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SHORT COMMUNICATION

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Canine adenovirus type 1 (CAdV-1) in free-ranging European brown bear (*Ursus arctos arctos*): A threat for Cantabrian population?

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Abstract

Canine adenovirus type 1 (CAdV-1) is responsible for infectious canine hepatitis. The disease has been described in captive American black bear (*Ursus americanus*) and European brown bear (*Ursus arctos arctos*), with just one recently reported case in a cub of a free-ranging brown bear (*Ursus arctos horribilis*) from Alaska. The aim of this work is to summarize findings related to presence and associated mortality of CAdV-1 in 21 free-ranging Cantabrian brown bears (*Ursus arctos arctos*) submitted to necropsy in Asturias and Castilla y León (northwestern Spain) from 1998 to 2018. On the basis of the anatomopathological findings and laboratory results three free-ranging brown bears died due to infectious canine hepatitis, which is to our knowledge the first description of death due to this disease in free-ranging bears in Europe. Gross lesions consisted of petechial haemorrhages and congestion in different internal organs, haemorrhagic fluid in internal cavities, friable and yellowish liver and thickening of gall bladder. Microscopic lesions were observed mainly in liver, kidney and brain and consisted of multifocal necrosis of cells with presence of basophilic intranuclear inclusions. Immunohistochemical (IHC) and real-time polymerase chain reaction (qPCR) techniques were used to assess the presence of CAdV-1 in paraffin-embedded liver samples. Viral antigens were detected by IHC labelling within hepatocytes and Küppfer cells in the three animals. The presence of viral DNA was confirmed by qPCR in one of them. In order to evaluate the circulation of CAdV-1 in brown bears, a retrospective study was performed using both IHC and qPCR techniques in 11 and 12 additional brown bears, respectively. An extra brown bear was found positive by IHC. This study shows that CAdV-1 surveillance of brown bears and sympatric carnivores should be considered as major concern for the monitoring the population evolution throughout time in this endangered species.

KEYWORDS

canine adenovirus type 1, free-ranging brown bear (*Ursus arctos arctos*), immunohistochemistry, management, pathology, qPCR

1 | INTRODUCTION

The European brown bear (*Ursus arctos arctos*) population located in the Cantabrian Range (northwestern Iberian Peninsula) represents the southwestern limit distribution for this species in Europe. As with other remnant ursid populations on the continent, it underwent a dramatic decline in the second half of the twentieth century (Martínez-Cano, Taboada, Naves, Fernández-Gil, & Wiegand, 2016). This decline, together with human-caused mortality as a key factor (González et al., 2016; Naves, Wiegand, Fernández, & Stephan, 1999), reduced the Cantabrian brown bear population to a nadir of less than 100 individuals in the 1990s and divided it into two subpopulations (western and eastern) favoured by geographical barriers, thus putting the species in serious danger of extinction in the Cantabrian region (Wiegand, Naves, Stephan, & Fernández, 1998). As for other European large carnivore species, the establishing of protective legislation, supportive public opinion and a variety of practices (monitoring and conservation plans) enabling large carnivore–people coexistence (Chapron et al., 2014), led to a recovery of the Cantabrian brown bear population over the last two decades, with an apparent steady increase in the number of individuals (nowadays estimated in 230–260 individuals, Fundación Oso de Asturias, 2018), bear dispersal and gene flow (González et al., 2016).

The identification of mortality causes in wild natural populations is of major concern not only for the detection and recognition of possible conservation problems or risks, but also for a correct design of conservation strategies and management programs (Almberg, Cross, & Smith, 2010). Nevertheless, mortality studies and sanitary data are scarce in the small and endangered Cantabrian brown bear population: the low number of individuals, their elusive behaviour and high mobility and the absence of GPS-collared bear monitoring programs to date in the area hamper the detection of dead bears, thus preventing in many cases the performance of necropsy and the determination of the cause of death if possible.

Canine adenovirus (CAdV), family Adenoviridae, genus Mastadenovirus, is a nonenveloped dsDNA virus which infects numerous mammalian carnivores (King, Adams, Carstens, & Lefkowitz, 2012). There are two types of canine adenovirus, CAdV type 1 (CAdV-1) and CAdV type 2 (CAdV-2), distinguishable by genetic, antigenic and pathogenetic characteristics. While CAdV-1 is responsible for infectious canine hepatitis, characterized by acute necrohaemorragic hepatitis, CAdV-2 is one of the viral agents implicated in the aetiopathogenesis of infectious tracheobronchitis in dogs, also known as kennel cough (Greene, 2012).

