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ALEUTIAN DISEASE SEROLOGY, PROTEIN ELECTROPHORESIS, AND PATHOLOGY OF THE EUROPEAN MINK (*MUSTELA LUTREOLA*) FROM NAVARRA, SPAIN

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Abstract: The European mink, *Mustela lutreola*, has suffered a dramatic decline in Europe during the 20th century and is one of the most endangered carnivores in the world. The subpopulation of European mink from Navarra, Spain, estimated to number approximately 420, represents approximately two thirds of the total number of mink in Spain. Aleutian Disease Virus (ADV) is a parvovirus with a high degree of variability that can infect a broad range of mustelid hosts. The pathogenesis of this virus in small carnivores is variable and can be influenced by both host factors (e.g., species, American mink genotype, and immune status) and viral strain. A cross-sectional study was conducted during the pre-reproductive period of February–March 2004 and 2005 and the postreproductive period of September–December 2004. Mink were intensively trapped along seven rivers that were representative of the European mink habitat in Navarra. Antibody counter immunoelectrophoresis against ADV was performed on 84 European mink blood samples. All the samples were negative. Protein electrophoresis was performed on 93 plasma samples. Nine of those samples (9.6%) had gamma globulin levels exceeding 20% of the total plasma protein. Complete necropsies were performed on 23 cadavers of European mink collected in the area between 2000 and 2005. Seventeen of the mink (74%) had traumatic and hemorrhagic lesions compatible with vehicular impact injuries. Although there were no histopathologic lesions associated with ADV, this study documents the first description of a naturally occurring canine distemper virus infection in a European mink. In addition, pulmonary adiaspiromycosis in three European mink from Spain was reported.

Key words: Aleutian disease, counter immunoelectrophoresis, European mink, *Mustela lutreola*, protein electrophoresis, pathology, seroprevalence, canine distemper virus, adiaspiromycosis.

INTRODUCTION

The European mink, *Mustela lutreola* (Carnivora, Mammalia), is one of the most endangered carnivores in the world.[17] Populations of these animals have suffered a dramatic decline in Europe during the 20th century.[20] The range of the European mink has been dramatically reduced and is actually fragmented into two distinct and geographically isolated population units: an eastern population and a western population.[20] The number of European mink in Navarra, Spain, is estimated to be 420, and this number represents approximately 66% of the animals in Spain and the largest percentage of animals in the western population.[3]

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The decline in the mink population has been attributed to a number of factors. On a larger scale, the decline is caused by loss of habitat, excessive hunting and trapping, pollution, and the competition from the introduction of the American mink (*Mustela vison*).[20,24] On a smaller scale, factors include vehicular impact injuries, predators (e.g., dogs), and pest control.[7]

The American mink, an invasive species on the Iberian Peninsula, has expanded into the remaining range of the European mink in Spain and is considered a direct competitor for food and habitat as well as a source of infectious disease for the European mink.[22,25,35,38] The subpopulation of European mink in Navarra is outside of the range of the feral population of American mink, where it is limited to fur farms.[3] Serologic evidence of Aleutian disease virus (ADV) infection has been reported in free-ranging American mink, European mink, polecats (*Mustela putorius*), stone martens (*Martes fiona*), pine martens (*Martes martes*), and common genets (*Genetta genetta*), as has been suspected in an Eurasian otter (*Lutra lutra*) as well.[6,22,43,44] Information concerning the prevalence, distribution, strains, and pathogenesis associated with ADV in the European mink is limited, and research to elu-

cidate this information is required when developing conservation plans for these animals.

