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Adenoviral hemorrhagic disease in California mule deer, 1990–2014

Leslie W. Woods,[1] Brant A. Schumaker, Patricia A. Pesavento, Beate M. Crossley, Pamela K. Swift

Abstract. We reviewed case records from the California Animal Health and Food Safety (CAHFS) laboratory and the California Department of Fish and Wildlife (CDFW) spanning 25 years (1990–2014) for all deer accessions submitted to CAHFS for pathology and/or histopathology, with and without a diagnosis of adenoviral hemorrhagic disease (AHD), in order to determine the prevalence of AHD in California. We also examined spatial and temporal distribution, age, and mule deer subspecies in deer that died from AHD. Of 483 deer submitted to CAHFS for diagnostic testing in 1990–2014, 17.2% were diagnosed with confirmed AHD, and 26.5% were confirmed plus suspected cases of AHD. Columbian black-tailed deer (*Odocoileus hemionus columbianus*), particularly fawns and juveniles, were most frequently affected. Deer adenovirus (*Odocoileus* adenovirus 1; OdAdV-1) was detected by immunohistochemistry in archived CDFW formalin-fixed, paraffin-embedded tissues from deer that died in mortality events in 1981, 1983, and 1986–1987. OdAdV-1 is a common cause of hemorrhagic disease mortality events in California deer, and mortality as a result of AHD is documented as early as 1981.

Key words: Adenovirus; deer; hemorrhagic disease; *Odocoileus* adenovirus 1.

Introduction

A newly recognized deer atadenovirus, *Odocoileus* adenovirus 1 (OdAdV-1; deer adenovirus 1, formerly Cervine adenovirus A), was identified as the cause of an epizootic of hemorrhagic disease that caused high mortality in resident and migratory mule deer (*Odocoileus hemionus*) in California in 1993 and 1994.[6,15,20] Mortality in deer during this event was estimated in the thousands, and more than 17 counties in California were affected. Male and female deer of all ages were affected, but fawns and juveniles appeared to be most susceptible to disease. Two manifestations of the disease occurred: systemic and localized infection. Deer with systemic infection had systemic vasculitis that was most prominently associated with pulmonary edema and/or intestinal hemorrhage. Animals with localized infection had severe necrosis and ulceration in the upper alimentary tract, infrequently with underlying deep vasculitis. Occasionally, deer had both manifestations of the disease. In deer with systemic infection, adenoviral intranuclear inclusions in the endothelium were always present microscopically. Only rarely were endothelial intranuclear inclusions present in deer with localized infection as a result of chronicity and obliteration of the lesion by secondary bacterial infections.

Both systemic and localized adenoviral hemorrhagic disease (AHD) were reproduced in subsequent experimental infection studies in Columbian black-tailed deer (BTD; *Odocoileus hemionus columbianus*) fawns[17] and yearlings,[16] and systemic disease was reproduced in white-tailed deer (WTD; *Odocoileus virginianus*).[18] Systemic infection with OdAdV-1 mimics the orbiviral hemorrhagic diseases, bluetongue (BT) and epizootic hemorrhagic disease (EHD) in WTD,[5] similarly causing pulmonary edema and intestinal hemorrhage, likely associated with disseminated intravascular coagulopathy resulting from vascular endothelial cell necrosis.[4] However, targeted endothelial cells are more frequent in medium-to-large vessels of deer with AHD compared to orbiviral diseases, in which microvascular endothelium is targeted.[8] Necrosis in the upper alimentary tract seen in localized infection is associated with vascular thrombosis and ischemic necrosis with ulceration in the oral cavity, esophagus, or forestomachs.

Deer adenoviral infection has since been diagnosed as a frequent cause of herd mortality in mule deer subspecies in the western United States and infrequently in mule deer, WTD, and moose in Iowa,[13] Idaho, Wyoming (LW Woods,

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pers. obs.), and Canada.[11] In our retrospective study of AHD in California, we examined the prevalence of OdAdV-1 infection in California deer as a cause of hemorrhagic disease. We also examined the spatial and temporal distribution of AHD in California herds as well as age and mule deer subspecies most commonly affected.

