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 typical 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 strains 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 were 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.

Key words: wild carnivores; canine adenovirus type 1; canine adenovirus type 2; zoological park.

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 et al., 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 gray 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., 2013; 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 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 et al., 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 (CAdV) (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 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/835/2015/FRA was annotated using the same CAdV-1 strain as reference using the Geneious software (version 10.1.3). The full-length genome of CAdV-1 strain Wolf/835/2015/FRA was deposited in the GenBank database under accession number MH048659.

2.7. Phylogenetic analysis

For a deeper molecular characterisation, the complete genome of the wolf CAdV-1 strain was aligned with the available sequences of CAdVs retrieved from GenBank using the MAFFT algorithm (Katoh et al., 2002) within the Geneious software package (version 10.1.3), including bat adenovirus as outgroup. Phylogenetic tree construction was performed using the neighbor-joining method with 1000 bootstraps and the Jones-Taylor-Thornton (JTT) substitution model with a gamma distribution among sites. However, since few complete genomes of CAdVs are currently

175 available in the databases, a comparative analysis based on the hexon and E3 genes was also carried
176 out, comprehensive of all the partial genomes deposited in Genbank.

177 ***2.8. Epidemiological surveillance on wolves and other carnivore species in the zoological park***

178 In order to collect data regarding the circulation of CAdVs in the zoological park, a serological
179 survey paired with a molecular investigation was conducted taking advantage of the samples
180 available from the archive and those collected on purpose. All the serum samples, as well as
181 archived tissue samples and biological materials, were collected from carnivores species by
182 veterinarians working at the Parc Animalier de Sainte-Croix or at the zoological park of origin,
183 from 2006 to 2016, mainly during animal capture or anesthesia for medical purpose or before
184 transfer.

185 ***2.8.1. Serological study***

186 A total of 52 sera belonging to 51 animals from different carnivore species were analysed for the
187 purpose of this study. Sera #12 and #13 belonged to the same animal but were collected at different
188 time points. Data regarding animals and sample collection are synthesised in Table 1, including date
189 and place of birth, vaccination status and any additional information. Sixteen sera (#1-16) belonged
190 to the archival collection of samples taken from Eurasian wolves living at The Parc Animalier de
191 Sainte-Croix and belonging to the two packs that had been originally residing in the park; in
192 addition, other 3 samples (#17-19) were from Eurasian wolves newly introduced in the park, which
193 have been sampled in their park of origin, before their arrival, and belonging to a third pack (Table
194 1). Thirty-three sera (#20-52) were available or promptly collected from different carnivore species
195 living in the park. In details, 22 archival sera collected between 2006 and 2016 belonged to 4
196 Alaskan tundra wolves *(Canis lupus tundrarum)* (sera #20-23), 10 arctic (Northewestern) wolves
197 (*Canis lupus arctos*) (#24-33), 2 timber wolves (*Canis lupus occidentalis*) (#35, #36), 1 European
198 pine marten *(Martes martes)* (#37), 1 raccoon (*Procyon lotor*) (#38), 1 brown bear (*Ursus arctos*)
199 (#43), and 3 arctic foxes (*Vulpes lagopus*) (#50-52). The 11 newly collected sera included 1 arctic

wolf (#34), 4 raccoons (#39-42), 2 red foxes (*Vulpes vulpes*) (#44, #45), and 4 arctic foxes (#46-49). All serum samples were tested for antibodies by virus neutralisation assay (VN), using CAdV-1 isolate 33/01 (Pratelli et al., 2001). For VN test, twofold dilutions of heat-inactivated serum (starting from dilution 1:2) were mixed with 100 $TCID_{50}$ of the virus in 96-well microtitre plates. After incubation at room temperature for 60 min, 2×10^4 MDCK cells were added to each well. Plates were read after 5 days of incubation at 37°C in a humidified 5% CO_2 atmosphere. A positive and negative controls were included for each test performed.