ADV is a parvovirus with a high degree of variability and can infect a broad range of mustelid hosts.[6,10,19] ADV infections have also been described outside the family Mustelidae, including in red foxes (*Vulpes vulpes*) and raccoons (*Procyon lotor*).[16] The pathogenesis of this virus in small carnivores depends on both host (e.g., species, American mink genotype, and immune status) and viral strain.[2,15]

Aleutian disease (AD) was first recognized in farmed American mink that were homozygous for the recessive Aleutian coat color gene.[14] Adult American Aleutian mink infected with a virulent strain of ADV develop the classical severe form of the disease: persistent, progressive infection characterized by hypergammaglobulinemia, plasmocytosis, and immunocomplex-mediated glomerulonephritis and arteritis.[32] The high levels of antibody that characterize AD are ineffective at eliminating the virus and are directly related to the pathology mediated by immunocomplexes. Adult American mink of another genotype may develop different forms of infection depending on the strain of the virus. Affected animals may have a persistent progressive infection similar to the Aleutian genotype (high antibody titer, severe lesions); persistent nonprogressive infection (high antibody titer, no lesions); or nonpersistent nonprogressive infection (low antibody titer, no lesions), with eventual clearance of the virus.[8,12,13] In persistently infected American mink, ADV has been reported to cause uveitis, nonsuppurative meningoencephalitis, reduced fertility and spontaneous abortions, and increased susceptibility to opportunistic bacterial infections.[2,4,11,15] In neonates, direct viral damage, rather than immune-mediated disease, causes an acute and often fatal interstitial pneumonia.[10]

AD has also been described in domestic ferrets (*Mustela putorius furo*), but ferret ADV strain differs from the American mink ADV strains.[19,27,28,32] A review of the ADV phylogenetics confirms that the ferret ADV strains belong to the same genetic group but have diverged from American mink ADV strains.[27,32] AD appears to progress more slowly in ferrets and produces less severe histopathologic lesions and disease than in the American mink.[9,28,32]

The purposes of this study were to characterize the seroprevalence of AD in the European mink subpopulation in Navarra, Spain, during the period extending from 2004 to 2005 and to determine the presence of lesions compatible with AD in cadavers collected during the 2000–2005 period. It was suspected that 20–25% of the samples would be seropositive for AD virus and that at least 5% of the cadavers collected would present lesions compatible with AD, as described in previous serologic surveys in Spain.[21] This study was part of a larger project aimed at estimating the distribution, density, and population composition of the European mink subpopulation in Navarra, Spain.[3]

MATERIALS AND METHODS

A cross-sectional study was conducted during the pre-reproductive period of February–March in 2004 and 2005 and the postreproductive period of September–December in 2004. Mink were intensively trapped along 180 km of seven rivers that were considered to be representative of the European mink habitat in Navarra, Spain. The animals were live trapped in 20 by 20 by 60–cm wire cages, transferred to a tubular canvas bag (20 × 20 × 60 cm), and injected with an anesthetic combination of ketamine hydrochloride (Imalgene 1000, Merial, Lyon, France; 10 mg/kg) and xylazine hydrochloride (Rompun 2%; Bayer AG, Leverkusen, Germany; 0.6 mg/kg) intramuscularly. Once anesthetized, the animals were examined for the presence of a microchip. Standard morphometric measurements, body weight, and biological samples were collected. Gender was determined by examining the external genitalia. Mink were classified into four estimated age groups based on their dental status: these groups included juvenile (milk teeth), subadult (adult teeth without wear and tartar), adult (teeth with partial wear and tartar), and geriatric (teeth with excessive wear and tartar). A blood sample was collected from the jugular vein or cranial vena cava using a 1-ml or 3-ml syringe and a 22- or 25-gauge needle, respectively. The sample was transferred into duplicated heparin and ethylenediaminetetraacetic acid–coated tubes (Microtainer; Becton Dickinson and Company, Franklin Lakes, New Jersey 07417, USA). If not previously implanted, a subcutaneous microchip was administered between the shoulder blades (Backhome; Virbac, 08950 Esplugues de Llobregat, Spain). Once the procedures were complete, the animals were allowed to recover in the trap in a dark and quiet room. After recovery, the animals were released at the capture site. The heparinized blood samples were transported with cold packs within 24 hr to the University of Leon School of Veterinary Medicine (Leon, Spain) for processing. Samples were centrifuged within 24 hr of collection at 4°C and 10,000 rpm for 30 min (Beckman, Microfuge® 18 Centrifuge; Beckman Coulter, Inc., Fullerton, California 92834, USA). The plasma was maintained at −30°C until testing was performed.

