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Original Contribution

Molecular Detection of Human Adenovirus and Rotavirus in Feces of White-Eared Opossums

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Abstract: The white-eared opossums (*Didelphis albiventris*) is the largest Brazilian marsupial and a great example of animal synanthropy. Considering the high potential as a carrier of viruses originating from environmental contamination, the presence of *Human adenovirus* (AdV) and rotavirus was investigated in the feces of rescued white-eared opossums, which were in the process of rehabilitation. The feces of 49 animals were initially investigated by immunochromatography, with three samples positive for AdV and one sample positive for rotavirus. When submitted to PCR and *nested* PCR, the samples of six animals were positive for AdV and three animals were positive for group A rotavirus. Two positive samples in the immunochromatographic assay were not confirmed by PCR. After sequencing and phylogenetic analysis of AdV samples, all were identified within the genus *Mastadenovirus*, one being HAdV-C, four HAdV-E, and one being similar to a *Mastadenovirus* found in primates. This is the first report of molecular confirmation of human adenovirus and rotavirus in white-eared opossums. These data could be important of anticipation some emerging diseases and their effects on ecosystems health.

Keywords: Didelphidae, Environmental virology, Zoonosis, Mastadenovirus, Rotavirus A, Brazil

Introduction

Some wild species have adapted to live with humans and have settled in urbanized areas, adapting to such conditions despite man's will. These species are classified as synanthropic (Daszak et al. 2000). The genus *Didelphis* is the example of synanthropic animal, being represented in Brazil by three species: the black-eared opossum (*Didelphis aurita*), the common opossum (*Didelphis marsupialis*), and the white-eared opossum (*Didelphis albiventris*) (Gonçalves et al. 2009).

The white-eared opossum (Lund 1840) is the largest of the Brazilian marsupials, inhabiting gallery forests as well as open areas, such as fields. Opossums act as effective seed dispersers, and the species is classified as generalist in relation to their diet and habitat selection. These facts enable them to obtain success in well-wooded rural and urban areas (Lessa and Geise 2010). There are indications that the

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white-eared opossum may be a reservoir and virtual transmitter of some pathogens, such as bacteria, parasites, protozoa, or viruses (Jikion et al. 2007).

Human mastadenoviruses (HAdVs) are classified in the family *Adenoviridae* and *Mastadenovirus* genus, which contains seven known HAdV species HAdV-A to HAdV-G (Huang and Xu 2013). HAdVs are double-stranded, non-enveloped, linear DNA viruses of 34–36 kbp length. To date, 88 genotypes of HAdV have been reported (Dhingra et al. 2019). Infections are predominantly subclinical, and overt disease develops upon the occurrence of immunosuppression or other cofactors (Lion 2014). HAdV genotypes classified as HAdV-B, HAdV-C, and HAdV-E are associated principally with respiratory diseases; HAdV-A, HAdV-D, HAdV-F, and HAdV-G with gastrointestinal diseases; and HAdV-D and HAdV-E with ocular diseases. The majority of HAdV types belong to species HAdV-D (57 types) followed by species HAdV-B (16 types) (ICTV 2019). Despite the fact that species HAdV-C comprises only 5 types so far (1, 2, 5, 6, and 57), these are clinically more significant than species HAdV-B and HAdV-D in causing severe manifestations in immunocompromised individuals (Lion 2014).

Member of the family Reoviridae, the genus *Rotavirus* (RV), has three main species: *Rotavirus A*, *Rotavirus B*, and *Rotavirus C*, each one containing 11 segments of double-stranded RNA. Since they are segmented, the genomes of RVs allow rearrangement events, producing new strains with different combinations of genome segments. Due to this mechanism, new strains emerge each year, being an important device for generating genetic diversity and boosting the evolution of RVs (Esona et al. 2010). RV strains are associated with acute diarrhea in humans and animals (Coria-Galindo et al. 2009). Although a large part of the studies reflect the restriction of these viruses to a host, inter-species transmission has been documented (Martella et al. 2010). Recently, new genotypes found in a variety of animals have been reported. Thus, monitoring rotaviruses in domesticated and wild animals can potentially identify emerging zoonotic pathogens (Esona et al. 2010). Considering the high potential of the species *D. albiventris* as a carrier of viruses originating from environmental contamination, the objective of this study was to evaluate the presence of different species of adenovirus (AdV) and rotavirus in the feces of the white-eared opossums, which were received at the Wildlife Rehabilitation Nucleus and Wild Animal Screening Center (NURFS/CETAS) of the Federal University of Pelotas (UFPEL) and were in the process of rehabilitation.