2.8.2. Molecular investigations

Molecular investigations were carried out on archival and newly collected samples. Animal living conditions in the park prevented an extensive sampling because of practical limitations, and only samples clearly identifiable were admitted to the analysis, which were collected from animals captured on purpose or located in individual boxes. Samples analysed and data collected are reported in Table 2. For red foxes, 3 urine samples from 2 captive animals (#44, #45) were available, including one sample (#53) collected from the ground and not clearly attributable to either animal. In addition, tissue samples were collected from one carcass of a free-ranging animal (#54) found dead in the enclosure of the Eurasian wolf pack no. 2 in December 2015. Samples were analysed by means of molecular tools, as described in paragraph 2.2. Nucleic acids were extracted using the commercial kit QIAamp cador® Pathogen Mini Kit (QIAGEN) and subjected to screening for common carnivore pathogens. Real-time PCR assays were performed for the detection and discrimination of CPV/FPLV (Decaro et al., 2005) and CAdVs (Dowgier et al., 2016). (RT-)PCR assays for carnivore coronaviruses (Gut et al., 1999), caliciviruses (Di Martino et al., 2007), haemoplasmas (Tasker et al. 2003), and *Erlichia* spp./*Anaplasma* spp. (Parola et al., 2000) were additionally carried out.

3. Results

3.1. CAdV-1 detection in the Eurasian wolf

Samples collected during necropsy tested positive for CAdV-1 by means of CAdV discriminating real-time PCR, with a titre of 9.75 x 10^7, 1.18 x 10^6, and 4.34 x 10^6 viral DNA copies μl^{-1} in the spleen, intestine and liver, respectively (Table 2). Screening for other selected viral pathogens did not give any positive result, as did not bacteriological investigations. By immunohistochemistry CAdV antigens were detected in the liver (Fig. 1A), whereas histopathology showed large basophilic intranuclear inclusions and necrotic areas in the same tissue.

3.2. Virus isolation

Virus isolation on MDCK cells from the spleen and liver of the infected Eurasian wolf resulted in the appearance of CPE at 48 h post-inoculation, showing rounding of the cells, increased granularity and detachment from the monolayer. By IIF assay, granular fluorescence areas were evident in the cell nuclei from cells infected with isolate Wolf/835/2015/FRA and infected cells stained with H&E showed large basophilic intranuclear inclusions (Fig. 1B).

3.3. NGS analysis and genome structure

NGS analysis provided the full-length genomic sequence of CAdV-1 isolate Wolf/835/2015/FRA. A total of 203,549 reads of 243.6 bps average length were mapped to the reference sequence CAdV-1 strain R1261 (GenBank accession number Y07760; Morrison et al., 1997) with a mean coverage of 1620.6, generating a consensus sequence of 30,534 bps covering 100% of the reference genome. The assembly was performed using the Geneious software package (version 10.1.3). The full-length genome of CAdV-1 isolate Wolf/835/2015/FRA revealed a structure similar to previously described CAdVs. The genome is flanked on both sides by inverted terminal repeats (ITRs) of 158 bps in length, as in the Italian wolf isolate ITL2015 (Pizzurro et al., 2017). Both wolf viruses showed a deletion of 41 bps compared to CAdV-1 vaccine strain CLL, which was 3-bps larger than that of CAdV-1 strain R1261 (Sira et al., 1987). The hexon gene revealed the same pattern of amino acid substitutions observed in previously characterised CAdV strains isolated in

