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Experimental Bluetongue and Epizootic Hemorrhagic Disease Virus Infection in California Black-tailed Deer

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ABSTRACT: Four adult black-tailed deer (*Odocoileus hemioneus columbianus*) and five fawns were inoculated with bluetongue virus (BTV) and one adult deer was inoculated with epizootic hemorrhagic disease (EHD) virus to produce clinical signs and lesions of hemorrhagic disease. Serologic response was monitored using the agar gel immunodiffusion (AGID) test and the competitive enzyme-linked immunosorbent assay (C-ELISA). Embryonating chicken eggs and vero cells were used to detect viremia. No animal exhibited clinical or pathologic signs of hemorrhagic disease. Bluetongue viremia was detected as early as 2 days post-inoculation (DPI-2) and in some animals, persisted until at least DPI-12. The earliest detection of BTV antibodies using the AGID was DPI-8. Two adult deer remained seropositive for BTV antibodies for >9 mo and 1 yr, respectively, using both the AGID and C-ELISA tests. We observed cross reactions between BT and EHD antibodies using the AGID tests. Also, the AGID test did not consistently detect exposure to BTV. Viremia was not detected in the deer inoculated with EHD although this animal was AGID positive between DPI-6 and DPI-49.

Key words: Bluetongue, epizootic hemorrhagic disease, black-tailed deer, infection, serology, *Odocoileus hemioneus columbianus*.

The causative agents of bluetongue (BT) and epizootic hemorrhagic disease (EHD) belong to two serogroups of double stranded RNA viruses of the genus *Orbivirus* (family Reoviridae) (Knudson and Shope, 1985). Bluetongue virus (BTV) has 24 serotypes (Gard et al., 1987) of which types 2, 10, 11, 13, and 17 occur in North America (Knudson and Shope, 1985). Epizootic hemorrhagic disease virus (EHDV) is classified into seven serotypes of which types 1 and 2 occur in North America (Knudson and Shope, 1985). Both viruses are transmitted by Cerotopogonid gnats of the genus *Culicoides* (Price and Hardy, 1954; Jones et al., 1977).

The white-tailed deer (*Odocoileus virginianus*) has been the most closely examined species in attempts to characterize BT and EHD in wild ruminants. Infections with BTV or EHDV in white-tailed deer cause similar gross lesions and clinical signs and are occasionally referred to as "hemorrhagic disease" because of the pathologic changes induced by these viruses (Karstad et al., 1961; Karstad and Trainer, 1967). Cause of death for both diseases is believed due to loss of vascular integrity and consequent disseminated intravascular coagulation (Karstad et al., 1961; Karstad and Trainer, 1967; Howerth et al., 1988; Howerth and Tyler, 1988).

The possibility that BTV or EHDV are responsible for "hemorrhagic disease" in California black-tailed deer (*Odocoileus hemioneus columbianus*) has been suggested (Jessup et al., 1984, 1990; Work and Jessup, 1990). However, clinical signs of BT or EHD are variable and often mimic other diseases (Luedke and Jochim, 1968). Accurate diagnosis of BT or EHD during suspected outbreaks in California is difficult due to lack of success in isolating virus from dying or dead deer. Interpretation of serology is complicated by lack of information as to how long deer seropositive to BTV or EHDV have been infected or how long antibodies remain detectable.

Our objective was to characterize BTV and EHDV infection in black-tailed deer under controlled conditions. The specific aims of this study were to infect deer with BTV or EHDV and describe clinical signs, gross and microscopic pathologic changes, serology and viremia.

We used five adult and five fawn black-tailed deer from various regions of northern California. All animals were housed in insect-proof enclosures according to University of California animal care and use

protocols. Fawns were removed from their mothers within 48 hr of birth, hand-raised in insect-proof enclosures, and exposed to test viruses at 6 mo of age. Three adult deer were removed from their enclosure 3 mo after inoculation and housed in covered pens near the northwest coast of California, an area deemed free of BT. The study lasted from 13 January 1989 to 16 May 1990.