Hepatitis caused by CAdV-1 is a common disease in dogs, easily controlled using vaccination (Greene, 2012). It also affects wild carnivores of the *Canidae* (including red fox and wolf), *Mustelidae* and *Ursidae* families (Woods, 2001), with prevalence over 70% in some cases (Millán et al., 2016). The disease has been described as the cause of death in captive American black bear (*Ursus americanus*) and European brown bear (Pursell, Stuart, Styer, & Case, 1983; Collins et al., 1984; Kritsepi, Rallis, Psychas, Tontis, & Leontides, 1996), with just one recently reported case in a free-ranging brown bear (*Ursus arctos horribilis*) from Alaska (Knowles, Bodenstein, Hamon, Saxton, & Hall, 2018). Bears usually die within 12 hr after the onset of illness, with clinical signs consisting of anorexia, ataxia, lethargy, nystagmus, padding legs, clonic seizures and convulsions (Pursell et al., 1983; Collins et al., 1984).

The aim of this study is to summarize findings related to circulation and associated mortality of CAdV-1 in free-ranging Cantabrian brown bears submitted to necropsy in Asturias and Castilla y León (northwestern Spain) over the past 19 years. These data could provide valuable information on the factors threatening this recovering population, and may help in the management and conservation projects and efforts carried out in the future.

2 | MATERIAL AND METHODS

2.1 | Study area

The Cantabrian brown bear is distributed in the Cantabrian Mountain Range (northwest Spain) in two populations (western and eastern) separated by about 50 km and are present in four regions: Asturias, Cantabria, Castilla y León and Galicia (Figure 1, Fundación Oso de Asturias, 2018).

The western population has an estimated census of 200 individuals and covers an area of approximately 2,800 square kilometres (Fundación Oso de Asturias, 2018). The eastern population, with about 25–30 individuals, occupies a small area in Asturias with the largest distribution area located in the mountains of Palencia, eastern León and Cantabria.

2.2 | Studied Cantabrian brown bears

Twenty-one free-ranging Cantabrian brown bears from Asturias and Castilla y León (Figure 1), of different ages and sex (see Table 1), were submitted to necropsy from 1998 to 2018. After the detection of one dead animal in the wild a complete postmortem examination of each carcass was conducted at the University of León or SERIDA (Asturias) in less than 24 hr. Tissues were taken for evaluation of the cause of death using standard methods in microbiology, virology, parasitology, toxicology and histopathology. A dental histological study (Klevezal, 1996) was performed when possible in order to determine the age of the bears. Two out of the 21 animals had insufficient available tissues due to predation and/or remains that were in bad conservation (advanced autolysis) to perform a complete necropsy.

Three free-ranging brown bears showed lesions compatible with infectious canine hepatitis, which was afterwards confirmed using laboratory techniques in all of them (Table 1). In order to evaluate the circulation of CAdV-1 in brown bears a retrospective study was carried out using both immunohistochemical and real-time polymerase chain reaction (qPCR) techniques in 11 and 12 additional brown bears respectively (Table 1).