Materials and methods

Case selection and diagnostic workup

Case records from the California Animal Health and Food Safety Laboratory (CAHFS) were reviewed for all deer accessions submitted for pathology and/or histopathology, with and without a diagnosis of AHD from 1990 through 2014. Postmortem examinations were performed at CAHFS or at the Wildlife Investigations Laboratory, California Department of Fish and Wildlife (CDFW; Rancho Cordova, CA), and all microscopic examinations were performed at CAHFS. Deer submitted to CAHFS as whole carcasses for postmortem examination received full diagnostic workups in addition to gross and microscopic examination of tissues, which may have included tests for detection of bacteria [culture, immunohistochemistry (IHC)], virus [fluorescent antibody test, PCR, electron microscopy (EM), IHC], antibodies [virus neutralization test (VN), ELISA, complement fixation], and tissue minerals and toxins. Ancillary testing was based on individual clinical history or herd history and gross and microscopic findings. Reverse-transcription quantitative (RT-q)PCR and serology tests for detection of infection with BT virus (BTV) and EHD virus (EHDV) were performed routinely after 2009 for all deer accessions whenever the target tissue (carcass or spleen/bone marrow submitted from field postmortem cases) and serum (respectively) were available for testing. In 2009, BTV and EHDV RT-qPCR were included in a test panel performed at CAHFS for endemic ruminant viral diseases that mimic foot-and-mouth disease. The test panel was validated according to American Association of Veterinary Laboratory Diagnosticians (AAVLD) accreditation standards and has been consistent since that time.[3,9] Prior to 2009, standard PCR was performed intermittently for detection of BTV, as previously described, when lesions were compatible with the orbiviral hemorrhagic diseases.[1,7]

IHC and/or PCR to test for OdAdV-1 were performed on samples submitted during the years after test development and retrospectively on all cases of systemic infection for validation studies when history and lesions were compatible with OdAdV-1 infection. Additionally, archived CDFW formalin-fixed or paraffin-embedded tissues from deer with similarly described lesions that died during high mortality events in 1981, 1983, and 1986 were retrieved from CDFW, examined microscopically, and tested for OdAdV-1 by IHC.

All confirmed and suspected cases of OdAdV-1 infection in deer were examined. Confirmed cases were defined as cases with gross and microscopic lesions compatible with AHD (endothelial intranuclear inclusions) and identification of OdAdV-1 by IHC, qPCR, or EM. Suspected cases were cases of deer with gross and microscopic lesions characteristic of localized AHD infection (necrotizing pharyngitis, stomatitis, glossitis), but in which virus could not be detected given chronicity and obliteration of the primary lesion by secondary bacterial infection. Additional cofactors were investigated in suspected cases, such as associated herd mortality, diagnosis of systemic infection within the same herd, or region/county and concurrent adenoviral mortalities throughout the state in order to determine the likelihood of adenovirus as the cause of the localized infection. Lesions of systemic and localized infection, mule deer subspecies, age, location of confirmed and suspected cases of OdAdV-1–infected deer (Deer Assessment Unit, deer hunt zone, county, town, deer herd: resident or migratory), and other cofactors were gleaned from case histories. Deer cases in which BTV or EHDV were detected in tissues by qPCR were reviewed, and lesions were compared to AHD cases.

Spatial and demographic analysis

A GPS data point was individually assigned for each case using Google Earth (https://www.google.com/earth/) as follows. When a street address of the collection site was provided, an accurate data point (latitude/longitude-WGS84 [https://goo.gl/6Q9k5b]) was recorded. Cases submitted with only the nearest town recorded were assigned a data point of the town city hall. Samples submitted with approximate location (mountain pass, valley, lake, or ranch) were assigned data points at the center of the area. Samples submitted with only the deer hunt zone were assigned a data point at the center of the hunt zone. Samples submitted with only the county recorded received a data point at the center of the known deer habitat in the county.

Deer submissions were analyzed for associations between adenoviral infection status and age (adult vs. juvenile) and the 6 native California mule deer subspecies [Columbian black-tailed deer (*Odocoileus hemionus columbianus*), California mule deer (*Odocoileus hemionus californicus*), Rocky Mountain mule deer (*Odocoileus hemionus hemionus*), southern mule deer (*Odocoileus hemionus fuliginatus*), Inyo mule deer (*Odocoileus hemionus inyoensis*), and desert mule deer (*Odocoileus hemionus eremicus*)] with the null hypothesis that there was no association. Additionally, the number of deaths associated with the submission was compared for adenovirus and other diagnoses with the null hypothesis being no association. Analysis was performed using the Fisher exact test.[14]

Primer design

Selection of primers for amplification of adenoviral sequences was based on conserved sequence domains within the hexon gene of deer and among all available hexon sequences of the genera *Mastadeno*- and *Atadenovirus* (Hex 28–196). The

forward (3'-GCAAATTGAAGATGGGCAAA-5') and reverse (3'-TTTCTAGCCCTTGTCCGAGA-5') primers are from the fiber portion of the virion and amplify a 187-bp fragment (DADV 810–997). Selection of primers for distinguishing *Atadenovirus* from *Mastadenovirus* (Fib 12–224), and OdAdV-1 from other members of the *Atadenovirus* (Fib 810–997), was based on non-conserved sequence domains within the fiber gene. Primers were designed (Primer v.3.0 software, Life Technologies, Grand Island, NY) and thereafter analyzed by the Lawrence Livermore National Laboratories (LLNL) Bioinformatics group and the KPATH signature design system.[12] A BLAST search (https://goo.gl/KsJFCM) of the primers and probes in GenBank indicated no detectable cross-reactions against unintended targets.