Prior to inoculation, we gave all animals a physical exam which included inspection of the mouth and hooves for lesions. We also screened blood from all animals for presence of BT and EHD virus and specific antibodies to these viruses. All virus isolations were made from heparinized red blood cells washed three times, immediately after collection, in physiological saline containing 2% penicillin (Gibco, Gaithersburg, Maryland, USA) and 2% streptomycin (Gibco), and stored at 4 C. Red blood cells were sonicated and injected intravenously (IV) into 10-day-old embryonating chicken eggs. Upon death, chick embryos were ground and passed through embryonating chicken eggs (Goldsmith and Barazilai, 1968). Virus from eggs was subsequently adapted to vero cell cultures (Wechsler and McHolland, 1988), with cytopathic effect (CPE) read at 7 days. Virus concentration was estimated by inoculating serial 10-fold dilutions of viral inoculum onto vero cells and observing for CPE at 7 days. Serotyping of viral strains was done using the plaque inhibition method (Stott et al., 1978).

Agar gel immunodiffusion (AGID) tests (Pearson and Jochim, 1979) were used to screen and monitor all animals for exposure to BT and EHD. A competitive enzyme linked immunosorbent assay (C-ELISA) kit (Blueplate Special, DiagXotics, Wilton, Connecticut, USA) was used retrospectively to detect antibodies to BT. The sera tested by C-ELISA included those collected at the time the animal was infected (for a negative control) and those collected at least 6 days after infection. The number of samples tested by C-ELISA was limited to the number of kits available, therefore attempts were made to test sera collected between days 6 and 12 post-inoculation (DPI-6 to DPI-12) or to test sera collected beyond DPI-12. A bluetongue antibody positive on the C-ELISA was determined according to the instructions supplied with the kit. Briefly, the optical density of the sample (ODS) was read on a Dynatech MR600 ELISA plate reader (Dynatech, Alexandria, Virginia, USA). The background OD (ODB) was substracted from the ODS to give an adjusted OD (ODJ). Finally, the ODJ was divided by the OD of the negative control serum and the result multiplied by 100 to give the percent OD of negative control (ODP). Test sera producing an ODP of 0 to 70% were considered positive. Optical density of the negative control was calculated using both the negative control sera supplied by the kit and negative (DPI-0) sera from the experimental deer.

In January 1989, 3 ml of whole washed blood infected with BT virus serotype 17 (BT-17) from a local domestic sheep were inoculated, half subcutaneously (SQ) and half intradermally, in the medial thigh in multiple sites (ID) into a second sheep (sheep B) that was serologically and virologically negative for BTV and EHDV. After inoculation, sheep B developed a fever on DPI-3 that lasted 8 days; this animal survived. Three ml of whole blood, collected DPI-7 from sheep B, were inoculated IV into an adult deer (deer #1). Five ml of whole blood from deer #1 collected on DPI-4 were inoculated, half SQ and half ID, into each of two adult deer (deer #2 and #3) and one fawn (deer #4). Separately, 9 ml of whole blood from deer #1, harvested on DPI-5, were inoculated into each of two fawns (deer #5 and #6), with half the dose going SQ and half ID in multiple sites in the medial thighs. The viral concentrations inoculated into these animals were not determined.