Materials and Methods

From November 2017 to October 2018, 49 free-living white-eared opossums were received at the Wildlife Rehabilitation Nucleus and Wild Animal Screening Center (NURFS/CETAS) of the Federal University of Pelotas (UFPEL). The NURFS is a reference in the care of wild animals in the south of Brazil (NURFS 2016). Data about the origin of the animals (municipalities, districts or highways), when available, were obtained from agents of the supervisory bodies or third parties, when the animals arrived. The animals were rescued in the municipalities of Pelotas (latitude: 31° 46′ 19″ S longitude: 52° 20′ 33″ W), Rio Grande (latitude: 32° 1′ 60″ S, longitude: 52° 5′ 55″ W), Capão do Leão (latitude: 31° 45’ 48″ S, longitude: 52° 29′ 02″ W), and Cristal (latitude: − 30.9901, longitude: − 52.038) (Fig. 1).

Fecal samples from the 49 white-eared opossums, which did not show clinical signs of disease, were collected in sufficient amount for analysis, stored in Eppendorf tubes®, and sent to the Virology Laboratory of the UFPEL and to the Molecular Microbiology Laboratory of Feevale University for processing and molecular analysis purposes. Feces were packed at − 80°C and thawed at the time of processing.

Initially, fecal specimens were screened using a lateral flow immunoassay for the presence of rotavirus and adenovirus (OnSite Rota/Adeno Ag Rapid Test, CTK Biotech).

Subsequently, the extraction of nucleic acids was performed with the Mini Spin Plus kit (BioPur®) for DNA and Trizol for RNA. Complementary DNA synthesis (cDNA) was performed with the GoScript™ Reverse Transcriptase kit(Promega®). Viral genome detection was performed by PCR and *nested* PCR using 20 µM of each oligonucleotides, 25 µl of GoTaq Colorless Master Mix (Promega®), 18 µl of nucleases-free water, and 5 µl of DNA or RNA sample. Positive and negative controls were used in all reactions. For the detection of AdV, a *nested* PCR was used to amplify the DNA polymerase region and for RV a PCR targeting the VP6 region of the viral genome, which is able to detect genomes belonging to group A of the genus Rotavirus. Details of the primers used are described in Table 1. For the detection of AdV, the reactions started at 94°C for 5 min, followed by 40 cycles at 94°C and 50°C for 30 s and 72°C

Figure 1. Map with the location of the municipalities (highlighted) of the state of Rio Grande do Sul, Brazil, where the specimens of white-eared opossums were found and then analyzed for the presence of adenoviruses and rotavirus. The municipalities of origin of the specimens are marked on the map: Rio Grande (dark blue circle), Pelotas (purple circle), Capão do Leão (green circle), and Cristal (light blue circle) (Color figure online).

Table 1. Oligonucleotides Used for Genome Detection of AdVs and RVs.

	Primer forward	Primer reverse	References
RV	5′-GATGTCCTGTACTCCTTGT-3′	5′-GGTAGATTACCAATTCCTCC-3′	Monteiro et al. (2015)
AdV	5′-CAGCCKCKGTTRTGYAGGGT-3	5′-GCHACCATYAGCTCCAACTC-3′	Li et al. (2010)
AdV-*Nested*	5′-GGGCTCRTTRGTCCAGCA-3′	5′-TAYGACATCTGYGGCATGTA-3′	Li et al. (2010)

for 1 min and one last step ending at 72°C for 10 min (Li et al. 2010). The RV reactions started at 94°C for 5 min, followed by 30 cycles of 94°C, 58°C and 72°C for 1 min each and ending at 72°C for 10 min.