DNA extraction for paraffin-embedded sections and fresh tissue

A total sectional width of 25–50 µm of paraffin-embedded tissue was subjected to a modified extraction procedure (QIAamp DNA mini kit 51106, Qiagen, Hilden, Germany) with the following modifications: after addition of ATL buffer, the sample was placed at 98°C for 15 min and cooled to room temperature for 5 min prior to addition of proteinase K. The sample was then placed at 68°C for 45 min. Precipitated DNA was suspended in 60 µL of 10 mM Tris–HCl (pH 8). Estimated yield was 1.8–2.2 ng of genomic DNA/5 µL. A total of 5 µL of template was used per reaction. Amplification of a 200-bp region of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used in each individual extraction as a control for DNA recovery and quality (GenBank accession AY650282).

PCR reaction conditions

For each specimen, 50-µL reactions were prepared by adding 5 µL of DNA extract to 45 µL of master mix. The master mix was 29.75 µL of nuclease-free water, 5 µL of 10× PCR buffer (containing 15 mM $MgCl_2$), 1 µL of each dNTPs, 2 µL each of the forward and reverse primers, and 0.25 µL of HotStarTaq (Qiagen). Amplification in a thermocycler (DNA engine PTC-200, Bio-Rad, Hercules, CA) consisted of an initial round at 95°C for 15 min, 60°C for 30 s, 72°C for 1 min, 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, followed by 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, then 72°C for 1 min, and 4°C hold. The PCR products were separated by electrophoresis in 1.5% agarose gel (Fisher Scientific, Pittsburgh, PA) containing ethidium bromide solution (Sigma-Aldrich, St. Louis, MO). The primers used for our study were tested on 100 known positive individual tissues in which OdAdV-1 was detected by IHC (which included multiple tissues from deer with natural systemic infection and experimentally inoculated deer)[16,17] and on 50 known negative tissues for OdAdV-1 (verified by clinical history, pathology, and IHC).

PCR validation

For screening of incoming pathology cases, the original PCR assay was adjusted to a 96-well plate format to fit the routine workflow. Quantitative PCR assay for OdAdV-1 was performed as described previously.[3,9] Validation of the assay was performed following the guidelines of the AAVLD for PCR assays available in 2011. Extraction of tissue (lung, liver, kidney, spleen, and intestine) was completed (MagMAX-96 viral isolation kit, Life Technology, Carlsbad, CA) according to manufacturer's recommendation. The extraction of brain was performed using Trizol (Invitrogen, Carlsbad, CA). Briefly, a 10% brain tissue suspension was prepared and homogenized under addition of 2.5 mm zirconia–silica beads (MagNA Lyser, Roche, Basel, Switzerland) on speed setting 6,500 for 45 s. The homogenized tissue was extracted following the recommendations of the manufacturer. From the extracted DNA material, 8 µL were added to a reagent mixture containing 1 µL of each previously described primer (final concentration of each primer: 40 µmol) and commercial enzyme-buffer concentration (Path ID multiplex one step PCR, Life Technology) to a total volume of 25 µL. Standard PCR was performed (ABI 7500 thermocycler, Applied Biosystems, Thermo Fisher, Fremont, CA) using the following thermo-profile: denaturation at 95°C for 900 s followed by 35 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 60 s. PCR products (5 µL) are subjected to 4% precast agarose gel (Invitrogen). Samples that produced a visible band of ~150 bp were cleaned up for Sanger sequencing (Amicon ultra centrifuge filters, Ambitec, Chicago, IL) following recommendations of the company, and sequenced for confirmation. OdAdV-1 is difficult to propagate in cell culture, therefore deer tissue samples with a confirmed OdAdV-1 status were used for the analytical validation. Diagnostic validation was performed on archived frozen and fresh diagnostic sample material. Confirmatory test methods used were either IHC or EM. For test performance calculation, only one tissue per animal was used.

Immunohistochemistry

IHC was performed as described previously, with some modifications.[17] Briefly, 4–5 µm, paraffin-embedded tissue sections were applied to charged slides, deparaffinized, rehydrated, and then incubated with 3% hydrogen peroxide for 5–10 min at room temperature to quench endogenous peroxidase activity. The specimens were digested with prewarmed 0.4% pepsin (s/v in 0.1 N HCL, pH 7.8, Sigma Chemical, St. Louis, MO) for 10 min at 37°C. Nonspecific (rabbit IgG, Vector Labs, Burlingame, CA) and specific antibodies (anti-deer adenovirus rabbit polyclonal antibody, provided by Dr. Howard Lehmkuhl, USDA-ARS National Animal Disease Center, Ames, IA) were applied for 30–60 min after rinsing with Tris buffered saline–Tween buffer (Biocare, Pacheco, CA) for 3–5 min. Rabbit-on-Farma HRP polymer (Biocare) was applied for 45 min and then rinsed