The plasma samples were evaluated for the presence of antibodies against ADV by counter immunoelectrophoresis (CIEP) using a commercial test antigen (United Vaccines, Inc., Madison, Wisconsin 53744, USA). The samples were submitted to Harlan UK Ltd. (Loughborough, United Kingdom) and processed simultaneously. The samples were considered negative when there was no detection of antibodies against AD. The heparinized samples were submitted to the Laboratorio de Analisis Veterinarios (Madrid, Spain) for plasma protein electrophoresis using the cellulose acetate method. Hypergammaglobulinemia was considered when gamma globulins exceeded 20% of the total plasma protein.

Twenty-five frozen European mink cadavers were submitted to the Pathology Laboratory of the School of Veterinary Medicine, University of Leon, for necropsy. The cadavers were collected during the 2000–2005 period and were stored frozen until submission. The cadavers were defrosted at 4°C. After gross examination, samples for histology were obtained from the liver, kidney, spleen, lung, heart, esophagus, stomach, intestine, trachea, lymph nodes, pancreas, brain, muscle, and testicle. The samples were fixed for 48 hr in 10% buffered formalin and processed for histopathology. Sections (4 μm) were cut from each sample and stained with hematoxylin and eosin (H&E), periodic acid–Schiff (PAS), and Grocott and Gram stains. Sections, stained with H&E, that revealed lesions consistent with canine distemper virus (CDV) infection were further examined by immunohistochemistry using the avidin–biotin–peroxidase complex (ABC) method, which utilizes 3,3′-diaminobenzidine as the chromogen substrate (Vector Laboratories, Inc., Burlingame, California 94010, USA) and a mouse anti-CDV monoclonal antibody (1:200; Serotec Ltd., Kidlington OX5 1GE, United Kingdom). Additional samples from the liver in a European mink were frozen at −20°C for bacterial isolation.

RESULTS

A total of 90 European mink were captured during the study. Of those, 15 were captured in both the pre-reproductive and postreproductive periods. The use of CIEP for the detection of antibodies against ADV was performed on 84 European mink. All of the samples were negative (95% confidence interval [CI], 0–4%).[41] Protein electrophoresis was performed on 93 plasma samples from 82 European mink. Eleven plasma samples submitted for protein electrophoresis represented recaptures in a different period of time. Nine of the samples (9.6%) had gamma globulin levels exceeding 20% of the total plasma protein (95% CI, 3–15%). Only one recaptured European mink had an increase in gamma globulin levels exceeding 20% of the total plasma protein.

Complete necropsies were performed on 23 European mink cadavers. Two out of the 25 mink cadavers collected were autolyzed to derive any information. Seventeen of the animals had traumatic and hemorrhagic lesions compatible with vehicular impact injuries. Six different animals had lung lesions. In three mink, grayish-white nodules measuring 0.5 to 1 mm in diameter were found in both lungs. Microscopically, the lung showed numerous granulomas, each containing one spherical corpuscle measuring up to 60 μm in diameter, surrounded by a chronic inflammatory reaction. These structures, limited by a thick trilaminar PAS- and Grocott-positive wall, were identified as adiaspores of *Chrisosporium* spp. (Fig. 1). Occasionally spores also were observed in the bronchial lumen without granulomatous reaction. In one mink, interstitial pneumonia, marked depletion of lymphocytes in the splenic white pulp, splenic hyalinosis, and multifocal demyelination of the white matter in the cerebellum (pons and cerebellar peduncles) were diagnosed. In the same animal with interstitial pneumonia, CDV antigen was found in the different organs examined (lung, spleen, kidney, and cerebellar cortex) using immunohistochemical techniques (Fig. 2). In two mink, pulmonary lesions consisted of moderate granulomatous parasitic infection with viviparous nematodes, presumably *Filaroides* spp. (Fig. 3). In one mink, suppurative multifocal hepatitis, characterized by compact clusters of gram-negative bacteria within areas of necrosis and surrounded by a granulocytic infiltration, was observed. Unfortunately, no bacteria were isolated from aerobic cultures in this individual. Diffuse hepatic cellular swelling indicative of severe cellular distress and disseminated intravascular coagulation secondary to severe trauma were seen in the livers of two mink. In one mink, chronic interstitial nephritis with fibrosis and the presence of lymphoid aggregates in the intertubular connective tissue were noted. Finally, *Sarcocystis* infections affecting different striated muscles were observed in four mink. Numerous basophilic intracellular *Sarcocystis* cysts (3–8 cysts per field), with massive involvement of the diaphragmatic muscle (14–28 cysts per field) (Fig. 4), were observed. A few cysts were noted in the heart of one animal.

