Adenoviruses of canine and human origins in stool samples from free-living pampas foxes (*Lycalopex gymnocercus*) and crab-eating foxes (*Cerdocyon thous*) in São Francisco de Paula, Rio dos Sinos basin

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Abstract

The spread of enteric viruses of domestic animals and human beings to wild species can be facilitated by the resistance of these viruses on the environment and their ability to be transmitted by water and contaminated food. The health status of the populations of pampas foxes (*Lycalopex gymnocercus*) and crab-eating foxes (*Cerdocyon thous*) is largely unknown and the landscapes occupied by these animals in southern Brazil have been threatened by human occupation and expansion of agriculture. In this work, the search of genomes of human and canine adenoviruses in feces from these wild carnivores was used to track the dissemination of domestic animals and human pathogens to the free-living populations in a wildlife reserve located in southern Brazil. This was performed by virus-specific differential real-time polymerase chain reactions (qPCR) on stool specimens, avoiding capture and additional stress to the animals. Genus-specific conventional reverse-transcriptase PCR (RT-PCR) was complementarily performed aiming the detection of enteroviruses (EV) and rotaviruses (RV) on these same samples. HAdV genomes were found on 14 out of the 17 (82.35%) stool samples analysed, whereas CAV was found co-infecting 5 of these samples. RV genomes were detected on 7 of the 17 samples (41.18%) and all samples were negative for EV. The results point to the dispersion of HAdV and RV at a high rate to these species of South American wild carnivores, which can be an effect of growing anthropisation of the habitat of these animals.

Keywords: Lycalopex gymnocercus, *Cerdocyon thous*, adenovírus, rotavirus, enterovirus.

Adenovírus de origens canina e humana em fezes de graxains (*Lycalopex gymnocercus*) e cachorros-do-mato (*Cerdocyon thous*) de vida livre em São Francisco de Paula, bacia do Rio dos Sinos

Resumo

A disseminação de vírus entéricos de animais domésticos e seres humanos para espécies selvagens pode ser facilitada pela resistência desses vírus no ambiente e sua capacidade de ser transmitida por água e alimentos contaminados. O estado de saúde das populações de Graxains-do-campo (*Lycalopex gymnocercus*) e Cachorros-do-mato (*Cerdocyon thous*) é em grande parte desconhecida e as paisagens ocupadas por estes animais no sul do Brasil têm sido ameaçadas pela ocupação humana e a expansão da agricultura. Neste trabalho, utilizou-se a pesquisa de genomas de adenovírus humanos (HAdV) e caninos (CAV-1 e -2) em amostras fezes desses carnívoros selvagens com vistas a diagnosticar a disseminação de patógenos de animais domésticos e seres humanos às populações de vida livre em uma reserva de vida selvagem, localizado no sul do Brasil. Foram realizadas reações em cadeia da polimerase diferenciais e em tempo real (qPCR) de adenovírus específicos em amostras de fezes, evitando a captura e estresse adicional para os animais. PCRs gênero-específicas convencionais com transcrição reversa prévia (RT-PCR) foram ainda realizadas visando a detecção de enterovírus (EV) e rotavírus (RV) nestas mesmas amostras. Genomas de HAdV foram encontrados em 14 a 17 amostras de fezes (82.35%) analisados, Considerando que o CAV foi encontrado coinfectando 5 destas amostras. Genomas de RV foram detectados em 7 das 17 amostras (41.18%) e todas as amostras foram negativas para EV. Os resultados apontam para a dispersão de HAdV e RV em uma taxa elevada para estas espécies de carnívoros selvagens sul-americanas, que podem ser um efeito da crescente antropização do habitat desses animais.

Palavras-chave: Lycalopex gymnocercus, *Cerdocyon thous,* adenovírus, rotavírus, enterovírus.

1. Introduction

The conservation of free-living wild animals is influenced by several factors and the dissemination of pathogens from domestic animals and human beings to these populations may hamper conservation efforts (Murray et al., 1999). In rapidly-developing countries, like Brazil, deforestation, expansion of agriculture, and proximity of forests to urban areas may generate conflicts that jeopardise the health of the environment, but also may result in the dispersal of vectors and adaptation of pathogens to new hosts (Alexander et al., 2010; Daszak et al., 2001; Whiteman et al., 2008).