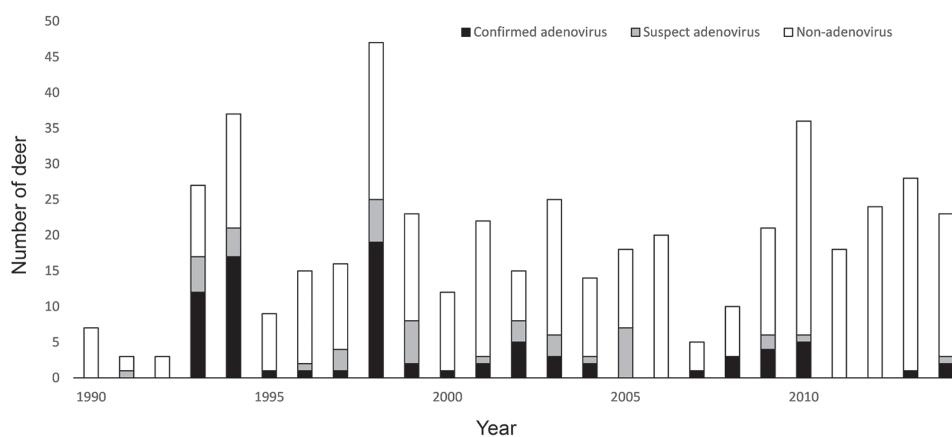

Figure 1. Confirmed and suspected adenoviral hemorrhagic disease (AHD) cases by year and total deer submissions for 1990–2014 (n = 483). AHD was confirmed in 1993–2004, 2007–2010, 2013, and 2014. Suspected cases of AHD occurred in 1991, 1993–1994, 1996–1999, 2001–2005, 2009–2010, and 2014. Large epizootics of AHD with high herd mortality in more than one county occurred in 1993–1994, 1998–1999, 2002–2003, 2008, 2009, 2010, and 2014.

with buffer for 3–5 min; 3-amino-9-ethylcarbazole (AEC RTU, Thermo Fisher) was applied for 10–20 min and then rinsed with distilled water. Slides were then placed in Mayer hematoxylin for 1–5 min, rinsed in distilled water and then tap water, after which aqueous mounting medium was applied and slides coverslipped.

Results

Mortalities and postmortem examinations

From 1990 through 2014, 483 deer carcasses or tissues were submitted to the CAHFS laboratory system for gross postmortem examination and/or histopathology. Eighty-three of 483 deer were diagnosed with systemic OdAdV-1 infection (17.2% confirmed cases). Forty-five of 483 deer had lesions that were suspect but unconfirmed AHD (9.3%). Suspect cases were characterized as likely adenovirus localized infection based on cofactors described previously. Combined adenovirus-confirmed and -suspected cases of AHD comprised 26.5% of the total deer cases submitted to CAHFS during the study period.

Cases of confirmed (systemic) AHD were submitted to the CAHFS Davis laboratory every month except for February, March, and November, with the majority of cases occurring May–October. Suspected cases of AHD were more evenly scattered throughout the year, but with the majority of cases in July and August. AHD was confirmed in 1993–2004, 2007–2010, 2013, and 2014 (Fig. 1). Suspected cases of AHD occurred in 1991, 1993–1994, 1996–1999, 2001–2005, 2009–1010, and 2014. Large epizootics of AHD with high herd mortality in multiple counties occurred in 1993–1994, 1998–1999, 2002–2003, 2008, 2009, 2010, and 2014. During 2010 and 2014, AHD was second only to hair loss syndrome as the most common diagnosis as a cause of mortality in mule deer.

Spatial and demographic analysis

California deer live throughout the state with minimal numbers in the Central Valley and Mojave Desert areas, and are managed in 45 hunt areas. Prevalence of adenoviral infection in CAHFS submissions was higher in northern hunt areas (Fig. 2). Submissions of adenovirus-infected deer were more likely to be associated with multiple deaths (72 of 129, 55.8%) than deer diagnosed with other causes of death (126 of 347, 36.3%; $p < 0.05$). Adenovirus submissions were more likely to be juvenile animals (59 of 82, 72%) than other causes of death (84 of 225, 37.3%; $p < 0.05$). There was a significant association of adenovirus by deer subspecies ($p < 0.05$), with Columbian black-tailed deer over-represented among adenovirus cases versus non-cases (89.3% of cases but only 70.4% of non-cases) and California mule deer under-represented (6.9% of cases but 23.1% of non-cases). Infection with OdAdV-1 was not diagnosed in the 3 remaining mule deer subspecies in California: southern mule deer, desert mule deer, and Inyo mule deer.