Italy from two dogs (strains 574-2013-RS and 417-2013-L) and a red fox (strain 113-5L) (Balboni et al., 2017). Specifically, amino acid mutation from asparagine to serine at position 388 was shared by those Italian isolates, the wolf isolate from this study (Wolf/835/2015/FRA) and the recent Italian wolf isolate (Pizzurro et al., 2017), defining a clear distinctive pattern of substitution. Similarly, in the fibre protein, amino acid substitutions were exactly as described for the Italian isolates, matching with the wolf isolate ITL2015, with the only exception at residue 110, where isolate Wolf/835/2015/FRA retained the same amino acid observed for other CAdV-1 strains. A unique feature to isolate Wolf/835/2015/FRA emerged at amino acid position 49 of the E1B 55 kDa protein, where a change from proline to serine occurred. Additional mutations occurred at positions 310 and 326 of the same protein, at position 73 of pIX and at position 293 of pIIIa, with all the changes being shared with CAdV-2 Toronto 26/61 (GenBank accession number CAU77082) and BatAdV PPV1 (JN252129). Strain Wolf/835/2015/FRA displayed a unique triplet inserted at position 475 of the pIIIa protein and another unique change at position 363 of the DNA binding protein encoded by gene E2A. Common features with strain ITL2015 appeared at position 388 of pVa, and at the N-terminus of E4 ORF3 where an additional methionine was observed.

3.4. Sequence and phylogenetic analyses

Genomic analysis of isolate Wolf/835/2015/FRA showed a 99.8% nucleotide (nt) identity with a CAdV-1 strain recently isolated from a wolf in Italy (Pizzurro et al., 2017). However, both wolf isolates were closely related to other CAdVs detected in dogs, displaying a 99.75% and 99.7% nt identity with CAdV-1 field strain RI261 (accession number Y07760) and vaccine strain CLL (accession number U55001), respectively, whereas only a 85.8% nt identity was found to CAdV-2 strain Toronto A26/61 (accession number U77082). The phylogenetic tree based on CAdV full-genomes available in GenBank clearly locates the wolf isolate within the CAdV-1 clade, segregating with the Italian wolf isolate CAdV ITL2015 (Fig. 2A). Analysis of the E3 (Fig. 2B) and hexon (Fig. 2C) genes confirmed this pattern of segregation, indicating that isolate Wolf/835/2015/FRA clusters with other CAdV-1 strains. Phylogeny clearly demonstrates that this

isolate is particularly close to other CAdV-1 strains from dogs, foxes and wolves detected in Italy, namely ITL2015 (KX545420), 574-2013-RS (KP840549), 417-2013-L (KP840547), 113-5L (KP840545) and Fox/ITA/466/2017 (MH399790), the last recently recovered from a free-ranging fox cub with neurological signs in Apulia (N. Decaro, unpublished).

3.5. Serological survey

A total of 29 out of 52 sera (55.76%) resulted positive by the VN test, including 8 samples collected from vaccinated animals (15.38%). Among Eurasian wolves, 12 out of 19 (63.15%) sera tested seropositive for CAdVs, of which only 1 animal (#19), introduced in 2015 in pack 3, had been previously vaccinated (Table 1). Seroprevalence resulted in 100% for pack 1, which included 10 wolves not previously vaccinated, whereas CAdV antibodies were detected in a single animal from pack 2 (#13), which had been sampled the day of death presumably caused by a clostridium infection from an old wound (data not shown). Noteworthy, sera #12, collected from the same animal more than 1.5-year before, tested negative, thus accounting for seroconversion of this animal after the occurrence of the CAdV index case. Results from the serological survey conducted on other carnivore species in the park are displayed in Table 1. Out of 33 animals, 17 tested positive for CAdVs (51.51%), but only 7 of these seropositive animals had been previously vaccinated. The 5 raccoons, the single brown bear and the European pine marten tested all seronegative, while the presence of specific antibodies in the 2 timber wolves and 1 arctic wolf could be due to the CAdV-2 vaccination carried out 2.5 and 3 years before sample collection, respectively. Conversely, a large proportion (8/11 animals) of the arctic wolves tested seropositive, although animals #28 to 33 had been sampled in their park of origin, in Austria, thus accounting for silent CAdV circulation in another zoological park.