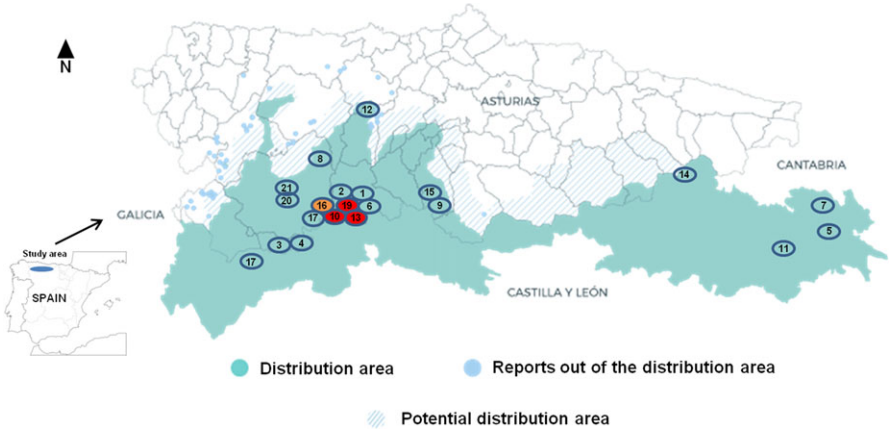

FIGURE 1 Studied Cantabrian brown bears (*Ursus arctos arctos*) in Asturias and Castilla y León (northwestern Spain) from 1998 to 2018 (1-21, see Table 1). Red circles represent animals that died with lesions compatible with infectious canine hepatitis. Orange circle represents animal without lesions compatible with infectious canine hepatitis but positive by anti-canine adenovirus type 1 (CAdV-1) immunohistochemical technique. Brown bear identified as number 10 was also positive for CAdV-1 by real-time polymerase chain reaction (qPCR). Cantabrian brown bear geographical distribution map was obtained from Fundación Oso de Asturias (2018) [Colour figure can be viewed at wileyonlinelibrary.com]

2.3 | Immunohistochemistry (IHC)

4-μm sections from paraffin-embedded liver samples acquired from 14 brown bears were used for the immunohistochemical study (Table 1). The remaining seven animals had livers in bad conservation, thus IHC was not conducted. IHC was performed following manufacturer's instructions using a commercial monoclonal anti-CAdV-1 antibody (Mybiosource, San Diego, United States) diluted 1:200 and the avidin–biotin complex (ABC) kit (Vector Laboratories, San Diego, United States). Positive (dog affected by CAdV-1) and negative (additional slide of each sample with omission of the primary antibody) controls were used during each immunohistochemical run.

2.4 | Molecular genetics

Total DNA was isolated in duplicate from 15 brown bears' paraffin-embedded liver samples using the NucleoSpin® FFPE DNA kit (Macherey-Nagel, Bethlehem, United States) according to the manufacturer's instructions. Fourteen out the 15 brown bears were also used for IHC studies. The remaining six animals had livers in bad conservation, thus DNA extraction was not performed. The detection included two qPCR protocols. The first amplified a 160-base pair (bp) long fragment of the ZFX gene of brown bear DNA (Bidon et al., 2013), and was used to avoid false negatives. The second consisted on the amplification of a 160-bp long fragment comprising part of the E3 and U exon genes of CAdV (Balboni, Dondi, Prosperi, & Battilani, 2015). Both PCRs were performed, using the Quantimix easy Kit (Biotools, Madrid, Spain), in a StepOne Plus (Life technologies, Carlsbad, United States). Cantabrian brown bear liver DNA sample and CAdV-1 extract DNA were used as positive controls respectively.

A 508-bp long fragment of CAdV-1 E3 gene and flanking regions was amplified using primers described in Balboni et al. (2013), and sequenced in both strands using the BigDye Terminator v.1 Cycle Sequencing Kit (Applied Biosystems, Madrid, Spain). To compare the phylogenetic relationships of the brown bear CAdV-1, an unweighted pair group method analysis tree and the Jukes–Cantor method was constructed in MEGA 7 (Kumar, Stecher, & Tamura, 2016), by using published CAdV-1 sequences detected in different geographical regions and host species: UK red fox (KU755718, KU755712), Italy red fox (JX416838), Italy wolf (KX545420) and domestic dog from Australia (KT853097), China (KJ451612) and Portugal (KC577558).

3 | RESULTS

Gross lesions compatible with CAdV-1 infection were observed in three male bears (Collins et al., 1984; Knowles et al., 2018; Kritsepi et al., 1996; Pursell et al., 1983), one adult and two cubs (identified as brown bears number 10, 13 and 19, Table 1). They consisted of petechial haemorrhages in thymus (in cubs), lungs, heart, gastric mucosa, gall bladder and mesentery; haemorraghic fluid in thoracic and abdominal cavities; friable and yellowish liver; hepatomegaly; thickening of gall bladder and congestion of spleen, kidney and meninges. Microscopically the main pathological findings appeared in liver, kidney and brain. Liver showed mild centrolobular multifocal degeneration and necrosis of hepatocytes (Figure 2a), with the presence of basophilic intranuclear inclusions bodies in hepatocytes and Küpffer cells and low inflammatory infiltration mainly of lymphocytes (Figure 2b). The main lesion in kidney was tubulonephrosis with multifocal necrosis of tubular epithelial cells which showed cariorrexis, cariolisis and basophilic intranuclear inclusions (Figure 2c). The same lesions were observed in few endothelial glomerular cells. The brain showed nonpurulent encephalitis, oedema, congestion and areas of demyelination. Foci