Pathology

Pathology findings of all deer with a diagnosis of confirmed and suspect AHD were reviewed for distribution of lesions. Of 83 deer with systemic infection, all had pulmonary edema (Fig. 3A) and 22 also had hemorrhage in the intestinal tract (26.5%; Fig. 3A, 3D). Twenty of these 83 deer (24%) with OdAdV-1 systemic infection also had lesions in the upper alimentary tract (10 with stomatitis, 5 with necrosis in the forestomach, 4 with esophageal necrosis, and 1 with abomasal necrosis). Of 45 cases of likely localized infection, all had necrotizing stomatitis, which most commonly affected the pharynx or tongue (Fig. 3B, 3C). Four of those also had osteomyelitis (Fig. 3C) associated with deep necrotizing lesions in the mandible or pharynx, and 7 had necrotizing

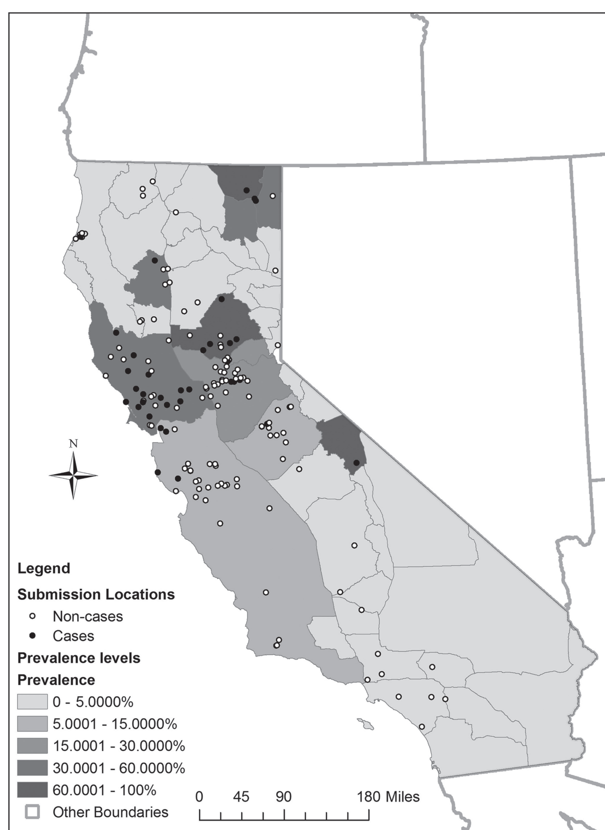

Figure 2. Percentage of deer submitted to California Animal Health and Food Safety with confirmed or suspected adenoviral hemorrhagic disease (AHD) by hunt zones. Note higher prevalence of AHD in the northern hunt zones. Regions of higher prevalence are also regions with more concentrated populations of Columbian black-tailed deer.

lesions in the forestomach(s) (Fig. 3D). Secondary bacterial infections with multiple aerobic and anaerobic bacteria within the necrotizing lesions were common.

Microscopically, deer with systemic infection had pulmonary edema that was most prominent in the interlobular septa (Fig. 4A). Vasculitis was most frequently subtle and characterized by endothelial hypertrophy with intranuclear inclusions and margination of leukocytes (Fig. 4C). Florid vasculitis was infrequent. Vasculitis was most often present in the large vessels of the lungs and large serosal vessels of the intestines (Fig. 4C). Endothelial intranuclear inclusions ranged from infrequent to frequent but could always be detected (Fig. 4C inset). IHC demonstrated that virus was most frequent in endothelium in large and medium vessels, but could be detected in microvasculature infrequently (Fig. 4D). When hemorrhagic enteropathy was present, hemorrhage was detected in the lamina propria and intestinal lumen without associated necrosis or inflammation (Fig. 4B).

OdAdV-1 was detected by IHC in CDFW archived, formalin-fixed tissues or paraffin blocks of tissues from deer that died in high mortality events in 1981, 1983, and 1986–1987. Some of these cases of AHD had been diagnosed as BT based on gross anatomic lesions.

PCR validation

Of the 100 OdAdV-1 IHC–positive tissues from deer with systemic infection that were tested by standard PCR, 100% were positive by PCR for OdAdV-1. All 50 known negative tissues tested negative by PCR. For the RT-qPCR validation assay, 38 tissues were taken from deer in which affected tissues were positive for OdAdV-1 by IHC. OdAdV-1 was detected in 32 (of 38, 84%) of those samples by RT-qPCR. Of the 32 OdAdV-1 RT-qPCR–positive samples, 31 samples were taken from deer with systemic OdAdV-1 infection. The 6 samples that did not test positive by RT-qPCR were lung samples taken from OdAdV-1–infected animals with localized infections (without systemic infection) confirmed as adenovirus by experimental inoculation or association with deer with systemic infection in a closed herd. A total of 62 samples were tested by RT-qPCR, with 100% agreement between the RT-qPCR results and the confirmatory test, including final assessment by the pathologist.