3.6. CAdV molecular survey

Results of the molecular survey conducted to assess the circulation of CAdVs in the zoological park among wolves and other carnivore species are represented in Table 2, which includes also data of

the deceased wolf 835/2015/FRA. While no CAdV-1 detection was obtained following this index case, a certain circulation of CAdV-2 was demonstrated among different carnivore species in the park. In particular, the urine of 1 timber wolf (#35), 1 red fox (#53) and 1 raccoon (#42) were found positive. Interestingly, sample #53 belonged to either foxes #44 or #45 that tested negative 3 days later. CAdV-2 was also detected in the internal organs of red fox #54, found dead in the wolf enclosure. Notably, samples #35 and #53 were from animals that had been vaccinated against CAdV-2 at least 3 years before sampling.

4. Discussion

The present study reports the consecutive appearance of CAdV-1 and CAdV-2 infections in wild canids of a French zoological park. CAdV-1 was isolated from a case of fatal infection in a Eurasian wolf maintained in a large natural enclosure in the Parc Animalier de Sainte-Croix. To the best of our knowledge, this is the first published case of CAdV-1 infection in a captive wolf. By whole genome sequencing and subsequent sequence analysis, the wolf CAdV-1 isolate was found to be genetically related to a strain recently retrieved from a free-ranging wolf in Italy (Pizzurro et al., 2017), displaying a nt identity of 99.8%. A high genetic relatedness (~99% nt identity) was also evident to CAdV-1 strains recovered from other wild and domestic carnivores, confirming that the virus is genetically stable even across different host species. The limited availability of complete CAdV genomes prevented a more in-depth comprehension of the virus genetic relationship, thus leading to restrict the analysis to shorter genomic fragments in order to include strains detected in different carnivore species and geographic areas. Phylogenetic analysis performed on the E3 and hexon genes showed that isolate Wolf/835/2015/FRA segregated with recent Italian CAdV-1 strains detected in dogs, red foxes and wolves (Fig. 2). An in-depth analysis revealed that several nt and aa mutations were scattered through all the genome, which were mostly shared by Italian strains (Balboni et al., 2013; Pizzurro et al., 2017). Interestingly, both wolf strains, Wolf/835/2015/FRA

and ITL2015 displayed shorter ITRs compared to extant CAdVs. The ITRs have an essential function in virus replication, taking part in the protein-primed DNA replication mechanism, thus constituting an important junction in virus evolution and recombination. Similarly, both wolf strains showed a duplication of the starting methionine of ORF3 in the E4 region and an aa mutation at position 388 of pVa, traditionally involved in duplication or deletion events in *Mastadenovirus* evolution (Davison et al., 2003). These common features may represent an evolutionary adaptation of CAdV-1 to the wolf species, but they need to be supported by sequence data from additional wolf isolates. Most of the genus-specific genes in adenoviruses are located near the ends of the genome, many of these captured from the host and involved in host interaction and adaptation to biological niches. Mutations in these genus- and virus-specific genes are therefore relevant for viral fitness and host adaptation in vivo. The genus-specific E1B 55 kDa protein of isolate Wolf/835/2015/FRA displayed a unique mutation at position 49 and additional mutations unexpectedly shared with CAdV-2 Toronto 26/61 and BatAdV PPV1. Few mutations also occurred in the E2 region, which is implicated in virus structure and replication, whereas an additional asparagine unique to this strain was introduced at position 292 in the pIIIa protein and an aa substitution occurred in the DNA binding protein. However, further studies would be necessary to evaluate to which extent these mutations are involved in virus-host interaction and adaptation to different hosts.

No other sample from the same zoological park tested positive to CAdV-1, whereas a certain circulation of CAdV-2 was observed. Interestingly, the source of CAdV-1 was not recognised, while free-ranging red foxes could have carried the CAdV-2 strain into the zoological park. In a recent study (Walker et al. 2016), red foxes had inapparent infections with CAdV-1, but none tested PCR positive for CAdV-2. Since most animals were vaccinated using CAdV-2 formulations, the shedding of the vaccine virus from immunised animals could not be ruled out. Unlike modified-live CPV, whose shedding pattern has been evaluated in domestic dogs (Decaro et al, 2014; Decaro and Buonavoglia, 2017), no data are available about the shedding of the CAdV-2 vaccinal strain

