (2/4)	0% (0/4)	100% (4/4)	75% (3/4)	25% (1/4)	50% (2/4)	50% (2/4)	100% (4/4)	0% (0/4)
Puma yagouaroundi (n = 5)	VU/LC	0% (0/5)	100% (5/5)	60% (3/5)	40% (2/5)	80% (4/5)	20% (1/5)	0% (0/5)	100% (5/5)	100% (5/5)	0% (0/5)
Mustelidae											
Galictis cuja (n = 3)	LC/LC	0% (0/3)	100% (3/3)	100% (3/3)	0% (0/3)	66.7% (2/3)	33.3% (1/3)	33.3% (1/3)	66.7% (2/3)	100% (3/3)	0% (0/3)
Lontra longicaudis (n = 1)	NT/DD	0% (0/1)	100% (1/1)	100% (1/1)	0% (0/1)	100% (1/1)	0% (0/1)	100% (1/1)	0% (0/1)	100% (1/1)	0% (0/1)
Procyonidae											
Nasua nasua (n = 2)	LC/LC	50% (1/2)	50% (1/2)	100% (2/2)	0% (0/2)	100% (2/2)	0% (0/2)	0% (0/2)	100% (2/2)	100% (2/2)	0% (0/2)
Procyon cancrivorus (n = 9)	LC/LC	33.3% (3/9)	66.7% (6/9)	66.7% (6/9)	33.3% (3/9)	100% (9/9)	0% (0/9)	22.2% (2/9)	77.8% (7/9)	100% (9/9)	33.3% (3/9)
Total		28.8% (15/52)	67.3% (35/52)	73.1% (38/52)	26.9% (14/52)	90.4% (47/52)	9.6% (5/52)	36.5% (19/52)	59.6% (31/52)	96.2% (50/52)	3.8% (2/52)

* Conservation status of the studied species (DD = Data Deficient; LC = Least Concern; VU = Vulnerable and NT = Near Threatened) according to the Chico Mendes Institute for Biodiversity Conservation (ICMBio) and the International Union for Conservation of Nature (IUCN).

constructed using Mega 7.0 and compared to previously described sequences available at GenBank/EMBL/DDBJ databases, using the BLAST search. Nucleotide and deduced amino acid identities between the obtained sequence and those present in public databases were calculated based on p-distance. Phylogenetic trees based on nucleotide and deduced amino acid sequences were constructed using the maximum likelihood algorithm, with a bootstrap value of 1000 replications. All bootstrap frequency values less than 70 were omitted.

2.3. Histopathological and immunohistochemical evaluation

All available tissue samples from adenovirus-positive animals were formalin-fixed and embedded in paraffin, cut at 3 μm, stained with hematoxylin-eosin and analyzed by light microscopy.

For immunohistochemistry, histological sections were enzymatically digested with proteinase K (20 mg/ml) for antigen retrieval. Subsequently, endogenous peroxidase and nonspecific protein blocking with non-immune serum of the same species was used to raise the primary antibody (monoclonal antibody against adenovirus, 1:1000 dilution; Light Diagnostics/Millipore, 3105/500, CA, USA), which was incubated overnight at 8 °C. The signal was amplified and visualized by two different systems: Novolink Polymer Detection System (Leica Biosystems, Newcastle, UK) with diaminobenzidine chromogen (DAB; D-5637; Sigma, MO, USA) conjugated with phosphatase and Polink-2AP Broad Kit (GBI Labs, D24–110, WA; USA) with Fast Red Chromogen (Permanent Red Kit, PRD-61, UT, USA) conjugated with peroxidase, followed by counterstaining with Harris' hematoxylin. All immunohistochemistries were processed with positive and negative controls (nonimmune homologous serum used instead of the primary antibody).