Orbiviruses

From 2009 through 2016, 133 BTV RT-qPCR tests were performed on deer tissue, of which 7 were positive. EHDV RT-qPCR was performed 121 times, of which EHDV was detected 12 times. In one deer with systemic OdAdV-1 infection, a few copies of BTV were detected (indeterminate); in none of the remaining deer with systemic or localized OdAdV-1 infection were either of the orbiviruses detected. Whereas all deer with AHD had pulmonary edema, this lesion was prominent in only 2 deer with orbivirus infection. Intestinal luminal hemorrhage was seen in 26% of the deer with AHD, but was only observed in 2 orbivirus-infected deer (1 EHD and 1 BT). Three orbivirus-infected deer had hemothorax and/or hemoabdomen (1 BT, 2 EHD); 4 orbivirus-infected deer had subcutaneous hemorrhage (1 BT, 3 EHD). Alimentary ulcers evident in orbivirus-infected deer were superficial and seen in 4 orbivirus-infected deer (2 BT, 2 EHD), whereas deer infected with OdAdV-1 had deep necrosis in the oral cavity and forestomach. Myonecrosis in the esophagus was present in 3 BTV-infected deer, and myocardial necrosis was evident in 5 orbivirus-infected deer (3 BT, 2 EHD); 4 of these had very low liver selenium, compatible with deficiency. Pigmentary nephrosis was seen in 5 deer with orbivirus infection (2 BT, 3 EHD). Meningoencephalitis with subtle lymphocytic vasculitis was apparent in 2 EHDV-infected deer. Other deer in which RT-qPCR detected orbivirus were diagnosed with bacterial bronchopneumonia, pleuritis, fungal rumenitis, tubulointerstitial nephritis, and peritonitis.

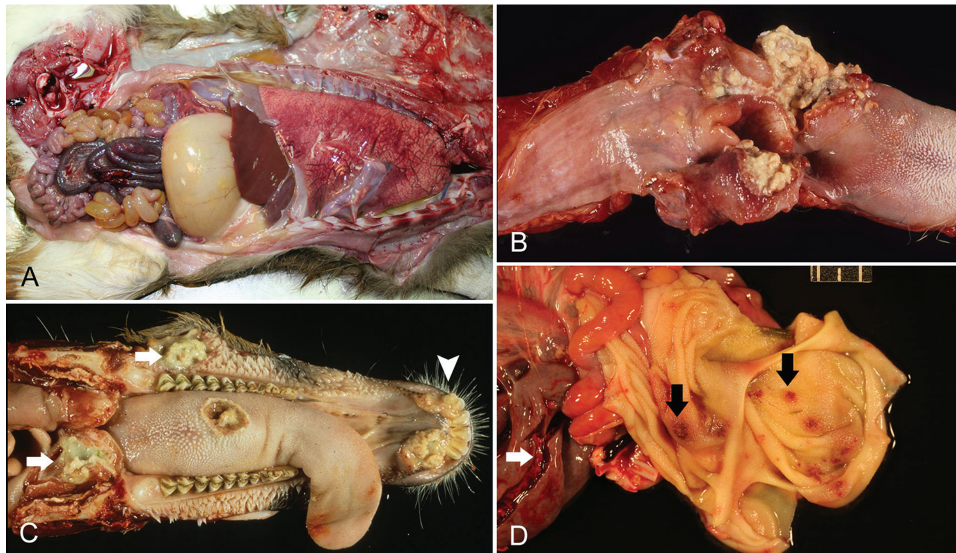

Figure 3. Mule deer with adenoviral hemorrhagic disease (AHD). **A.** Acute systemic AHD with pulmonary edema and hemorrhagic enteropathy. **B.** Necrotizing pharyngitis, glossitis, and laryngitis. Deep necrosis is associated with vasculitis of large vessels with subsequent ischemic necrosis and secondary bacterial infection. **C.** Deer mandible with necrosis of the buccal mucosa and pharynx (arrows) and the tip of the mandible (arrowhead), and deep focal necrosis in the tongue. Secondary infection with numerous aerobic and anaerobic bacteria typically obliterates the primary viral vasculitis-associated ischemic necrosis. **D.** Multifocal ulceration in the rumen (black arrows) and frank blood in the intestine (white arrow) of a deer with lesions of both localized and systemic infection.