especially in wild carnivores. Unfortunately, the viral loads detected in urine of live animals and in tissues of the dead animal prevented the sequencing of large genomic regions that could have been useful to address whether the shed virus was a vaccine or a field strain. However, the detection of CAdV-2 in carnivores vaccinated 3 years before supports the circulation of a field rather than the vaccine virus. This hypothesis is corroborated by the virus detection even in unvaccinated animals, since the vaccine virus is unlikely transmitted from immunised to other carnivores. Anyway, only future studies will help assess the extent and duration of the shedding of CAdV-2 vaccine strain in wild animals, thus ruling out definitively the possible transmission of this virus from vaccinated to CAdV naive animals. The absence of specific clinical signs and gross lesions in live and dead carnivores of the zoological park suggests that CAdV-2 circulate in wild animals without inducing any disease. This scenario is also supported by the fact that serological studies carried out in wild carnivores of different countries indicate a widespread exposure to CAdV infection, while detection of either CAdV type in ill or dead wild animals is only sporadic (Decaro et al., 2012).

Zoological parks represent an assortment of several carnivores living in a restricted environment that provides the opportunity to observe how dynamics of infections may evolve across different species and in animals those infections are usually not observed in their natural environment. In the Parc Animalier de Sainte-Croix a single Eurasian wolf displayed a severe CAdV-1 disease, although some animals of the same pack (*pack 2*) and all animals of a previously established pack (*pack 1*) were retrospectively found to have high CAdV antibody titres. Since those packs had not been vaccinated at the time of sampling, antibodies were the consequence of direct exposure to a field CAdV, but it was not possible to assess whether CAdV-1 or CAdV-2 was responsible for the seroconversion. Subsequent detection of CAdV-2 in the urine of 3 live captive carnivores and in internal organs of 1 dead free-ranging red fox without any CAdV-related gross lesions suggests a subclinical circulation of either or both CAdVs in the park. CAdV-2 natural infections were sporadically described in wolves (Millan et al., 2016), but only recently, Balboni et al. (2013) reported the first cases of CAdV-2 infection in healthy red foxes, which reinforces the evidence for

a subclinical circulation of this adenovirus in wild carnivores. Interestingly, in our study CAdV-2 was also retrieved from internal organs of foxes, thus accounting for a systemic infection, which had been previously reported only in domestic dogs (Decaro et al., 2004). Wild foxes represent a ready interface between domestic animals and wildlife, and could therefore play an important role in CAdV epidemiology. Additional data are needed about ecology of both CAdVs in wildlife in order to better understand the potential threat represented by these canine pathogens for the conservation of endangered carnivore species.

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Figure legend.

Fig. 1. Isolation of CAdV-1 from the infected wolf. A. Immunohistochemistry performed on liver: intranuclear reacting inclusions in infected MDCK cells are shown by arrows (400×). B. Histopathology from liver: basophilic intranuclear inclusions, compatible with type B Cowdry bodies (arrows) in infected MDCK cells (H&E, 400 ×).