DISCUSSION

Using CIEP, there was no detectable serologic evidence of ADV exposure in the European mink

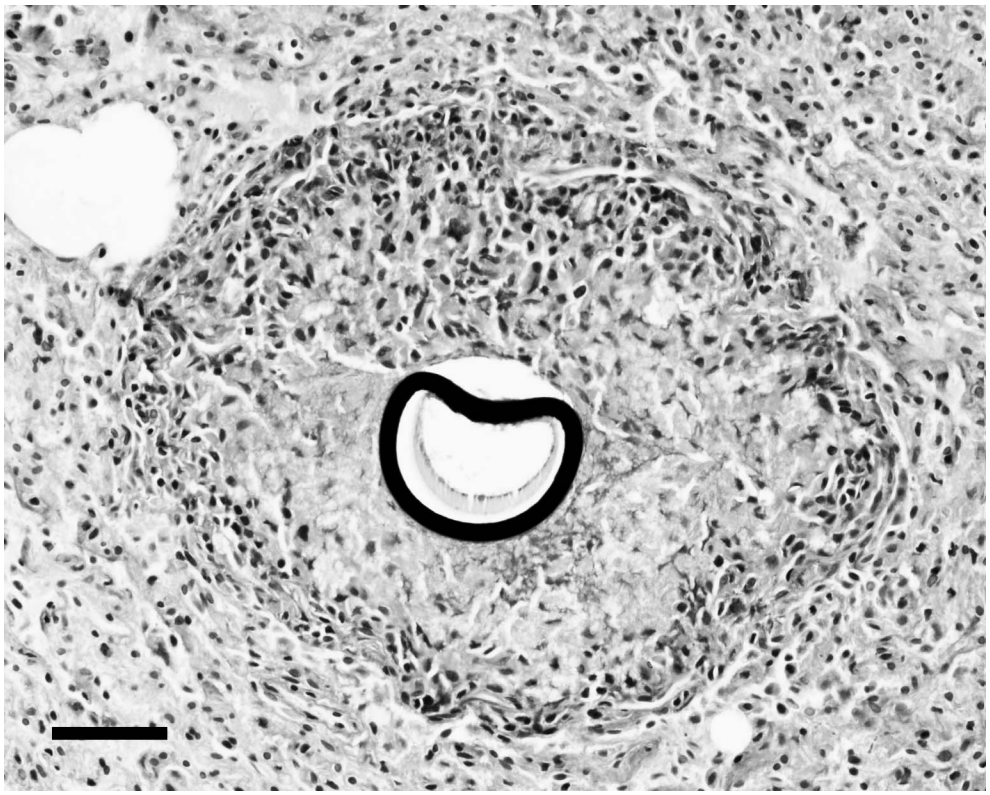

Figure 1. Mycotic granuloma containing a thick-walled adiaspore in the lung parenchyma. Grocott stain. Bar = 50 μm.