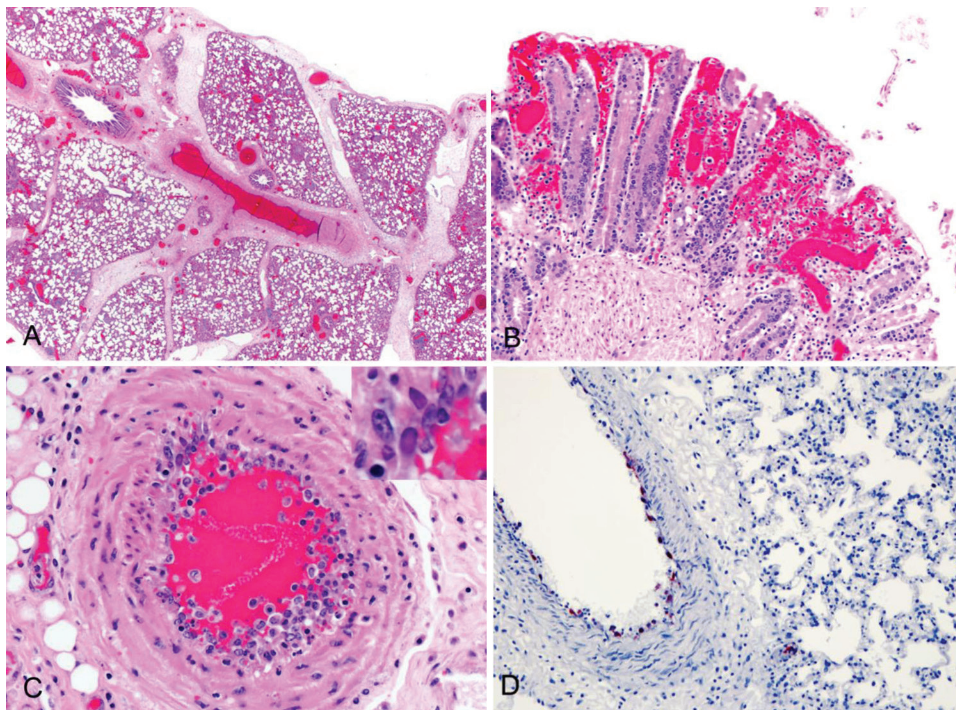

Figure 4. Black-tailed deer with adenoviral hemorrhagic disease (AHD). **A.** Interlobular septal edema separates pulmonary lobules. Cellularity of large vessels demonstrates vasculitis. H&E. **B.** Lamina propria hemorrhage with no associated mucosal necrosis is seen with acute systemic AHD (hemorrhagic enteropathy). H&E. **C.** Vasculitis of a serosal blood vessel, with endothelial necrosis, leukocytic inflammatory cell infiltrates in the intima, and margination of leukocytes. H&E. Inset: higher magnification of vessel with endothelial cell intranuclear inclusions. H&E. **D.** Immunohistochemistry demonstrates most frequently labeled cells infected with *Odocoileus* adenovirus 1 are in larger vessels, with less frequently infected cells in the microvasculature of the lungs. IHC.

Discussion

Adenoviral hemorrhagic disease was diagnosed in deer every year but 3 since the 1993–1994 epizootic when the disease was first described.[15] The outbreaks occurred in resident and/or migratory deer mostly affecting counties in which AHD was confirmed or suspected during the 1993–1994 epizootic. Most mortality events involved many animals in a single county or few contiguous counties. High herd mortalities occurred in 1998–1999, 2002–2003, 2008–2010, and 2014 but none as large as the epizootic in 1993–1994 that spread to over 17 counties with greater than a thousand deer affected. IHC demonstrated OdAdV-1 in CDFW archived tissues from 1981, 1983, and 1987, all associated with large mortality events, indicating OdAdV-1 has been present and associated with high mortalities in California deer at least since 1981. The 1986–1987 event was particularly large per historical records. The reason for the periodic large epizootics is unknown and needs to be elucidated.

The definition of confirmed cases required demonstration of endothelial intranuclear inclusions and detection of OdAdV-1. OdAdV-1 cannot be detected in the unaffected lungs of animals that die from localized infection (as demonstrated in the RT-qPCR validation assay), and in many cases, OdAdV-1 cannot be demonstrated in affected upper alimentary tissues of deer with localized infection, likely because of obliteration of the primary vasculitis by necrosis and secondary bacterial infection or because of the transient nature of the virus in localized infections. Although diagnosis of AHD in deer with localized infection is not definitive in many cases, many of the localized infection cases in our study can be included in the total number of AHD cases based on a history that deer came from herds in which systemic infection was confirmed. Although OdAdV-1 antigen was not demonstrated in these animals, it has been clearly demonstrated in experimental infection studies, as well as in a case study of natural infection in a captive group, that the lesions used in our study to define suspect cases are produced by infection with OdAdV-1, and that bacterial infections are secondary and typically mixed.[2,16,17] Other differential diagnoses of localized infection could be foxtail migrations with secondary bacterial infections or *Fusobacterium necrophorum* infection. Tests for BTV and EHDV were performed in many of the localized cases in which tissues were available and neither was detected in any of these cases that were considered suspect cases. Additionally, none of the orbivirus cases diagnosed in California deer had lesions similar to deer with localized AHD. Lesions of orbiviral ulcerative stomatitis were more superficial compared with deep necrosis in deer with AHD. As in the lungs, the orbiviruses target the microvasculature of the alimentary tract, whereas OdAdV-1 more frequently targets larger vessels, therefore causing deep ischemic necrosis. However, there is some overlap, and diagnosis of AHD localized infection in free-ranging deer should be made based on typical lesions and concurrent mortality in the herd because of confirmed systemic infection or high antibody titer to OdAdV-1.

