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Sequential circulation of canine adenoviruses 1 and 2 in captive wild carnivores, France

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ABSTRACT

Scarce data are currently available about the ecology of canine adenoviruses (CAdVs) in wild carnivores. In this paper, the consecutive circulation of CAdV-1 and CAdV-2 in wild carnivores maintained in a French zoological park is reported. A fatal CAdV-1 infection was observed in a Eurasian wolf (*Canis lupus lupus*), which displayed gross lesions, histopathological changes and immunohistochemical findings suggestive of CAdV-1 infection. The virus was isolated on cell cultures and its genome was determined through next-generation sequencing, resulting genetically related to a recent Italian CAdV-1 strain detected in an Italian wolf. Subsequently, subclinical circulation of CAdV-2 was demonstrated by molecular methods in wild carnivores maintained in the same zoological park, some of which had been previously vaccinated with a CAdV-2 vaccine. Virus detection at a long distance from vaccination and by unvaccinated animals was suggestive of infection by a CAdV-2 field strain, although no data are available about the extent and duration of shedding of CAdV-2 modified-live virus in wild or domestic carnivores. The present paper provides new insights into the CAdV ecology in wildlife, although future studies are needed to fully understand the pathogenic potential of both CAdVs especially in endangered carnivore species.

1. Introduction

Infections by canine adenovirus type 1 (CAdV-1) have been reported worldwide from several free-ranging and captive carnivore species included in the *Canidae, Ursidae* and *Mustelidae* families (Woods, 2001). Whilst the infection is well described in dogs, causing a systemic disease known as infectious canine hepatitis (ICH), mainly characterised by acute necrohaemorragic hepatitis, uveitis and interstitial nephritis (Decaro et al., 2008; Green, 2006), clinical signs and pathogenetic features are poorly defined in wild canids. The first cases in wildlife were described in 1930 in silver foxes (*Vulpes vulpes*) from North America, and the disease was defined "epizootic fox encephalitis" based on the neurological signs encountered (Green et al., 1930). Later reports have suggested the role of CAdV-1 in inapparent infections in foxes (Balboni et al., 2013; Walker et al., 2016a), with sporadic fatal cases reported in wild carnivores, such as fennec fox (*Vulpes zerda*) (Choi et al., 2014), red fox (*Vulpes vulpes*) (Walker et al., 2016b), gray fox (*Urocyon cinereoargenteus*) (Gerhold et al., 2007), black bear (*Ursus americanus*) (Pursell et al., 1983), Eurasian river otter (*Lutra lutra*) (Park et al., 2007), and Eurasian wolf (*Canis lupus lupus*) (Pizzurro et al., 2017). Most data currently available rely on serological studies, showing that circulation of CAdVs is relevant in several countries, with prevalences up to 97% in island foxes (*Urocyon littoralis*) (Garcelon et al., 1992) and 88% in grey foxes from California (Riley et al., 2004) and 94.7% in wolves (*Canis lupus*) from Alaska (Stephenson et al., 1982). In red foxes, CAdV seroprevalence was 19% to 64.4% in UK (Thompson et al., 2010; Walker et al., 2016a), 3.5% in Germany (Truyen et al., 1998), 59.6% in Scandinavia (Akerstedt et al., 2010) and 23.2% in Australia (Robinson et al., 2005). However, serological studies do not provide information about the disease, and, moreover, do not distinguish between CAdV-1 and the strictly related canine adenovirus type 2 (CAdV-2), one of the causative agents of the canine infectious respiratory disease (CIRD), a multifactorial disease of dogs (Decaro et al., 2008). Recently, circulation of CAdVs in domestic dogs has

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dramatically decreased, at least in developed countries, due to the extensive vaccination of dogs using cross-protective CAdV-2 modified-live virus. Nonetheless, re-emergence of adenovirus infections in dogs has been documented worldwide (Decaro et al., 2004; Benetka et al., 2006; Müller et al., 2010; Balboni et al., 2014; Pintore et al., 2016). Recent evidences suggest the role of foxes as reservoir of CAdV-1 (Balboni et al., 2017; Walker et al., 2016a). Red foxes are the most significant free-ranging wild species in Europe and represent a sympatric species with the domestic dogs, thus playing an important role in disease ecology, due to their high population density and intrusive behaviour (Bateman and Fleming, 2012; Gehrt et al., 2010). The threat of disease transmission from domestic animals to wildlife has generated a growing concern with respect to species conservation and disease ecology (Fiorello et al., 2006; Knobel et al., 2014). Though the scarce information available does not allow defining a clear picture on infection dynamics in the wildlife, a more complex interaction is feasible among all the carnivore species susceptible to CAdVs.

Here, we report the consecutive circulation of CAdV-1 and CAdV-2 in wild carnivores of a zoological park in France. A fatal CAdV-1 infection occurred in a Eurasian wolf, which was followed by CAdV-2 detection in some wild carnivores maintained in the same zoological park.