The amplified samples were purified using the PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen®) and sequenced by the Sanger method. The editing of the *contigs* was performed through the BioEdit program, and the construction of the phylogenetic tree was made through the Mega 7 program, the maximum likelihood method, based on the Tamura 3-parameter model (Tamura 1992). The tree was constructed using AdV

Table 2. Entry and Exit Log of 49 Specimens of *D. albiventris* at the NURFS/CETAS-UFPEL, Whose Stool Samples Were Collected in 2017 and 2018 for Molecular Detection of Adenoviruses and Rotavirus.

Identification	Date of entry	Origin	PCR diagnosis	Exit date	Progress
1130/17	–	–	Negative	–	–
1191/17	11/06/2017		Negative	–	Death
1192/17	11/06/2017	–	Negative	–	Death
1371/17	–	–	Negative	–	–
LMM 5102	–	–	Positive RV-A	–	–
74/18	02/09/2018	Pelotas	Negative	02/24/2018	Rehabilitation
78/18	02/09/2018	–	Negative	02/26/2018	Rehabilitation
90/18	02/18/2018	Capão do Leão	Negative	04/09/2018	Rehabilitation
94/18	02/18/2018	Capão do Leão	Negative	04/09/2018	Rehabilitation
130/18	03/07/2018	–	Negative	03/08/2018	Euthanasia
161/18	03/29/2018	Rio Grande	Negative	04/09/2018	Rehabilitation
181/18	04/06/2018	Pelotas	Negative	05/03/2018	Rehabilitation
183/18	–	–	Negative	–	–
187/18	04/07/2018	Pelotas	Negative	04/24/2018	Euthanasia
LMM5101	04/11/2018	Pelotas	Positive HAdV-E	04/11/2018	Rehabilitation
200/18	04/13/2018	Pelotas	Negative	04/15/2018	Rehabilitation
201/18	04/13/2018	Pelotas	Negative	04/15/2018	Rehabilitation
LMM5078	05/22/2018	Pelotas	Positive HAdV-C and RV-A	–	Rehabilitation
260/18	05/24/2018	Pelotas	Negative	05/24/2018	Rehabilitation
265/18	05/29/2018	Rio Grande	Negative	06/22/2018	Rehabilitation
270/18	06/06/2018	Rio Grande	Negative	07/27/2018	Rehabilitation
271/18	06/06/2018	Pelotas	Negative	06/07/2018	–
272/18	06/09/2018	Pelotas	Negative	06/10/2018	–
316/18	07/18/2018	Pelotas	Negative	07/17/2018	–
323/18	07/18/2018	Pelotas	Negative	08/18/2018	Death
324/18	07/20/2018	Pelotas	Negative	07/27/2018	–
326/18	07/26/2018	Pelotas	Negative	08/05/2018	–
356/18	05/30/2018	Capão do Leão	Negative	09/19/2018	–
367/18	09/05/2018	–	Negative	09/05/2018	–
464/18	10/02/2018	Capão do Leão	Negative	10/02/2018	Euthanasia
482/18	10/08/2018	–	Negative	–	–
521/18	10/17/2018	Pelotas	Negative	11/11/2018	–
LMM 5082	10/17/2018	Pelotas	Positive RV-A	10/30/2018	–
523/18	10/17/2018	Pelotas	Negative	10/30/2018	–
524/18	10/17/2018	Pelotas	Negative	10/30/2018	Release
528/18	10/17/2018	Pelotas	Negative	10/30/2018	Release
LMM5070	10/20/2018	Pelotas	Positive HAdV-E	11/16/2018	Release
548/18	10/21/2018	Pelotas	Negative	11/10/2018	Release
549/18	10/21/2018	Pelotas	Negative	11/10/2018	Release
LMM5104	10/22/2018	Pelotas	Positive monkey Mastadenovírus	10/22/2018	Release
565/18	10/26/2018	–	Negative	11/16/2018	Release
LMM5061	10/26/2018	Pelotas	Positive HAdV-E	12/01/2018	Release
594/18	10/29/2018	Cristal	Negative	11/21/2012	Release
602/18	10/29/2018	Capão do Leão	Negative	11/21/2018	Release
611/18	11/01/2018	Rio Grande	Negative	12/01/2018	Release
657/18	11/09/2018	Pelotas	Negative	11/09/2018	Release

Table 2. continued

Identification	Date of entry	Origin	PCR diagnosis	Exit date	Progress
LMM5071	11/13/2018	Rio Grande	Positive HAdV-E	11/24/2018	Release
M31	–	–	Negative	–	–

sequences obtained by Sanger sequencing, and reference strains from the NCBI GenBank database. Bootstrap values are indicated at each tree root (Kumar et al. 2016).