The number of deer submissions to CAHFS was high during the 1993–1994 outbreak when AHD was first discovered, reflecting the wide distribution of the epizootic in northern and central California. Of 128 confirmed and suspected cases, 39 clustered around 1993–1994. Typically, 1–3 deer from a mortality event in a specific herd were submitted for a definitive diagnosis of the cause of mortality in that county. All deer from a natural disease outbreak in a university deer herd were submitted to CAHFS for postmortem examination in 1998 to study natural adenoviral infection in a closed captive herd[2]; therefore, the number of cases in 1998 was higher than the numbers that represented free-ranging herds. The deer in this captive herd were included in our study because there were concurrent mortalities in adjacent and distant counties during this time period and these deer mortalities represented a natural outbreak of systemic and localized infection in Yolo County.

Adenoviral hemorrhagic disease was more commonly diagnosed in non-migratory BTD that reside in the coastal, more populated regions of the state. Experimental studies have demonstrated that OdAdV-1 is very infectious and easily transmitted by contact.[17] Nose-to-nose contact, and therefore transmission of the virus, is more likely as residential herd populations increase in size and urban boundaries are more restricted. The higher percentage of BTD submitted to CAHFS with and without AHD may also be the result of greater visibility of mortalities in these more urban populations.

All native deer in California are mule deer subspecies, which includes Columbia black-tailed deer, California mule deer, Rocky Mountain mule deer, southern mule deer, desert mule deer, and Inyo mule deer. AHD was not diagnosed in southern mule deer, desert mule deer, and Inyo mule deer during the study period or in the non-native fallow deer (*Dama dama*). The reason is currently unknown. Possible factors could be that these deer subspecies are less susceptible to infection and/or disease associated with OdAdV-1, mortalities are not as visible, or locations are not as accessible to retrieve carcasses to submit for diagnostic investigation. California does not have WTD, and importation of WTD into the state is restricted.

Differential diagnoses of lesions attributed to OdAdV-1 include the orbiviral diseases, BTV and EHDV. In the report of the 1993 epizootic, BTV was not isolated from 7 of 13 deer with AHD that were tested for BTV. BTV was detected by standard PCR in 1 of 3 deer with AHD that were tested for BTV, whereas OdAdV-1 was demonstrated in all 13 deer with systemic infection.[15] BTV was not detected by standard PCR in any of the deer that died in the captive herd in 1998.[2] In our study, BTV was only detected in one deer with AHD (2009–2014), and EHDV was not detected by RT-qPCR in any. Of 254 RT-qPCR tests for detection of BTV or EHDV, 19 were positive. Two had hemorrhagic enteropathy and 2 had pulmonary edema. Ulceration in the upper alimentary

tract was superficial in the 2 deer with BTV infection in contrast to the deep necrosis seen in deer with AHD. Although lymphocytic encephalitis was seen infrequently in deer with AHD, it was always accompanied by endothelial intranuclear inclusions and systemic vasculitis, in contrast to the deer in our study diagnosed with EHD in which the only lesion was subtle lymphocytic vasculitis in the brain. Hemorrhage that may be associated with disseminated intravascular coagulopathy, as demonstrated in WTD experimentally infected with the orbiviruses,[4] was only seen in 2 mule deer (subcutaneous hemorrhage and hemothorax and/or hemopericardium) and only in deer infected with EHDV. Our study demonstrates that lesions in mule deer infected with OdAdV-1 are not typically identical to lesions in mule deer infected with orbiviruses.

A study on the seroprevalence of BTV and EHDV in California indicated 13.4% seroprevalence for BTV and 16.8% seroprevalence for EHDV statewide, with seroprevalence as high as 49–51% for both BTV and EHDV in some of the northern counties of the state.[10] Seroprevalence studies indicate that a high percentage of deer, particularly in the northern hunt zones, are exposed to orbiviruses without apparent clinical disease given that most samples were collected from healthy live deer during capture events.[10] Studies have demonstrated that white-tailed deer are susceptible to experimental and natural infection with BTV and EHDV.[4] BT and EHD have not been reproduced in mule deer in experimental infection studies.[19] These previously published reports on seroprevalence and experimental infection in conjunction with our retrospective study suggest that mule deer may be more resistant to the orbiviral hemorrhagic diseases than are WTD, and that the most common cause of hemorrhagic disease epizootics in California deer characterized by pulmonary edema, intestinal luminal hemorrhage, and necrotizing stomatitis is OdAdV-1.

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