TABLE 1 Data of 21 free-ranging Cantabrian brown bears (*Ursus arctos arctos*) studied for detection of canine adenovirus type 1 (CAdV-1) infection from 1998 to 2018 in Asturias and Castilla y León (northwestern Spain)

Brown bear	Date	Age	Sex	CAdV-1 IHC/qPCR
1	08/05/1998	Adult	Male	Not performed[b]/Negative
2	12/06/1998	Cub	Female	Not performed[b]/Not performed[b]
3	10/06/2000	Adult	Not determined	Negative/Negative
4	06/06/2005	Cub	Not determined	Not performed[a]
5	26/09/2005	Adult	Male	Negative/Negative
6	19/11/2005	Adult	Not determined	Not performed[b]/Not performed[b]
7	14/06/2008	1 year	Male	Not performed[b]/Not performed[b]
8	27/08/2012	Adult	Male	Not performed[b]/Not performed[b]
9	12/06/2014	1 year	Male	Negative/Negative
10	15/06/2014	5 years	Male	**Positive/Positive**
11	12/12/2014	Adult	Male	Negative/Negative
12	29/04/2015	20 years	Female	Negative/Negative
13	23/05/2015	Cub (4 months)	Male	**Positive**/DNA not isolated
14	16/10/2015	Adult	Male	Negative/Negative
15	05/03/2016	Adult	Male	Not performed[a]
16	09/09/2016	Adult	Male	**Positive**/Negative
17	27/11/2016	6 years	Female	Negative/Negative
18	07/01/2017	6 years	Male	Negative/Negative
19	03/04/2017	Cub (3 months)	Female	**Positive**/DNA not isolated
20	21/04/2017	19 years	Male	Negative/Negative
21	21/04/2017	20 years	Male	Negative/Negative

Notes. IHC: Immunohistochemistry; qPCR: Real-time polymerase chain reaction. [a]Animals in which a complete necropsy could not be performed due to insufficient available tissues due to predation and/or remains in bad conservation (advanced autolysis). [b]Liver in bad conservation to perform the diagnostic technique. Bears 10, 13 and 19 showed lesions compatible with infectious canine hepatitis. Positive results appear in bold type.

of gliosis (Figure 2d) and perivascular cuffing were observed mainly located in the brainstem. In the cerebellum, foci of gliosis were also present in the molecular layer of the folia (Figure 2e). Changes in the Purkinje cells varied from mild degeneration of a few cells to widespread necrosis and depletion variably affecting different cerebellar folia (Figure 2e). In these cases there was often proliferation of Bergmann glial cells and in some instances the loss of Purkinje cells was marked by empty spaces. Loss of neurons was observed in several nuclei in the brain including the olivary, medial accessory olivary and rostral trigeminal, accessory cuneate, reticular formation and nuclei of the vagal and hypoglossal nerves. Lung showed congestion and oedema. The gall bladder showed oedema of the wall.

Immunohistochemical technique performed on paraffin-embedded liver samples detected the presence of CAdV-1 antigen in the three brown bears (see Figure 1 and Table 1). Positive immunolabel was observed within hepatocytes and Küppfer cells (Figure 2f). The presence of viral DNA was identified by qPCR in brown bear number 10. An extra brown bear (identified as number 16), which did not show either gross or microscopic lesions compatible with infectious canine hepatitis, and killed due to poaching, was positive by IHC.

A 437-bp long sequence (accession number MH469715) was obtained from the positive sample (brown bear number 10), corresponding with nucleotides from 40 to 476 in KU755718. The sequence was 100% identical to the strain from domestic dogs, as well as to Italian red fox and one red fox from UK, and 99% identical to both Italian wolf and UK red fox CAdV. Phylogenetic analysis tree is shown in Figure 3.

The adult brown bear and the two cubs that died with lesions compatible with infectious canine hepatitis were found in Villablino (Castilla y León) and La Llamera (Somiedo, Asturias), respectively (Figure 1), two locations 45 km one from each other. The additional animal positive by IHC was found in Moal (Cangas de Narcea, Asturias), also close to Villablino and Somiedo.