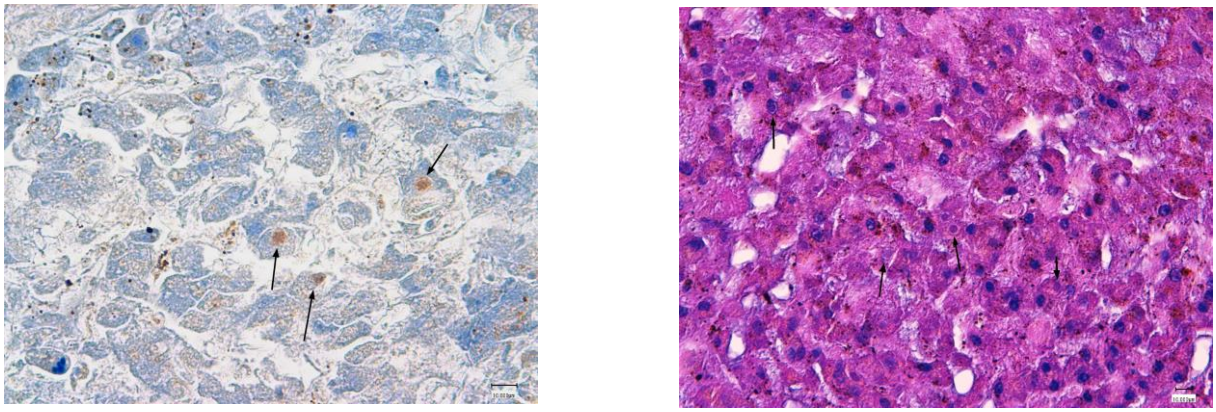

Fig. 2. Phylogenetic analysis of CAdV-1 wolf/835/2015/FRA and reference adenoviruses (AdVs). Trees were generated using the neighbor-joining method and the p-distance model, supplying a statistical support with bootstrapping of 1000 replicates. Asterisks denote the nucleotide sequence of strain Wolf/835/2015/FRA, while scale bars indicate the consensus support (%). A. Tree constructed with the CAdVs full-length genomes available in Genbank and bat adenovirus strain PPV1 as outgroup. The genomes included in the phylogenetic analysis, with the GenBank accession number displayed in brackets, are as follows: BatAdV-2 PPV1 (JN252129), CAdV-1 ITL2015 (KX545420), CAdV-1 RI261 (Y07760), CLL (U55001) and CAdV-2 Toronto 26/61 (CAU77082). B. Tree constructed with the E3 gene of canine and bat adenoviruses. Genomes of other AdVs (and their GenBank accession numbers) included in the comparative analysis were as follows: BatAdV-2 PPV1 (JN252129), CAdV-1 B579 (GQ340423), CAdV-1 ITL2015 (KX545420), 09-13F (JX416838), 113-5K (JX416840), CAdV-1 RI261 (Y07760), CAdV-1 GLAXO (M60937), CAdV-1 India1 (EF057101), CAdV-1 India2 (EF090910), CAdV-1 CLL (U55001), CAdV-1 Utrecht (S38238), CAdV-2 CC0710 (GQ241864), CAdV-2 Manhattan (S38212), CAdV-2 HB1 (GQ915311), CAdV-2 Toronto 26/61 (CAU77082), CAdV-2 113-3F-c01 (JX416841) and CAdV-2 113-3F-c04 (JX416842). C. Tree constructed with the hexon gene of canine, bat and skunk adenoviruses. The triangle denotes the nucleotide sequence of a CAdV-1 isolated from a fox in Italy (Fox/ITA/466/2017, GenBank accession number MH399790). Genomes of other AdVs (and their GenBank accession numbers) included in the comparative analysis were as follows: Bat AdV-2 PPV1 (JN252129), Skunk AdV PB1 (NC_027708), CAdV-2 YCA (EF508034), CAdV-2 Toronto 26/61 (CAU77082), CAdV-2 IN2006 (DQ839392), CAdV-2 CC0710QB (EU717145), CAdV-2 CC0710QZ (EU794687), CAdV-1 IN2007 (EF206692), CAdV-2 CCC-V6 (EF559262), CAdV-1 RI261 (Y07760), CAdV-1 CLL (U55001), CAdV-1 ITL2015 (KX545420), CAdV-1 574-2013-RS (KP840549), CAdV-1 417-2013-L (KP840547), CAdV-1 113-5L (KP840545).