from Navarra, Spain, surveyed in this study. The use of CIEP has long been considered to be the standard for detecting anti-ADV antibodies in American mink and ferrets.[32] It is both a simple and rapid test when compared to complement fixation (CF) and immunofluorescence (IF), and it is also considered consistently specific.[15] Even when CIEP appears to be less sensitive than CF or IF, the advantages mentioned above have made it the assay of choice for routine detection of American mink and ferret anti-ADV antibody.[15] In addition, CIEP has also been found to be more effective (97%) than polymerase chain reaction (PCR) (62%) in identifying the presence of ADV infection from 94 mink serum samples.[10] Currently, enzyme-linked immunosorbent assay tests are available to detect anti-ADV antibodies with theoretical higher sensitivity, but there are no published reports regarding this data.[39]

Hypergammaglobulinemia was identified in 9.6% (9/93) of the samples submitted for protein electrophoresis. The presence of hypergammaglobulinemia with the concurrent presence of ADV antibodies in an American mink or ferret is strongly suggestive of AD.[30,39] None of the samples processed in this study indicated ADV antibody presence, and the results of the protein electrophoresis have to be interpreted cautiously, as many other disease processes can cause elevations in the gamma globulins.

In France, the most recent data appear to indicate an important reduction in the seroprevalence of ADV antibodies in France, when compared with the results of a previous published report, with samples obtained from 1996 to 2002.[6] The seroprevalence of ADV in the European mink was found to decrease from 11% to 4%, while the ADV seroprevalence in the polecat and American mink decreased from 10% to 2% and from 23% to 12%, respectively. In that study, CIEP and modified CIEP were used to estimate the prevalence of ADV.[6] The reduction in seroprevalence in both cases coincided with the beginning of an intensive control program for feral American mink. In this program, ADV-negative animals were sterilized and released, while the ADV-positive animals were euthanized (Fournier, pers. comm.).[7] In Spain from 1999 to 2003, the seroprevalence of ADV in the European mink