3. Results

One out of 52 animals (1.9%, CI 95%, 0.0–5.7%) tested positive to adenovirus; a roadkill well-nourished male adult ocelot, found dead on a highway at Barra do Turvo municipality (24°45′23“ S, 48°30’17” W), São Paulo state, in October 2018 (Fig. 1). The same adenovirus sequence was obtained in brain, skeletal muscle, spleen, mesenteric lymph node, stomach, and duodenum samples. The kidney, cecum, lung, liver, and tongue samples were negative.

The amplified ocelot adenovirus sequence presented highest nucleotide (68.6%) and deduced amino acid (78.1%) similarities to adenovirus sequences described in pinnipeds from Peru (MF175103), and the United States of America (JX244189), respectively. The obtained sequence clustered with others of the genus *Mastadenovirus* (Fig. 2) and was submitted to GenBank under accession number MZ147500.

This ocelot died as a consequence of trauma caused by motor vehicle collision (Fig. 1). The main gross lesions were: rupture of internal organs (cava, esophagus, heart, lungs, tongue, trachea), bone fractures (cervical vertebrae, frontal, jaw, maxillae, metacarpus, skull, ribs, scapulae, teeth, and thoracic vertebrae), skin lacerations and muscular rupture in

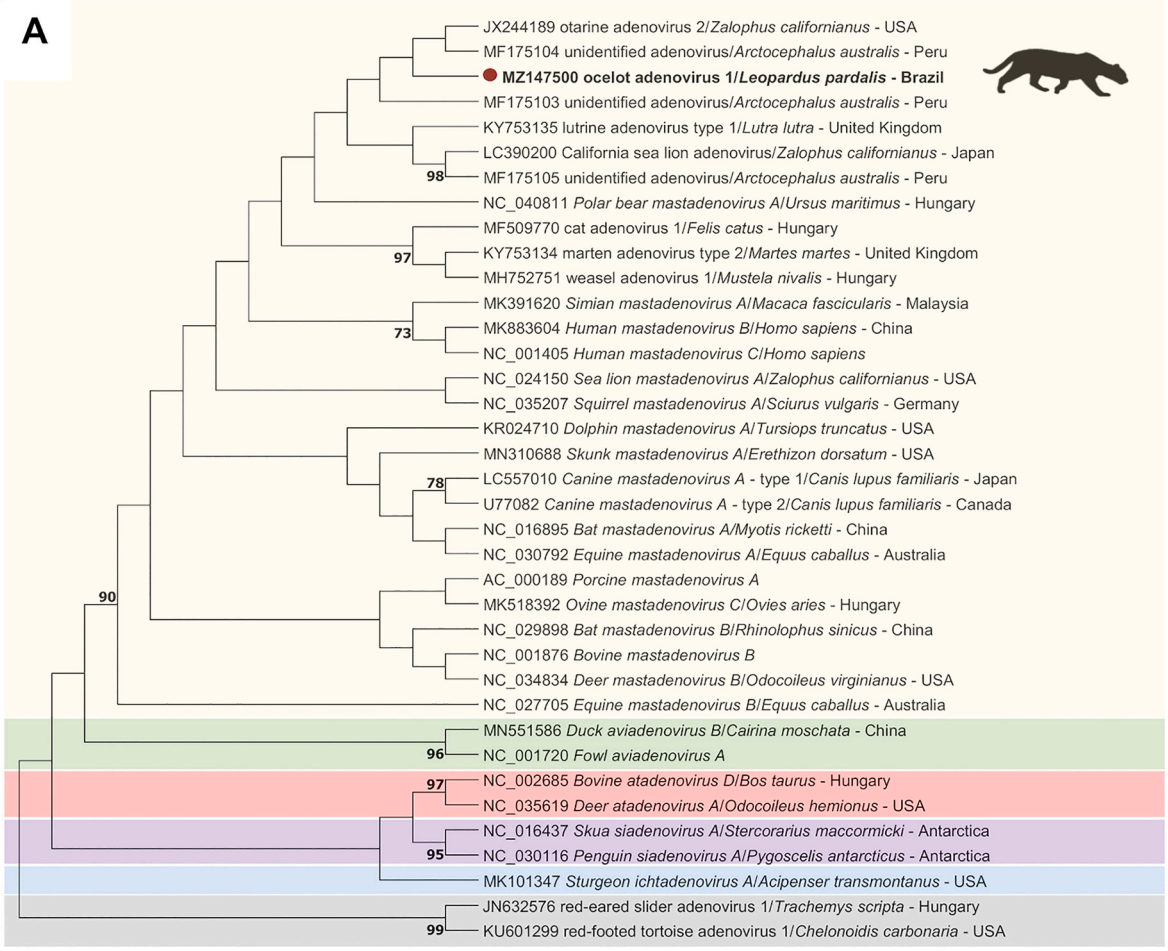

Fig. 2. Maximum likelihood phylogram of the alignment of deduced amino acid (A) and nucleotide (B) DNA polymerase sequences: (i) of the adenovirus identified in an ocelot (*Leopardus pardalis*) in Brazil (red dot); (ii) from other carnivores (genus *Mastadenovirus*); (iii) accepted adenovirus species of the genera *Mastadenovirus* (yellow color), *Aviadenovirus* (green color), *Atadenovirus* (violet color), *Siadenovirus* (red color), *Ichtadenovirus* (blue color), and sequences of *Testadenovirus* (gray color). Tree reliability was tested by bootstrap analysis with 1000 replicates. Bootstrap values lower than 70 were omitted. The branch length is also shown in the nucleotide phylogenetic tree. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