Two species of wild canids inhabit the south of Brazil: pampas foxes (*Lycalopex gymnocercus*) and crab-eating foxes (*Cerdocyon thous*) (Di Bitetti et al., 2009; Faria-Corrêa, 2004). These carnivores are considered opportunistic and generalist, thus benefitting in part from some anthropogenic changes, such as the extra supply of food generated by the accumulation of wastes or even by plantations and livestock (Faria-Corrêa, 2004). These behavioural characteristics, associated with the fragmentation and loss of habitats, can be considered as the most significant negative factors for the conservation of wild carnivores, and may thus facilitate the meeting of these animals with individuals of *Canis familiaris*, thereby increasing the possibility spill-over of new microorganisms from these species to wild animals (Alexander et al., 2010; Courtenay et al., 2001; Daszak et al., 2001; Hübner et al., 2010; Mattos et al., 2008).

Although some studies have been conducted on captive animals (Batista et al., 2005), there are few reports on clinical or sub-clinical viral infections in free-ranging wild carnivores from South America (Giannitti et al., 2012; Hübner et al., 2010). Data on the mortality of neotropical wildlife and their possible causes are still poorly assessed, possibly because of the difficulty in conducting longitudinal studies, considering the high cost and time needed for such purpose (Curi et al., 2010; Deem e Emmons, 2005). Especially for the pampas and crab-eating foxes, the literature is scarce, and there are only a few reports of the presence of viral and parasitic infections (Fiorello et al., 2007; Giannitti et al., 2012; Majláthová et al., 2007). However, the occurrence of other viruses on these populations is unknown.

Two types of canine adenovirus (CAV), genus *Mastadenovirus*, family *Adenoviridae* are double-stranded DNA, non-enveloped viruses, are described infecting domestic dogs, canine adenovirus types 1 and 2 (Decaro et al., 2008). CAV-1 is the agent of canine infectious hepatitis, while the CAV-2 is one of the agents involved in the etiology of the kennel cough syndrome (Decaro et al., 2008). A number of adenoviruses were also described in human beings and lately being used as reliable markers of fecal contamination of the environment (Silva et al., 2011). Not only adenoviruses. Those viruses are non-enveloped, being highly resistant on the environment; its genomes may be readily detectable on environmental samples (Miagostovich et al., 2008; Silva et al., 2011; Wolf et al., 2010). Other viruses which may be transmitted by the fecal-route are enteroviruses (EV, genus *Enterovirus*, family *Picornaviridae*) and rotaviruses (RV, genus *Rotavirus*, family *Reoviridae*), two non-enveloped RNA viruses. Since they are host-specific viruses and excreted in high titers by the fecal route, its detection and identification may allow the tracking of sources involved on fecal contamination of water and other environmental matrices (Silva et al., 2011; Wolf et al., 2010).

The major goal of the present study was to investigate the presence of viruses typical from domestic dogs and human beings in stool samples from pampas fox collected in a natural reserve from southern Brazil. This was an effort to examine whether there are transmission of these viruses to the wild animals as an effect of the growing urbanisation and agricultural activities in this particular geographical area. The presence of other enteric viruses, namely EV and RV was also investigated using conventional reverse-transcriptase PCR (RT-PCR).

2. Material and Methods

2.1. Study area

Samples were collected at the Parque Natural Municipal da Ronda (PNMR), a natural reserve inside the urban perimeter of the municipality of São Francisco de Paula, the middle portion of Rio dos Sinos basin, RS, Brazil.

The nature reserve is located in the Western region of the state of Rio Grande do Sul, southern Brazil, in the north of the Rio dos Sinos watershed. The PNMR has a total area of 1200 ha, covered by areas of grassland and Mixed Ombrophilous Forest (Fraga et al., 2008). Since it is a region of transition between two biomes, the area attracts numerous representatives from the fauna of the Atlantic forest and its associated ecosystems (Fontana et al., 2003). The surrounding areas are characterised by the presence of monocultures of *Pinus sp*. which have disrupted the flow of species from outside the park. A landfill for municipal solid waste disposal was located within the natural reserve area for 20 years and was disabled by the year 2005. However, there are until nowadays illegal and inappropriate disposal of waste in some locations of the PNMR, which lead to contamination of soil, water and can characterise an important source of transmission of pathogens to the fauna, especially carnivores that are often observed looking for food on these wastes.

2.2. Sampling

Seventeen (17) stool samples were collected during the months of April and September 2011. The stools were identified based on the behavioural characteristics of the pampas fox: these carnivores evacuate preferably at clean sites, on trails and above rocks. The points of each collection were demarcated with GPS (Global Positioning System). The identification of the feces was complemented by the visualisation of remnants of food characteristic of the pampas fox diet. The material was washed, filtered and observed under a magnifier for the identification of the components of the diet. The weather was dry during the collections, which facilitated the direct identification

of the material. The sampling was performed using sterile gloves and the samples were transported in a thermal box (4°C) to the laboratory and stored at in -80°C freezer until processing.