Results

The animals screened in this study were either juveniles (n = 22; release weight 160 g), or adults (n = 27) with no distinction between sexes. For the analysis, the collection of biological material of animals of different litters was prioritized in order to avoid vicious samples. Whenever possible the animals received were rehabilitated and released, when they did not die. Data related to the origin and destination of the animals, as well as the dates of entry and exit, were compiled into a table with the intention of facilitating the epidemiological and contextual understanding of the process (Table 2).

In the qualitative immunochromatographic assay, three samples (1130/17, 161/18, and LMM5071) were positive for AdV and one positive for RV (1130/17) (data not shown). When submitted to analysis by PCR and *nested* PCR, two positive samples in the immunochromatographic assay were not confirmed (161/18 and 1130/17). In total, feces of six animals (LMM5078, LMM5061, LMM5101, LMM5070, LMM5071, LMM5104) tested positive for AdV by *nested* PCR. The animals LMM5101, LMM5078, LMM5070, LMM5104, and LMM5061 were rescued in three geographically distinct areas (Laranjal, Areal, and Fragata neighborhoods) in the municipality of Pelotas. The animal LMM5071 was rescued in the municipality of Rio Grande. Since the animals were received at NURFS-CETAS on different dates, they belong to different litters.

After amplification of the viral genome and sequencing of the AdVs, the phylogenetic tree classified all the positive samples to the genus *Mastadenovirus*. They were LMM 5078—HAdV-C; LMM 5061, LMM 5101, LMM 5070 and LMM 5071—HAdV-E; and LMM 5104—monkey *Mastadenovírus* (Fig. 2). For Group A of the genus Rotavirus, three samples were positive in PCR (LMM 5078, LMM 5082, and LMM 5102).

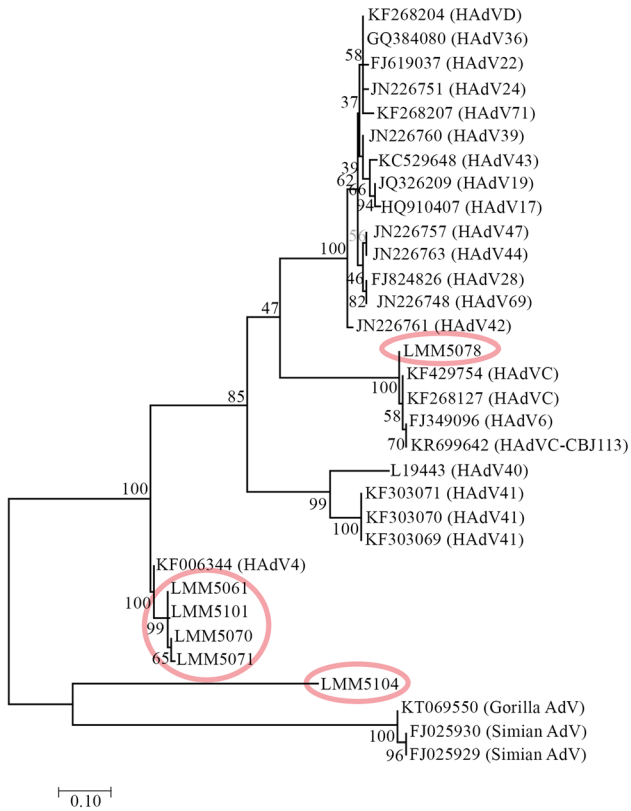

Figure 2. The phylogenetic analysis was carried out by the maximum likelihood method based on the Tamura 3-parameter model. The tree was constructed using AdV sequences obtained by Sanger sequencing and reference strains from the NCBI GenBank database. Bootstrap values are indicated at each tree root. The evolutionary analyses were conducted using MEGA7 software. Positive samples for AdVs are highlighted in red circles (LMM 5078—HAdV-C; LMM 5061, LMM 5101, LMM 5070, and LMM 5071—HAdV-E; LMM 5104—monkey *Mastadenovírus*) (Color figure online).