4 | DISCUSSION

On the basis of the anatomopathological findings and laboratory results it can be concluded that three bears died due to infectious canine hepatitis, suggesting that this disease has been an important cause of death on the Cantabrian brown bear population, especially from 2014 onwards (see Table 1). To our knowledge this is the first description of fatal disease in free-ranging bears due to CAdV-1 infection in Europe. The presence of this virus and its capacity to produce disease (and even death) has been recently confirmed in a

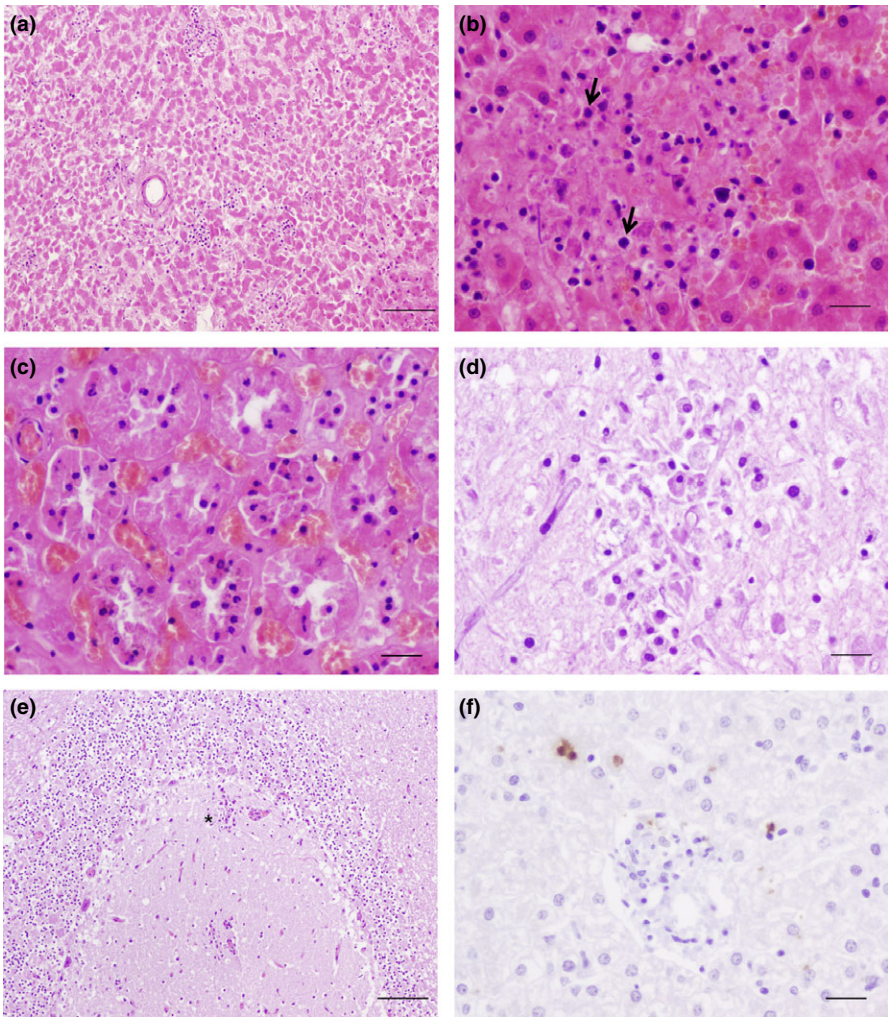

FIGURE 2 Histopathological and immunohistochemical features found in free-ranging Cantabrian brown bears (*Ursus arctos arctos*) that died with lesions compatible with infectious canine hepatitis. (a) Liver showing centrolobular degeneration and necrosis of hepatocytes. Haematoxilin–eosin stain, bar = 100 microns. (b) Focal necrosis in liver is observed with the presence of basophilic intranuclear inclusions bodies in hepatocytes (arrows) and low inflammatory infiltration mainly of lymphocytes. Haematoxilin–eosin stain, bar = 20 microns. (c) Congestion and necrosis of tubular epithelial cells is observed in kidney. Haematoxilin–eosin stain, bar = 20 microns. (d) Small focus of gliosis is observed in midbrain. Haematoxilin–eosin stain, bar = 20 microns. (e) Cerebellum with a focus of gliosis present in the molecular layer of the folia (asterisk). Degeneration and necrosis of Purkinje cells is also observed with proliferation of Bergmann glial cells. Haematoxilin–eosin stain, bar = 100 microns. (f) Positive immunolabel is observed within hepatocytes. Anti-canine adenovirus type 1 (CAdV-1) immunohistochemical technique, bar = 20 microns. Photomicrographs a to e correspond to brown bear number 10 and photomicrograph f to brown bear number 19 (see Table 1) [Colour figure can be viewed at wileyonlinelibrary.com]