2.3. Molecular detection of viral genomes and differentiation of adenoviruses

The commercial kit RTP DNA / RNA Virus Mini Kit (Invitek™, STRATEC® Molecular, Berlin, Berlin 13125, Germany) was employed to extraction of viral nucleic acids (DNA, HAdV-C and TTV; RNA, EV and RV) from 400 mg of fecal material diluted 10X (v/v) in Eagle's Minimal Essential Medium (E-MEM), according to the manufacturer›s instructions. The viral DNA or RNA obtained was stored in a freezer at –80 °C for later processing.

Real-time PCR (qPCR) was applied for the detection human adenovirus (HAdV) genomes using oligonucleotides described previously (VTB2-HAdVCf 5'-AGACGTACTTCAGCCTGAAT-3'; VTB2-HAdVCr 5'-GATGAACCGCAGCGTCAA-3') (Wolf et al., 2010). Nucleotides for the specific amplification of CAV-1 and -2 genomes were originally designed, the forward primer was CAV-F1: 5´-CACGATGTGACCACTGAGAG-3´; reverse: CAV-R1 5´-GGTAGGTATTGTTTGTGACAGC-3´), whose target is a fragment of 300 to 350 base pairs in the gene that encodes the hexon protein. The qPCR reactions had been optimised and carried out under the same conditions, using as controls for absolute quantification of viral DNA from prototype samples of HAdV-2, HAdV-5, CAV-1 and CAV-2, whose amount was correlated with the equivalent of genomes on viral titres determined by microplate titration in cell cultures. qPCR reactions were conducted in a thermal cycler iQ5™ Bio-Rad (Biorad™, Hercules, California 94547, USA) using commercial kit SYBR®Green Platinun® qPCRSupermix-UDG (Life Technologies™ Corporation, Carlsbad, California 92008, USA) in accordance with the manufacturer›s instructions. For each 25 µL reaction, 12.5 µL of the mix were used, 1.0 mL aliquots of each oligonucleotide at a concentration of 20 pmol, 5.5 µL of DNAse/RNAse free sterile water and 5.0 uL of the nucleic acid extracted from each sample. Each reaction was composed of a denaturation cycle of 95°C by 10 min., followed by 50 cycles composed of one step of 95°C for 20 s, and one annealing step at 55°C to HAdV-C or 54°C for CAV-1/2. After, a denaturing curve was made to check the specificity of amplification products; no inhibitory effects were observed using experimentally contaminated control feces. The oligonucleotides chosen for the amplification of the HAdV genome did not give a positive reaction for CAV and vice-versa.

For EV and RV, cDNA synthesis was obtained using High Capacity cDNA Reverse Transcription™ commercial kit (Life Technologies™ Corporation, Carlsbad, California, 92008, USA), with the aid of random primers, following the manufacturer›s instructions. The RT-PCR conditions were optimised and reactions were standardised as follows: (a) RV: 50 µL reaction mixtures consisting of 25 µL of GoTaq® Green Master Mix (Promega™ Corporation, Madison, Wisconsin 53711, USA), 18 µL of nuclease-free water, 1 µL of each primer for the VP6 gene (20 pmol, forward ROTAFEEVALE –FW: 5'-GATGTCCTGTACTCCTTGT-3'; reverse ROTAFEEVALE –REV: 5'-GGTAGATTACCAATTCCTCC-3', pan-specific for the Group A of genus *Rotavirus*) and 5 µL of nucleic acid; (b) *EV:* 25 µL of GoTaq® Green Master Mix (Promega™ Corporation, Madison, Wisconsin 53711, USA), 18 µL of nuclease-free water, 7.5 µL of nuclease-free water, 1 µL of each primer (20 pmol, forward ENT-F1: 5'-CCTCCGGCCCCTGAATG-3'; reverse ENT-R2: 5'-ACACGGACACCCAAAGTAG-3', specific for human EV) and 3 µL of cDNA. DNase/RNase free water was used as a negative control during all PCR assays. Amplification of the target genomic fragments was performed using a thermal cycler (MultiGene™, Labnet International, Edison, New Jersey 08837, USA). The PCR conditions were optimized for each virus group and were as follows: (a) RV: 94 °C for 5 min, 40 cycles of 94 °C for 1 min, 54 °C for 1 min (which was decreased by 0.5 °C at each of the 39 subsequent cycles), 72 °C for 1 min; (b) EV: 98°C for 5 min, 35 cycles of 94 °C for 1 min, 56°C for 1 min, 72 °C for 1 min.