2. Materials and methods

2.1. Case report and sample collection

In May 2015, a 5-year-old female Eurasian wolf (*Canis lupus lupus*), housed in a large natural enclosure in the Parc Animalier de Sainte-Croix, Rhodes (France), was found in a coma state and died shortly afterwards. She had presented with an intermittent head shaking for 2 days and weakness and anorexia for 1 day. The carcass, designated as Wolf/835/2015/FRA, was submitted to necropsy and investigated for infectious causes of disease. Necropsy showed haemorrhagic enteritis as the main gross lesion, along with petechia at the coronary heart, a firm and slightly discoloured liver, haemorrhagic mesenteric lymph nodes and superficial wounds. Sera and tissues from intestine, liver, spleen and kidney were collected and submitted to molecular investigation and histopathology. The wolf belonged to a pack of eight established in 2006 by three German individuals, herein referred as pack 2, where she occupied the lowest rank in the pack hierarchy, representing an omega member. A pack of Eurasian wolves had been living in the park since 1986, referred as pack 1, initially founded by three French wolves, and later increased to 12 individuals. The last three wolves of this pack were transferred to another zoological park at the beginning of May 2015. In 2013 an exchange of enclosure between the two packs occurred. Further on, in December 2015 three individuals from a French zoo had been newly introduced generating a new pack, namely pack 3.

2.2. DNA extraction, amplification and screening for carnivore pathogens

Nucleic acids were extracted from frozen collected samples using the commercial kit QIAamp cador® Pathogen Mini Kit (QIAGEN) and were subjected to a screening for common carnivore pathogens by means of molecular assays. Real-time PCR TaqMan assays were performed for the detection of canine and feline parvoviruses (CPV/FPLV) (Decaro et al., 2005) and canine adenoviruses (CAdVs) (Dowgier et al., 2016). PCRs for carnivore coronaviruses (Gut et al., 1999), caliciviruses (Di Martino et al., 2007), haemoplasmas (Tasker et al., 2003), *Erlichia* spp. and *Anaplasma* spp. (Parola et al., 2000) were additionally carried out. Standardized procedures were used for in vitro isolation of common pathogenic bacteria. Samples were plated out on 5% sheep blood agar and cultured aerobically at 37 °C for 24 h for detection of aerobic pathogens. Bacteriological investigations were carried out by standard biochemical procedures and analytical profile index (API, BioMérieux Italia S.p.A., Rome, Italy).

2.3. Histopathology and immunohistochemistry

Tissues from liver and kidney were provided for histopathological examination and fixed in 10% buffered formalin. The samples were embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) following standard protocols. For immunohistochemistry, sections were treated with an anti-CAdV-1 polyclonal antibody collected from a convalescent dog (Pratelli et al., 2001).

2.4. Virus isolation

For virus isolation, Madin-Darby Canine Kidney (MDCK) cells were used (ATCC® CCL-34), which were grown in Dulbecco's modified minimum essential medium (D-MEM) supplemented with 10% foetal bovine serum (FBS). Tissues from spleen and liver, revealing the highest CAdV-1 DNA loads in real-time PCR analysis, were homogenised in D-MEM (10%, w/v) using a TissueLyser II (Qiagen, Hilden, Germany) and centrifuged at 8000 × *g* for 10 min. Supernatants were treated with antibiotics for 30 min (penicillin 5000 IU/ml, streptomycin 2500 µg/ml, amphotericin B 10 µg/ml), inoculated on partially confluent cell cultures and incubated at 37 °C in a 5% CO_2 incubator. After an adsorption time of 45 min, inocula were removed and D-MEM was added to reach the final volume. Cells were observed daily for the occurrence of cytopathic effect (CPE). H&E staining and indirect immunofluorescence (IIF) assays were performed to confirm virus isolation. On this purpose, cells grown on coverslips placed in 12-well plates were mock- or virus-infected accordingly, and coverslips were harvested at 72 h post infection. For detection of inclusion bodies, cells were fixed in Bouin's solution for 2 h and stained with H&E. For IIF assay, inoculated cells were fixed with acetone 80% for 30 min. Coverslips were rinsed twice with PBS and incubated 30 min in humidified chamber at 37 °C with a CAdV-positive dog serum diluted 1:50. Following incubation, coverslips were washed twice with PBS and incubated with goat anti-dog IgG conjugated with fluorescein isothiocyanate (Sigma Aldrich srl, Milan, Italy).

2.5. Next-generation sequencing

DNA for next-generation sequencing (NGS) was prepared from viral stocks obtained from semi-purified viral particles with the aim of sequencing the full-length genome of the isolated virus. Briefly, MDCK cells were infected with isolate Wolf/835/2015/FRA and at 48 h post-infection cell medium was collected and clarified by centrifugation at 1000 × *g* for 10 min at 4 °C. Supernatant was treated with DNase I (100 U/200 µl sample) and the resulting virion-enriched sample was subjected to viral DNA extraction using the QIAamp Pathogen Mini Kit (Qiagen), according to manufacturer's instructions. DNA was carefully quantified using the fluorometric Qubit dsDNA HS (High Sensitivity) Assay kit. Genomic DNA library was prepared using the Nextera DNA Sample Prep Kit (Illumina, San Diego, CA) following the manufacturer's protocol. Size selection step was done manually with Ampure XP magnetic beads (Beckman Coulter). Quality control analysis of the sample library was carried out using the QIAxcel Advanced system with the QIAxcel ScreenGel Software 1.4.0. Library samples were normalised as suggested by the manufacturer's instruction and sequencing was performed on the Illumina MiSeq instrument, version 2 (Illumina, San Diego, CA, USA), using MiSeq reagent kit.

2.6. Genome annotation and comparison

The total paired reads obtained by the NGS sequencing were checked for quality control using FastQC (Andrews, 2010) and sequence trimming was performed using the plugin Trim Ends in Geneious software package v10.1.3. The NGS sequences were mapped to CAdV-1 strain R1261 (GenBank accession number Y07760) as reference. The full-length genome sequence of the isolate Wolf/835/