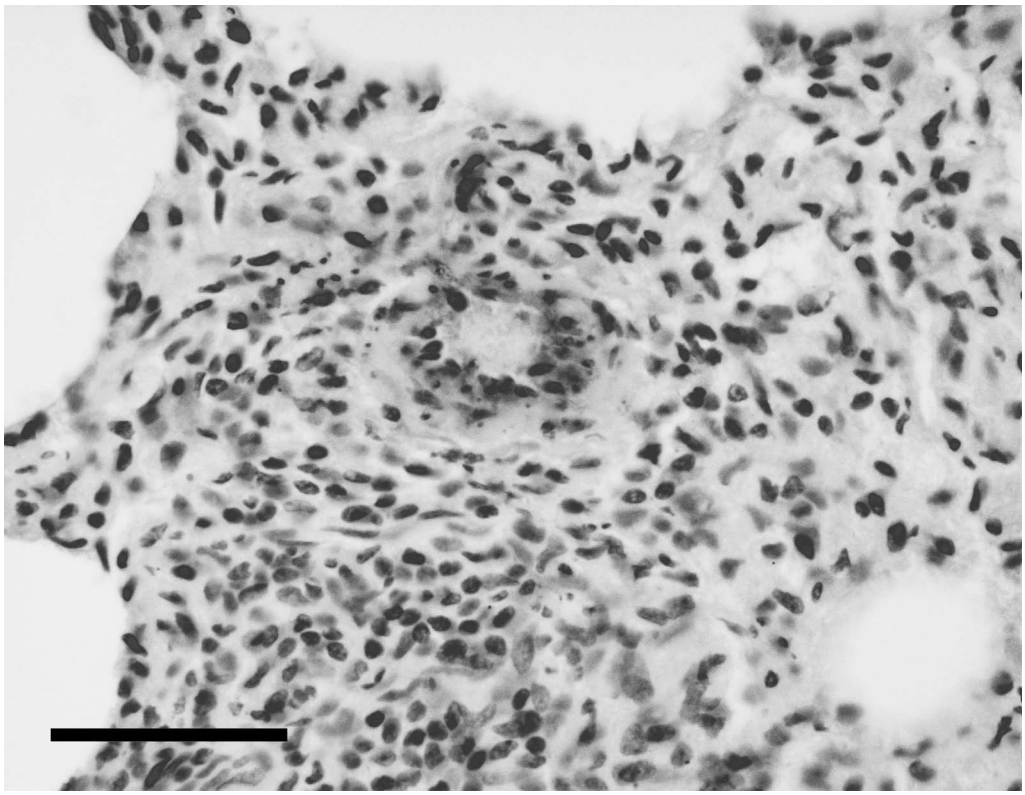

Figure 2. Lung. Canine distemper virus (CDV) antigen is found predominantly in the bronchiolar epithelium ABC system. Hematoxylin counterstain. Bar = 50 μm.

was found to be 25.2% (51/202), and seroprevalence of ADV was found to be 20.4% (44/216) in the American mink. No significant variations in the seroprevalence were detected over the duration of the study, which also used CIEP.[23] Currently, an American mink control program similar to the one in France is being executed in Spain.

In the only previously published data from Navarra, in 2000 the seroprevalence was found to be 21.4% (6/28) (95% CI, 6.3–36.5%).[21] The samples were processed by CIEP in the same laboratory as was used in this current study. The difference in seroprevalence values between the previous study and this present study could be attributed to seropositive animals being removed from the population, which thus rendered them unavailable for testing; clearance of the infection; or a laboratory error. There is no evidence of ADV cycling, and serial testing would be required to study this hypothesis. The absence of American mink in Navarra could explain the absence or low presence of ADV in the European Mink, since the American mink is considered the principal reservoir of the virus (Ceña, pers. comm.).[6,22] The origin of the ADV in the animals is uncertain.

Histopathologic lesions consistent with ADV infection were not found in any of the mink necropsied in this study. Although there is limited information regarding histopathologic lesions of ADV in European mink, ADV infection is thought to cause similar lesions to those observed in the American mink.[21–23] American mink infected with ADV can have a variety of histopathologic changes, including lymphoplasmacytic cellular infiltration in the liver, kidney, spleen, and lymph nodes; glomerulonephritis; and arteritis.[32] Depending on the viral strain, it is possible that the virus could demonstrate a similar behavior to that observed in ferrets. Ferrets that were experimentally infected with the American mink strains of ADV have been found to have replicating and persistent infections. The ferrets did seroconvert, but they were found to have lower levels of both ADV antibodies and gamma globulin levels when compared to mink. In addition, they produced more moderate histopathologic lesions when compared to mink. Ferret chal-