3. Results and Discussion

Several studies suggested the transmission of viral agents between domestic and wild carnivores (Alexander et al., 2010; Batista et al., 2005; de Almeida Curi et al., 2010; Fiorello et al., 2007; Fletcher et al., 1979). Adenoviruses and other canine viruses are found infecting domestic dogs in the state of Rio Grande do Sul (Dezengrini et al., 2007). There is also serological evidence for infection by CAV on Brazilian maned wolves (de Almeida Curi et al., 2010). HAdV is ubiquitous in human populations (Eick et al., 2011; Ersching et al., 2010), as well as the virus often being found in environmental samples contaminated by human feces (Miagostovich et al., 2008; Silva et al., 2011; Wolf et al., 2010). HAdV-C has occurred in 14 of the 17 samples (82.35%) and 5 samples were co-infected by CAV (29.41%). RV was detected in seven of 17 samples (41.18%) and all resulted negative for EV (Table 1). The food content analyses carried out from the feces revealed the presence of fragments from small vertebrates, by the presence of teeth, nails, hair, bones and remains of adipose tissue and muscles, plus remnants of vegetation and seeds, as expected for the diet of these animals. Fragments of plastic garbage bags in two of the 17 samples tested, pointing to the opportunistic consumption of human waste by these animals. The close contact of the free-ranging pampas foxes with domestic dogs at the edges of the natural reserve, as well as the evidence obtained from the analysis of stool samples regarding the consumption of garbage by these wild animals, may explain the presence CAV in 5 of the stool samples and the high rates of circulation HAdV (14/17) among these individuals.

On the other hand, the presence of CAV in approximately a third of the samples analysed points to the danger of the transmission of domestic dog diseases to the pampas

Table 1. Results for the detection of enterovirus (EV), rotavirus (RV), canine adenovirus (CAV) and human adenovirus (HAdV) in pampas and crab-eating fox stool samples collected at the Parque Natural Municipal da Ronda (PNMR), Rio dos Sinos basin, Brazil.

Stool Sample	EV	RV	CAV	HAdV
1G	–	+	–	+
2G	–	+	+	+
3G	–	+	+	+
4G	–	–	–	–
5G	–	+	–	+
6G	–	+	–	–
7G	–	–	+	+
8G	–	–	–	+
9G	–	–	–	–
10G	–	–	–	+
11G	–	–	–	+
12G	-	+	-	+
13G	-	+	+	+
14G	-	-	-	+
15G	-	-	-	+
16G	-	-	-	+
17G	-	-	+	+

–=negative; +=positive

fox population. As an example, the emergence of a well-documented CAV outbreak in island foxes (*Urocyon litoralis*) may have occurred through the interspecies passage of domestic dogs viruses introduced by the previously infected sympatric populations of ferrets (*Spilogale gracilis amphiala*). CAV mortality was observed in free-living silver foxes (*Vulpes vulpes*), with high incidence of diseases especially in young individuals (Clifford et al., 2006).

HAdV may not constitute itself as a threat to the health of the animals, since it is more likely that the virus was ingested and eliminated in feces but not replicated in the gut of those animals. However, the passage of this virus of human origin by a wild canid species eventually constitute a marker that points to the contamination of food or water supplies by invasive anthropic activity on this ecosystem.

Enterovirus are not often found in dog populations, and as expected, no EV positive samples were found in the present study. RV was detected in 7 out of 17 stool samples collected. Canine rotavirus (CRV) most often causes mild enteritis, especially in puppies with less than two weeks, but the virus is also found in healthy animals (Can Sahna et al., 2008; Kang et al., 2007; Yeşilbağ et al., 2007). Pets are continually exposed to infection by rotavirus, and yet that did not show clinical signs, they can host the virus in your intestinal epithelium, or carry the same through their snouts and paws and transmit it to other species likely, including human beings (Matthijnssens et al., 2011; Song e Hao, 2009). Dogs infected by RV may act as a reservoir and source of disseminating viruses, maintaining their spread in the environment (Gabbay et al., 2003; Tarsitano et al., 2010). These infections may also have some deleterious effects on the health status of free-living populations of pampas foxes and crab-eating foxes.

There is a concern in detecting such viral agents to which populations of free-living wild carnivores may be exposed due to increased contact with pets. The disposal of solid wastes containing remnants of food is also very attractive to the pampas foxes and may be a source not only for the ingestion of human viruses but for toxic pollutants as well.

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