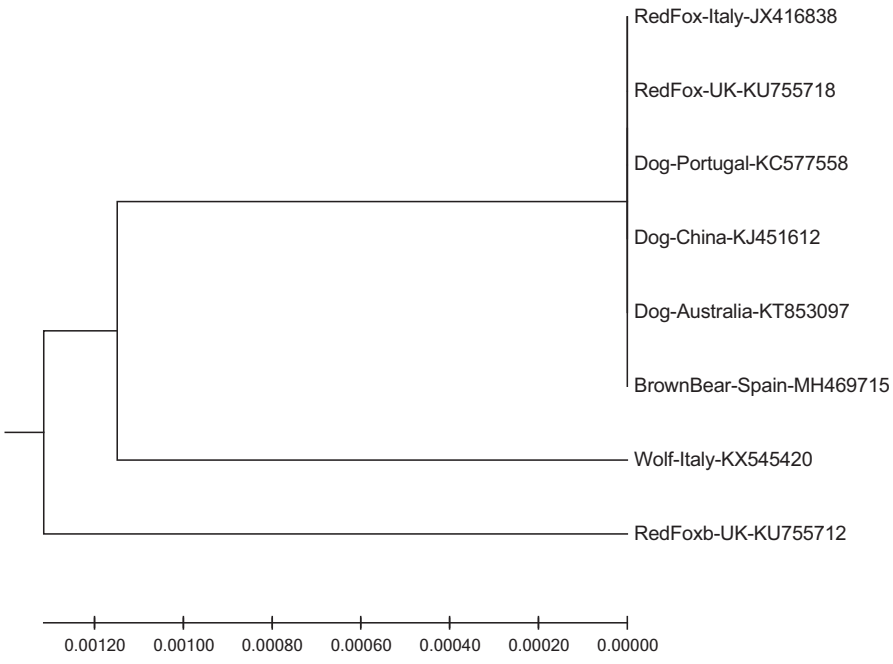

FIGURE 3 Phylogenetic relationships of the Cantabrian brown bear canine adenovirus type 1 (CAdV-1) with other CAdV-1 found in domestic and wild animals. Phylogenetic and molecular evolutionary analyses of the CAdV-1 were conducted using the UPGMA tree and the Jukes–Cantor method included in MEGA version 7. Scale bar indicates branch length, proportional to the number of nucleotide substitutions

free-ranging cub of brown bear in Alaska (Knowles et al., 2018) and in few captive American black bears (Collins et al., 1984; Pursell et al., 1983) and one European brown bear (Kritsepi et al., 1996; Ramsay, 2003) which presented gross and microscopic lesions similar to those found in this study. Serological surveys have also demonstrated the circulation of CAdV-1 in the wild in 12% of free-ranging Alaska grizzly bears (Zarnke & Evans, 1989) and in 6% of free-ranging Florida black bears (Dunbar, Cunningham, & Roff, 1998).

A widespread distribution of CAdV-1 infection in wild wolves has recently been reported in a study carried out in Asturias and Galicia (northwest Spain) by Millán et al. (2016), where it was detected using molecular techniques in 70% of studied wolves and antibodies against adenovirus were confirmed in 75% of them. These data suggest that CAdV-1 might be enzootic in Iberian wolves (Millán et al., 2016). The detection of deaths of three brown bears due to infectious canine hepatitis reported in this article and one more positive against CAdV-1 infection by IHC, which was a likely subclinical carrier (Woods, 2001), suggests a worrying degree of circulation of the virus in this species and confirms its pathogenic effect in the Cantabrian brown bear population. Those four bears were found within a radius of 45 kilometres in different years (2014, 2015, 2016 and 2017), suggesting a high level circulation of the virus in that particular region (Villablino, Somiedo and Cangas del Narcea, see Figure 1). The direct source of CAdV-1 infection might have been carriers such as wolves, domestic dogs or even red foxes (*Vulpes vulpes*), although in the case of dogs and red foxes the prevalence and role in the epidemiology of this virus remains unknown in this study area. Vaccination against CAdV-1 is included in the Spanish dog vaccination schedule (Colvema, 2018), therefore it is unlikely to find infectious canine hepatitis in this species in urban areas. However, in rural areas dogs are usually not sanitarily controlled and unvaccinated (Official Veterinary Services of the Principality of Asturias, personal communication) and become a possible source of CAdV-1 infection.