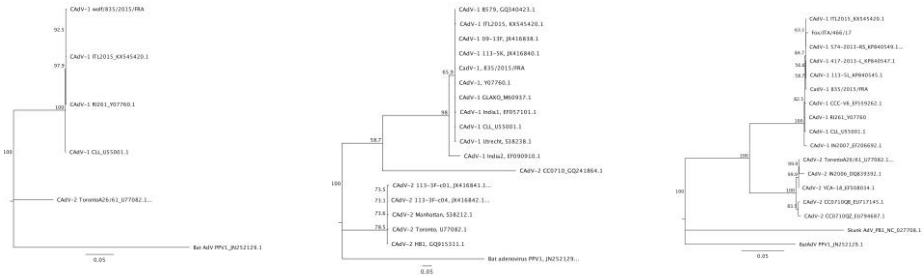

Table 1. Data collection and serology results from different carnivore species living at Sainte Croix Zoological park

Species	No. (pack)	Sex	Place, date of birth	Sampling	Vaccination	VN titre
Eurasian wolf *(Canis lupus lupus)*	#1(1)	M	Ste Croix, 05/5/01	3/11/09	No	>1:256
	#2(1)	M	Ste Croix, 30/4/03	21/10/09	No	>1:256
	#3(1)	F	Ste Croix, 30/4/03	8/8/14	No	>1:256
	#4(1)	M	Ste Croix, 30/4/03	5/3/13	No	1:128
	#5(1)	F	Ste Croix, 26/4/06	19/9/12	No	>1:256
	#6(1)	F	Ste Croix, 2/5/08	5/3/13	No	>1:256
	#7(1)	M	Ste Croix, 30/4/09	20/1/11	No	>1:256
	#8(1)	F	Ste Croix, 30/4/09	8/1/13	No	>1:256
	#9(1)	F	Ste Croix, 30/4/09	8/1/13	No	>1:256
	#10(1)	F	Ste Croix, 01/4/02	5/3/13	No	>1:256
	#11(2)	M	Ste Croix, 3/5/12	15/5/13	No	Negative
	#12(2)†	F	Ste Croix, May 2010	6/3/14	No	Negative
	#13(2)†	F	Ste Croix, May 2010	17/12/15	No	>1:256
	#14(2)	F	Ste Croix, 10/5/09	26/10/12	No	Negative
	#15(2)	F	Ste Croix, 10/5/09	19/1/11	No	Negative
	#16(2)	M	Germany, 3/5/05	13/2/14	Unknown	Negative
	#17(3)	M	France, 21/5/13	14/12/15 **	No	Negative
	#18(3)	M	France, 14/5/14	14/12/15 **	No	Negative
	#19(3)	F	France, 1/5/13	21/12/15 **	09/10/14[b]	1:4 *
Alaskan tundra wolf *(Canis lupus tundrarum)*	#20	M	Ste Croix, 15/5/03	19/11/14	No	1:64 ◆
	#21	F	Ste Croix, 15/5/05	18/3/06	No	1:32 ◆
	#22	M	Ste Croix, 15/5/09	11/4/10	No	Negative
	#23	M	Ste Croix, 15/5/09	30/11/10	No	Negative
Arctic	#24	M	Austria, 27/4/13	10/8/13	No	Negative