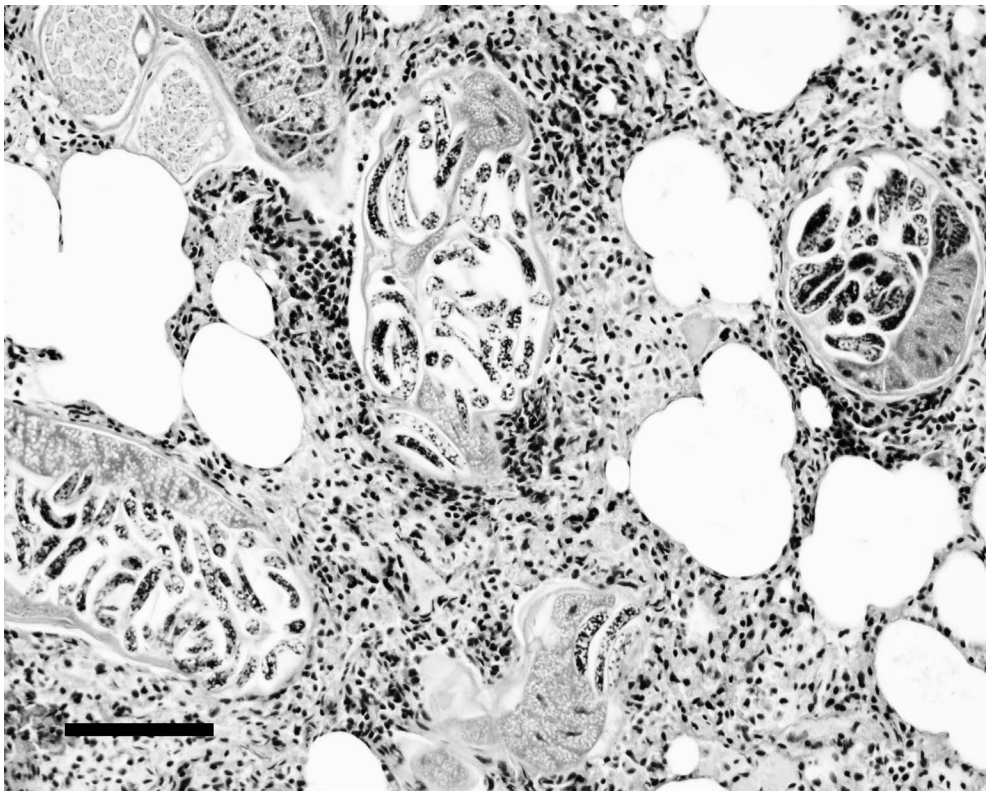

Figure 3. Filaroid nematodes containing larvae and surrounded by an inflammatory cellular infiltrate chiefly composed of eosinophils and lymphocytes. Hematoxylin and eosin (H&E). Bar = 100 μm.

lenged with ferret strains of ADV developed higher levels of ADV antibodies and gamma globulins and more severe histopathologic lesions when compared to ferrets challenged with American mink strains of ADV.[28,32] Mink that were experimentally inoculated with ferret strains of ADV produced antibodies but did not develop hypergammaglobulinemia or tissue lesions. In more recent reports of suspected cases of ADV in ferrets, lymphoplasmacytic hepatitis; interstitial lymphoplasmacytic nephritis; membranoproliferative glomerulonephritis; nonsuppurative meningitis; encephalitis; and astrocyte and plasma cell hypertrophy of the spinal cord were reported.[39,42]

The finding of adiaspiromycosis in two European minks at necropsy indicates that the fungus is present in the environment; this finding represents the first description of naturally occurring adiaspiromycosis in European mink in Spain. Pulmonary adiaspiromycosis occurs worldwide in small mammals, especially rodents, and humans can become infected by inhaling spores of the saprophytic soil fungus.[5,33,34] The diagnosis of adiaspiromycosis is usually based on the presence of large, thick-walled adiaspores on histopathologic examination; this finding of their typical structure is not exhibited by other fungi.[18,36] The lesions in the respiratory system, nervous system, and spleen, along with the immunohistochemical demonstration of CDV antigen in different tissues, indicate that this virus could be causing disease in European minks. The high susceptibility of mustelids (stone martens, polecats, badgers, and weasels), both juvenile and adult animals, with a seasonal prevalence in the summer that has been associated with the mustelid mating season, has been reported.[26] The presence, as in this study, of *Sarcocystis* spp. and filaroid nematodes has been observed in American mink and different mustelids, respectively.[26,33]

PCR and in situ hybridization (ISH) have been used to identify viral DNA from infected mink and ferrets.[10,22,27,31] In free-ranging small carnivores from Europe, at least three different strains of ADV have been identified.[21,22] Further studies are needed to characterize these viral strains and to determine their pathogenicity in the European mink and other small carnivores. In our study, PCR and ISH could not be performed because of budget limitations, but