CAdV-1 is a highly contagious virus, usually eliminated in urine, nasal and conjunctival secretions and faeces during disease and may be shed in the urine for several months after recovery. This virus can be transmitted through direct contact with infected animals or contaminated fomites, and is considered a stable virus in the environment (Woods, 2001). Indirect interspecies transmission could also occur through the persistence of the virus in urine within the environment, but this hypothesis needs to be study in the future. The low variability found in the sequenced 437-bp long fragment of the virus, and the unique CAdV-1 DNA sequence obtained in this study did not help to clarify the epidemiology of this virus. In order to confirm interspecies transmission or to determine if the same virus could be involved further studies such as sequencing of the virus in the affected species should be carried out. Recent studies have found specific areas of the CAdV-1 genome which could be studied to find sequence variation of the virus (Balboni et al., 2017). That would help to understand the epidemiology of the disease.

Another hypothesis of the source in the two affected cubs (3 and 4 months old) might have been a carrier mother, helped by the lack of a complete mature immune system in the cubs. Both cubs were found dead in April and May coinciding with the natural departure from the bear cave (Palomero et al., 2011), therefore their time in the environment outside the cave was presumably short with less probability of infection. Both cubs were found in the same area 100 m one from each other with an interval of 2 years. This will be the time it usually takes for a female to give birth again (Palomero et al., 2011), which might suggest the same infected mother. However, this hypothesis is difficult to verify. Cantabrian brown bear population has suffered a bottleneck bringing the species at high risk of extinction. Consequently, the genetic variability among the population is very low (Swenson, Taberlet, & Bellemain, 2011), being difficult to assign familiar relationship between two samples by means of genetic markers, even more when no parental DNA is available to be compared.

A serological survey in the Cantabrian brown bear for CAdV-1 would also help to increase the knowledge of both the circulation and epidemiology of the virus in the population.

Despite the fact that infectious diseases are not considered a global important cause of species extinctions or endangerment (Smith, Sax, & Lafferty, 2006), they are a more common driver of temporary or permanent population declines at local scales, especially among endangered, isolated or reduced populations (e.g., amphibian decline by *Batrachochytrium dendrobatidis* (Collins, 2010)). This could also occur in those reintroduced species in areas where they had been extinct (Heard et al., 2013; Pedersen, Jones, Nunn, & Altizer, 2007; Smith, Behrens, & Sax, 2009). The isolation of the Cantabrian brown bear from the rest of European brown bear population for at least 400 years (Nores & Naves, 1993), lead to its serious population decline during the late 1990s and the division in two genetically separated (western and eastern) populations (Martínez-Cano et al., 2016) severely affected the genetic features of the species in the Cantabrian mountains. A consequence is that the eastern Cantabrian subpopulation shows some of the lowest genetic variation among brown bear populations in Europe (Swenson et al., 2011). Depletion of genetic diversity within populations may make them more vulnerable to pathogens, with inbreeding depression and loss of variation at genes responsible for resistance to pathogens increasing the susceptibility to infection (Radwan, Biedrzycka, & Babik, 2010; Ross-Gillespie, O'Riain, & Keller, 2007; Sommer, 2005).

The apparent moderate but steady recovery experienced during recent decades provides a new outlook for this endangered population, where recovery and conservation studies will have to be accompanied by an increasingly important effort on surveillance and management programs of this species. In this way, the almost simultaneous occurrence of one brown bear death in Alaska due to CAdV-1 (Knowles et al., 2018) and three more in Europe in this study means that this disease could no longer be sporadic and that sanitary surveillance of brown bears and sympatric carnivores should be considered a major concern for the monitoring of bears worldwide for the detection of this disease as well as new possible threats. IHC and qPCR techniques have revealed as valuable diagnostic techniques for the detection of CAdV-1 in paraffin-embedded samples for retrospective and future studies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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