Discussion

The present study is the first to report the identification of HAdV in white-eared opossums. AdVs infect a wide variety of species, from fish to superior mammals (Carducci et al. 2009). The presence of an AdV (genus *Atadenovirus*) in a marsupial (*Trichosurus vulpecula*) inhabiting New Zealand was first described in 2002 (Thomson et al. 2002).

Adenoviruses are generally species-specific. However, they are also able to infect closely related species as predicted by phylogenetic data (Wevers et al. 2011). Recently, there was a report discussing the detection of HAdV-C in *Arctocephalus spp*. (Southern fur seals) and *T. vulpecula* (common brushtail possum), probably caused by the exposure of animals to contaminated water (Chiappetta et al. 2017). In our study, the molecular fragments of AdV detected in the LMM 5078 sample were similar to HAdV-C. This fact suggests that the detection of the viral genome in the feces of these animals was due to the probable ingestion of water, food, and/or other contaminated material, with subsequent fecal elimination of the virus (Victoria et al. 2010).

Monteiro et al. (2015) reported a similar occurrence in feces of *Lycalopex gymnocercus* (pampas fox) and *Cerdocyon thous* (crab-eating fox), where 82% of the samples (14/17) were contaminated with HAdV-C. The authors also reported that two samples contained remains of plastic bags, indicating that the animals may have ingested contaminated human waste. In our study, adenovirus fragments detected in samples LMM 5061, LMM 5101, LMM 5070, and LMM 5071 were similar to HAdV-E, suggesting the potential for exposure of white-eared opossums to contaminated water or food. AdVs are more resistant than other enteric viruses to water and sewage disinfection processes (Victoria et al. 2010), which promotes the presence of these microorganisms in high concentrations in wastewater, especially in endemic or precarious regions (Vieira et al. 2012). Due to the high frequency of HAdV detection in various types of aquatic ecosystems and their resistance to water and sewage treatment processes, these agents have been considered good indicators for assessing human viral contamination in the environment (Heller et al. 2003). These data point to the influence of anthropic action in relation to wild animals.

A few studies report the detection of RV-A in wild animal feces, such as the Monteiro et al. (2015) study where 41% (7/17) of the stool samples of *L. gymnocercus* and *C. thous* were considered positive for Rotavirus Group A. The presence of RV-A has also been reported in *Nyctereutes procyonoides* (raccoon dog), *Paguma larvata* (masked palm civet), and *Sus scrofa* (wild boars) (Abe et al. 2010; Okadera et al. 2013). In our study, only three samples were positive for RV-A (LMM 5078, LMN 5082, and LMM 5102), when analyzed by PCR. Contact with human feces during fluid or feeding intake provides a mechanism by which white-eared opossums can come into contact with RVs. Human rotaviruses were also detected in surface water and sewage, as well as isolated in drinking water (Rutjes et al. 2009). In addition, the small number of positive samples from the present study may be related to the sample size that was tested. It is also important to note that RNA is generally less stable than DNA and more susceptible to degradation, decreasing the potential for recovery of good quality RNA for testing (Chiappetta et al. 2017). Thus, further studies using broader assays would be needed to confirm the absence of rotaviruses in these samples.

Although some viral infections reported in marsupials are associated with clinical signs, there are many whose impact on animal health has not yet been determined (Nascimento and Horta 2014). Our results indicate the occurrence of HAdVs and RV-A in feces of white-eared opossum found in the extreme south of Brazil.

Conclusion

This is the first report of molecular confirmation of human adenovirus and rotavirus in marsupial white-eared opossums. These data lead us to develop studies and adopt measures that promote knowledge about the importance of these species as reservoirs of several etiological agents, being important to anticipate some emerging diseases and their effects on the health of ecosystems.

Acknowledgments

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Compliance with Ethical Standards

Ethical Approval All ethical procedures were respected, and the activities were officially authorized by the National Council for the Control of Animal Experi-

mentation (CONCEA), under Registration No. 23110.102426/2017-10.

Human and Animal Rights All applicable institutional and/or national guidelines for the care and use of animals were followed.

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