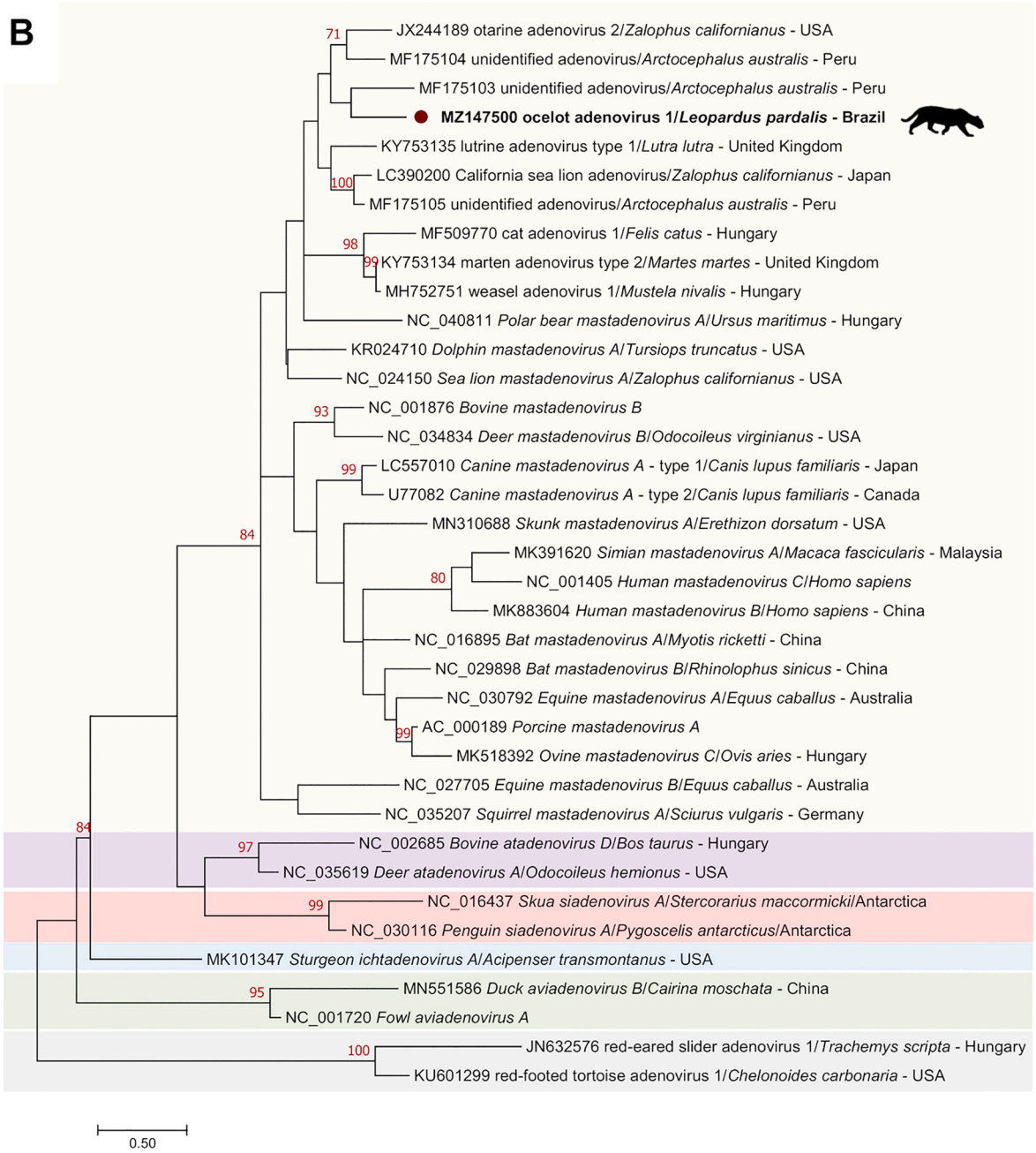

Fig. 2. (*continued*).

forelegs, head and neck, nail avulsion, luxation of carpo-radial joint, bilateral exophthalmia and hemothorax. Additionally, splenomegaly, mesenteric lymphadenomegaly and fat deposits in abdominal cavity were observed.

Histopathological analysis was partially impaired by the state of autolysis of the tissues. Nevertheless, we were able to observe moderate to marked pulmonary hemorrhage and edema; moderate medial hypertrophy of pulmonary arteries; moderate to marked, nodular, proliferative and sclerosing eosinophilic gastritis by spirurid nematodes, follicular hyperplasia of submucosal lymphoid aggregates (MALT) in small intestine, and mild to moderate, focal, granulomatous colitis with intralesional degenerate metazoan parasite structures. Additionally, there was cytoplasmic immunolabeling of the follicular lymphocytes, detected by amplification with polymers conjugated with peroxidase and phosphatase.

4. Discussion

We molecularly identified an adenovirus unique nucleotide sequence in several tissue samples from a wild free-ranging Neotropical felid. To the author's knowledge, potential adenovirus infections have been only reported once in wild felids - in a captive leopard (*Panthera pardus*) in India that presented intranuclear inclusion bodies and hepatitis (Gupta, 1978). In domestic cat (*Felis catus*), the first adenoviral infection was detected by light and electron microscopy in an individual co-infected with feline leukemia virus that presented intranuclear inclusion bodies in endothelial cells (Kennedy and Mullaney, 1993). This case was confirmed by PCR by Lakatos et al. (2017), who found DNA polymerase and hexon sequences more similar to adenoviruses identified in a marten and in a squirrel, respectively. Additionally, a different adenovirus was identified in rectal and pharyngeal swabs of a domestic cat with transient hepatic failure co-infected by feline immunodeficiency virus

(Lakatos et al., 1999). This strain was denominated feline adenovirus 1 and is highly similar to human adenovirus 1, with suggested transmission from human to cat (Ongrádi et al., 2019). Our findings expand the host range of adenoviruses in felids and report the first molecular description in wild felids worldwide.