When BT-17 from sheep A failed to produce clinical disease in deer #1 through

#6, we used two other strains of BT-17, one other serotype of BT and one strain of EHD. One adult (deer #7) received a 3 ml IV inoculum of tissue culture supernate containing $10^{4.0}$ tissue culture infective dose 50% per milliliter ($TCID_{50}$/ml) of BT-17 strain 62-45S (Mahrt and Osburn 1986). This supernate was harvested from vero cells incubated at 37 C for at least 6 days or until the CPE was >95%. The supernate was stored at −70 C until use. Two fawns were inoculated with heparinized blood originating from two white-tailed deer (#WTD5 and #WTD1) that were experimentally infected in Georgia with BT-10 and BT-17 on 2 February, 1990 and 3 January, 1990 respectively. Both white-tailed deer had gross lesions of BT and had demonstrable viral structures in the endothelium. These blood samples were stored refrigerated for two months. One fawn (deer #8) was injected SQ with 5 ml of heparinized whole blood containing $10^{5.5}TCID_{50}$/ml BT-10. Another fawn (deer #9) was injected SQ with 5 ml of heparinized whole blood containing $10^{3.5}TCID_{50}$/ml BT-17. One adult deer (deer #10) received 2.5 ml of heparinized sheep blood containing $10^{5.0}TCID_{50}$/ml EHD virus serotype 1 (EHD-1) SQ and 1 ml of tissue culture supernate containing $10^{3.8}TCID_{50}$/ml EHD-1 (isolated from the blood) ID. The blood originated from a local farm sheep exhibiting clinical signs of EHD during December of 1989. The EHDV supernate originated from vero cell cultures of the sheep blood and was treated in a similar manner as that containing BTV-17 strain 62-45-S.

Inoculated animals were observed daily, for at least 12 days, for clinical signs of BT as described by Pirtle and Layton (1961), Vosdingh et al. (1968) and Howerth et al. (1988). Physical exams, when possible, were done daily and animals were bled every 2 to 3 days post-inoculation for up to 13 days. Sera from three animals (deer #1, #7 and #10) were collected intermittently for over a year. Deer #1 was sampled six times between DPI-69 and DPI-273, deer #7 sampled eight times between DPI-248 and DPI-692, and deer #10 sampled seven times between DPI-49 and DPI-423. For blood sampling, physical and temperature assessment, deer were immobilized with intramuscular injections of xylazine hydrochloride (Mobay Corporation, Shawnee, Kansas, USA) at 0.9mg/kg and the effects of the drug reversed with an IV administration of yohimbine hydrochloride (Sigma Chemical Company, St. Louis, Missouri, USA) at 0.5mg/kg. In some cases, fawns allowed temperatures to be taken without immobilization. For this study, an elevated temperature was defined as 39.5 C.

Two adults (deer #2 and #3) and three fawns (deer #4, #5, and #6) were euthanized on DPI-12 or DPI-13 with a lethal injection of T-61 (American Hoechts Corporation, Sommerville, New Jersey, USA). Liver, intestine, spleen, rumen, reticulum, omasum, abomasum, lung, heart, great vessels, kidneys, lymph nodes, muscle, adrenals, skin, hoof corona, tongue, buccal papillae, brain and spinal cord were examined grossly. Samples of all organs were placed in 10% buffered formalin, sectioned, stained with hematoxylin and eosin, and examined microscopically for lesions of BT or EHD as described by Karstad et al. (1961), Howerth et al. (1988), and Howerth and Tyler (1988).

Prior to inoculation, all animals were serologically and virologically negative for BT and EHD viruses and antibodies, and had no clinical evidence of systemic disease. After inoculation, the most severe clinical sign manifested by any animal was elevated temperatures ranging from 40 to 41.2 C. No gross or histopathologic lesions suggestive of either BT or EHD were detected.

We recorded fevers in deer #1, #3, #4, #6, #8, and #9. Onset of fevers occurred generally between DPI-5 to DPI-8; however, deer #1 developed a fever on DPI-2. Fevers did not last more than three days except in deer #1 which had a fever for 6 days. Viremia was detected in all animals

except deer #2, #7 and #10. Onset of BT viremia for deer #1, #3, #4, #5, #6, #8 and #9 was DPI-2, DPI-6, DPI-3, DPI-6, DPI-9, DPI-3, and DPI-3 respectively. Duration of viremia for these seven animals was 7, 1, 10, 7, 1, 10, and 7 days, respectively. Bluetongue virus also was isolated from the spleen of deer #2 on DPI-12.