wolf (*Canis lupus arctos)*	#25	M	Austria, 27/4/13	10/8/13	No	Negative
	#26	M	Austria, 27/4/13	10/8/13	No	Negative
	#27	M	Austria, 27/4/13	19/11/14	10/08/12[b] 18/09/13[b]	>1:256
	#28	F	Austria, 24/4/14	2/12/14	No	1:128
	#29	M	Austria, 24/4/14	2/12/14	No	1:128
	#30	F	Austria, 24/4/14	2/12/14	No	1:64
	#31	M	Austria, 24/4/14	2/12/14	No	1:64
	#32	M	Austria, 24/4/14	2/12/14	No	>1:256
	#33	F	Austria, 24/4/14	2/12/14	No	1:128
	#34	F	Austria, 27/4/13	13/12/15	No	1:16
Timber wolf *(Canis lupus occidentalis)*	#35	M	Austria, 3/5/07	2/1/15	04/06/12[f]	1:32 *
	#36	M	Austria, 21/4/07	6/8/15	04/06/12[f]	>1:256 *
Pine marten *(Martes martes)*	#37	M	Germany, April 2006	29/10/12	Unknown	Negative
Raccoon (*Procyon lotor)*	#38	M	Netherland, 1998	1/4/14	Unknown	Negative
	#39	F	Luxembourg, 2014	1/11/15	Unknown	Negative
	#40	M	Luxembourg, 2015	1/11/15	Unknown	Negative
	#41	F	France, 6/8/01	23/12/15	Unknown	Negative
	#42	F	France, 22/6/02	23/12/15	Unknown	Negative
Brown bear *(Ursus arctos)*	#43	F	France, 6/1/98	30/4/15	No	Negative
Red fox *(Vulpes vulpes)*	#44	F	Germany, 8/4/12	31/7/15	20/06/12[d] 17/07/12[e]	1:8 *
	#45	F	Germany, 8/4/12	31/7/15	20/06/12[d] 17/07/12[e]	>1:256 *
Arctic fox *(Vulpes lagopus)*	#46	M	France, 11/4/14	12/8/15	12/08/15 [a] No	Negative **
	#47	M	France, 9/4/14	31/7/15	No	Negative **
	#48	M	France, 11/4/14	12/8/15	No	Negative **
	#49	M	France, 9/4/14	31/7/15	No	Negative **
	#50	M	Germany, 19/5/10	15/3/11	13/07/10[d] 16/08/10[e]	>1:256 *
	#51	F	Germany, 19/5/10	8/11/10	13/07/10[d] 16/08/10[e]	>1:256 *
	#52	F	Germany, 2009	5/8/10	No	1:32

564 Positive results at serological screening are enlightened in grey.* Vaccinated. ** Sampled before
565 vaccination. ◆Parents vaccinated in 2002. [†]Same animal sampled at two different time points.

[a] Eurican CHPPi2 – L (Merial); [b] Eurican CHPPi2 – LR (Merial); [c] Versican DHPPiL3 (Zoetis); [d] Virbagen canis SHAP/L (Virbac); [e] Virbagen canis SHAP/LT (Virbac); [f] Enduracell DA2PParvo-LR Zoetis).

Table 2. Molecular investigation on CAdVs circulation at Sainte Croix Zoological park

1. Species	2. Animal No.	3. Sex	4. Place, date of birth	5. Sample date	6. Sample type	7. CAdV type	8. Real-time PCR copy numbers μl^{-1} of template
Eurasian wolf *(Canis lupus lupus)*	835/15/FRA	F	Ste Croix, 10/5/10	15/5/15	Spleen Intestine Liver	CAdV-1 CAdV-1 CAdV-1	9.75×10^7 1.18×10^6 4.34×10^6
Timber wolf *(Canis lupus occidentalis)*	#35	**9.** M	Austria, 3/5/07	10/11/15	Urine	CAdV-2	5.74×10^3 * [f, a]
Raccoon (*Procyon lotor)*	#41	**10.** F	France, 6/8/01	23/12/15	Urine	CAdV-2	1.87×10^2
Red fox *(Vulpes vulpes)*	#44	**11.** F	Germany, 8/4/12	12/12/15	Urine	NA	Negative ** [a, b]
	#45	**12.** F	Germany, 8/4/12	13/12/15	Urine	NA	Negative ** [a, b]
	#53[†]	**13.** F	Germany, 8/4/12	9/12/15	Urine	CAdV-2	1.02×10^2 ** [a, b]
	#54	**14.** F	Unknown (free-ranging adult)	10/12/15	Bladder Spleen Kidney Liver	CAdV-2 CAdV-2 CAdV-2 CAdV-2	1.01×10^3 2.10×10^4 1.02×10^3 1.93×10^4

Positive results at molecular screening are enlightened in grey. * Vaccinated on 06/08/15. ** Vaccinated on 31/08/15
[a] Eurican CHPPi2 - L; [b] Eurican CHPPi2 - LR; [c] Versican DHPPiL3; [d] Virbagen canis SHAP/L; [e] Virbagen canis SHAP/LT; [f] Enduracell DA2PParvo-LR. [†]Urine from animal #44 or 45.