According to the adenovirus species demarcation criteria, two factors support our findings as a novel species (Harrach et al., 2011): (1) the deduced amino acid divergence for the DNA-dependent DNA polymerase of the ocelot adenovirus when compared to the most similar sequences from GenBank, detected in pinnipeds (over 20%, above the >5–15% specified by the International Committee on Taxonomy of Viruses as a cut-off point), and (2) its detection in a novel host. The branch length of the novel adenovirus also indicates its divergence when compared to other adenovirus species. Thus, the sequence obtained in this study was tentatively named ocelot adenovirus 1 and it seems to be a candidate for a member of a novel mastadenovirus species. A limitation of our study is the short length of the amplified DNA polymerase sequence (223 bp after primer exclusion). Further studies are required to sequence the complete genome of this novel virus representing a putative novel species, to elucidate important characteristics, such as its genome organization and nucleotide composition. Culture of the ocelot adenovirus 1 is also desirable.

Canine adenovirus 1 and 2 were not detected in any of the tested samples. This was an unexpected result, since these viruses are able to infect a wide range of carnivore species and have been previously observed in high prevalences in wild free-ranging carnivores (specially in canids) from other countries: in wolves from Spain (76% positive to CAdV, i.e., 70.3% [26/37] infected by CAdV-1 and 5.4% [2/37] by CAdV-2) (Millán et al., 2016), and in red foxes from northern Norway and arctic foxes (*Vulpes lagopus*) from Svalbard archipelago, Norway (70% positive to CAdV-1, including 7 out of 10 red foxes and 7 out of 10 arctic foxes) (Balboni et al., 2019). In Brazil, human adenovirus was molecularly detected in 82.4% (14/17) of evaluated foxes (pampas fox and crab-eating fox) from the southern region; 29.4% (5/17) of them co-infected with canine adenoviruses (Monteiro et al., 2015). Nevertheless, these authors did not specify the prevalence in each fox species, nor the specific human and canine adenovirus species detected. Despite the low adenovirus prevalence observed herein (1.9%, 1/52), continuous surveillance is required to evaluate its impact over Neotropical carnivores, especially due to the pathogenicity of certain species for wild carnivores.

The detection of a novel adenovirus in several internal organs of an ocelot indicates a systemic infection. The histopathological findings described herein are compatible with parasitic infection. The impact of the observed parasitosis and/or presence of other concomitant viral infections on the individual's immune system cannot be ruled out and requires further investigation. There was positive immunolabeling against adenovirus in a lymph node that belonged to the PCR-positive ocelot. Nevertheless, instead of the expected nuclear labeling, it was cytoplasmic. Although not conclusive, immunohistochemistry potentially indicated the viral antigen's presence. The lack of data regarding the pathogenicity of adenovirus infections in cats and other felids makes it difficult to link any of the histopathological findings (several of them related to the parasitic infections) described in the present study to adenovirus infection. Future research will be necessary to assess the impacts (if any) of the novel adenovirus detected in ocelots in felid species.

5. Conclusion

Herein we report a novel adenovirus species, the first in ocelots and in wild felids from South America. Although no adenovirus-associated lesions have been found, the virus' pathogenicity cannot be ruled out. Future studies will be conducted to obtain the complete genome of this novel adenovirus. Creation of an adenovirus surveillance program for wild carnivores in Brazil is also advised. Finally, herein we also demonstrate the epidemiological potential of viral surveillance surveys conducted in association with studies investigating the health status of roadkill wildlife.

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CRediT authorship contribution statement

Henrique Christino Lial: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft. **Pedro Enrique Navas Suárez:** Investigation, Methodology, Writing – original draft. **Ana Carolina Ewbank:** Investigation, Methodology, Writing – review & editing. **Helena Exposto Novoselecki:** Investigation, Writing – review & editing. **Eduardo Ferreira-Machado:** Investigation, Writing – review & editing. **Cinthya dos Santos Cirqueira:** Investigation, Methodology, Writing – review & editing. **Natália Coelho Couto de Azevedo Fernandes:** Investigation, Methodology, Writing – review & editing. **Fernando Esperón:** Investigation, Methodology, Writing – review & editing. **José Luiz Catão-Dias:** Funding acquisition, Project administration, Supervision, Writing – review & editing. **Carlos Sacristán:** Conceptualization, Data curation, Formal analysis, Investigation, Supervision, Writing – original draft.

Declaration of Competing Interest

None.

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