All but deer #2 and #3 seroconverted on the BT AGID test. Onset of AGID seroconversion for deer #1 and #4 through #10 was DPI-8, DPI-12, DPI-12, DPI-13, DPI-13, DPI-12, DPI-12, and DPI-6, respectively. Deer #1 remained seropositive through DPI-273, deer #7 was seropositive through DPI-692, and deer #10 through DPI-49. Deer #1 tested positive on the EHD AGID test on four occasions (DPI-69, DPI-90, DPI-162 and DPI-273), deer #4 was EHD AGID positive on DPI-12, and deer #10 was BT AGID positive on DPI-110. Deer #1 was consistently seropositive to BTV on each of six C-ELISA tests performed on sera collected between DPI-8 and DPI-273. Deer #7 was consistently positive of each of five C-ELISA tests performed on sera collected between DPI-9 and DPI-692. Deer #8 was C-ELISA positive on DPI-12, and deer #9 was C-ELISA positive on DPI-9 and DPI-12. Deer #10 was C-ELISA negative on DPI-9, DPI-13, DPI-18, DPI-110 and DPI-428. All other deer were C-ELISA negative between DPI-6 and DPI-12.

The period of BT and EHD viremia in black-tailed deer is similar to that reported for white-tailed deer (Vosdingh et al., 1968; Thomas and Trainer, 1970; Hoff and Trainer, 1974). Lack of virus isolation in deer #7 and #10 may have resulted because the techniques used to isolate the virus were insufficiently sensitive, the viremia was missed, or the animals were monitored too infrequently. In contrast to adults, all fawns exhibited a detectable viremia; thus, either BT virus may be more easily detected in this age group or black-tailed deer fawns, like white-tailed deer fawns, may be more susceptible than adults, respectively, to infection with BT virus. (Vosdingh et al. 1968).

Using the AGID test, we detected BT antibodies in black-tailed deer as early as DPI-8, and continued to do so for several months in two deer. However, in some BT-inoculated animals, antibodies cross-reacted with the EHD AGID test and antibody detection with the BT AGID was not consistent. This also happened with the EHD inoculated deer. Antibody cross reactivity between EHD and BT using the AGID test has been summarized by Campbell (1985) and should be considered when using the AGID test to monitor exposure in free-ranging black-tailed deer populations. Failure to detect seroconversion with the AGID test in deer #2 and #3 probably can be attributed to not monitoring these animals for more than 12 days. Although information regarding the efficacy of the C-ELISA to detect BTV exposure in deer was limited, the C-ELISA appeared comparable to the AGID for long-term detection of antibodies in deer.

The lack of demonstrable clinical signs or lesions of BT or EHD in experimentally infected black-tailed deer is problematic. That the animals were immune from earlier exposures is doubtful as none had detectable evidence of antibodies to either BTV or EHDV prior to inoculation. In animals inoculated with virus of tissue culture origin, there may have been some viral attenuation. However, even blood inocula containing viable BT virus from white-tailed deer exhibiting clinical and pathologic signs of BT were not sufficient to cause clinical disease in black-tailed deer. Pirtle and Layton (1961) observed that mule deer were refractory to EHD while white-tailed deer succumbed; this also may be the case with BT in black-tailed deer.

The experimental design may have also played a part in our inability to reproduce clinical signs, particularly in light of the small number of animals and variety of inocula used. Based on the failure to reproduce clinical disease in the first group

of six animals, perhaps the BT-17 strain used was incapable of producing disease in deer. Because we had a limited number of animals with which to work, we tried other viral strains and serotypes in attempts of achieving our primary goal of reproducing clinical hemorrhagic disease. It is unlikely that the routes of inoculations employed were responsible for lack of disease; such methods have produced clinical hemorrhagic disease in white-tailed deer (Pirtle and Layton, 1961; Vosdingh et al., 1968; Thomas and Trainer, 1970; Hoff and Trainer, 1974; Howerth et al., 1988).

In summary, it may not be possible to reproduce clinical hemorrhagic disease in captive black-tailed deer by methods used in this study. Experimentally infected black-tailed deer can maintain detectable BT viremia for 8 to 10 days, will become seropositive on the BT AGID and C-ELISA as early as DPI-8 and DPI-9 respectively, and can remain AGID and C-ELISA positive for >1 yr. Serology for BTV and EHDV using the AGID test should be interpreted with caution in light of cross reactions and intermittent detection of long term seropositivity.

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