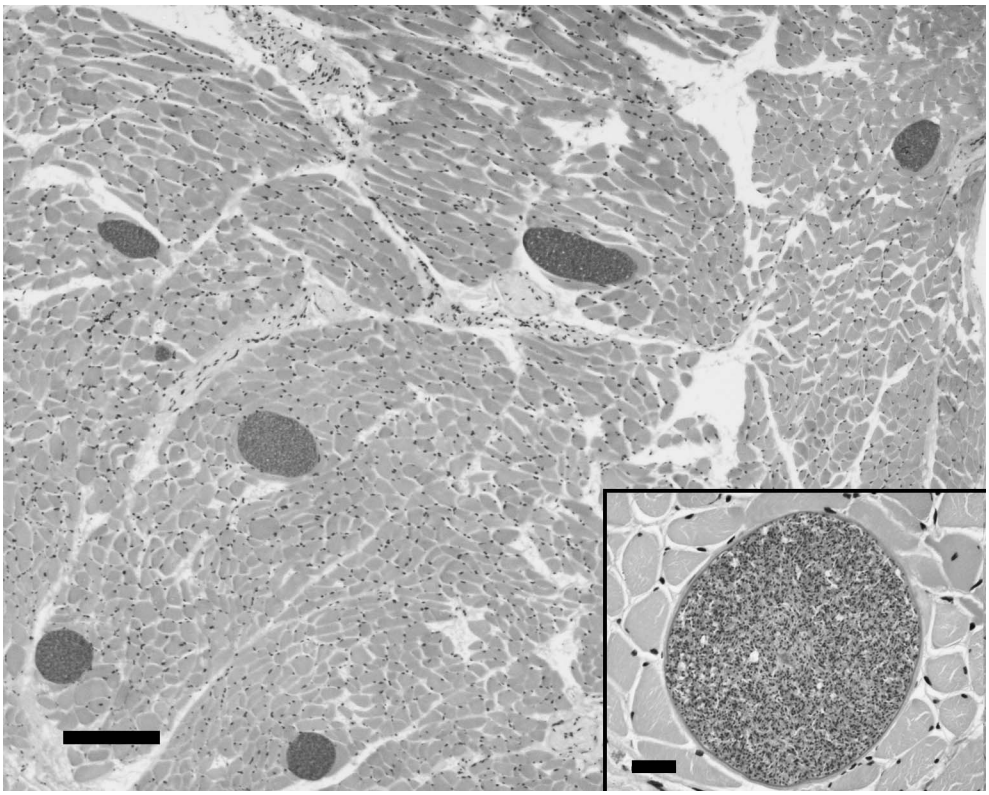

Figure 4. Sarcosporidian cysts in a skeletal muscle without any inflammatory reaction. Hematoxylin and eosin (H&E). Bar = 200 μm. The inset shows a cyst with numerous organisms. H&E. Bar = 25 μm.

these methods would have been ideal and are recommended in future studies.

The ADV can be transmitted horizontally through urine, feces, and saliva in the American mink and ferret and may also be spread by asymptomatic carriers.[1,31] Vertical transmission has also been documented in the American mink.[29] The ADV virus is highly persistent in the environment. Recent studies revealed that mink enteritis parvovirus under outdoor conditions can survive for 5–10 mo.[40] Although no data are available, ADV could survive in the environment in a similar fashion, and therefore any material contaminated with the virus may serve as a potential source of infection for uninfected animals. American mink and other small carnivores could thus act as a source of infection, since they share territory in the remaining range of the European mink.[28,32,35] Strict protocols for disinfecting equipment during trapping programs should be implemented. For disinfection of instruments, a minimum of 10 min of immersion in sodium hypochlorite (2%) is recommended; an initial cleaning to remove organic debris is advised. For the disinfection of cages and equipment, where contamination by organic matter is unavoidable, formalin (1% and 2%) and NaOH (2%, 1%, and 0.5%) are the disinfectants of choice.[37]

CONCLUSIONS

The results of this study document that ADV antibodies were not detected in the European mink from Navarra, Spain, using CIEP. The ADV may cause persistent subclinical infections in European mink, but highly virulent strains resulting from mutations of the virus could cause an outbreak of AD in areas of higher density. Because of the critical status of these animals, further studies are needed to characterize the epidemiology of ADV in the European mink. This study also describes the first report of a naturally occurring adiaspiromycosis and CDV infection in a European mink from Spain.

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