CROSS-PROTECTION BETWEEN EPIZOOTIC HEMORRHAGIC DISEASE VIRUS SEROTYPES 1 AND 2 IN WHITE-TAILED DEER

Authors: Gaydos, Joseph K., Davidson, William R., Elvinger, François, Howerth, Elizabeth W., Murphy, Molly, et al.

Source: Journal of Wildlife Diseases, 38(4) : 720-728

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-38.4.720

Journal of Wildlife Diseases, 38(4), 2002, pp. 720–728

CROSS-PROTECTION BETWEEN EPIZOOTIC HEMORRHAGIC DISEASE VIRUS SEROTYPES 1 AND 2 IN WHITE-TAILED DEER

Joseph K. Gaydos,[1,6,7] William R. Davidson,[1,4] François Elvinger,[5] Elizabeth W. Howerth,[3] Molly Murphy,[3] and David E. Stallknecht[1,2]

[1] Southeastern Cooperative Wildlife Disease Study, University of Georgia, Athens, Georgia 30602, USA
[2] Department of Medical Microbiology, University of Georgia, Athens, Georgia 30602, USA
[3] Department of Pathology, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602, USA
[4] D. B. Warnell School of Forest Resources, University of Georgia, Athens, Georgia 30602, USA
[5] Department of Large Animal Clinical Sciences, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute & State University, Blacksburg, Virginia 24061, USA
[6] Current address: Marine Ecosystem Health Program, U.C. Davis Wildlife Health Center, 982 Deer Harbor Road, Eastsound, Washington 98245, USA
[7] Corresponding author (email: jkgaydos@ucdavis.edu)

ABSTRACT: Viruses in the epizootic hemorrhagic disease (EHD) serogroup are the most frequent cause of hemorrhagic disease in the southeastern United States, but nothing is known about cross-protection between the two EHD serotypes (EHDV-1 and EHDV-2) present in this region. We experimentally tested whether deer surviving EHDV-2 infection would be protected against subsequent infection with EHDV-1, and used field data to examine the possibility of reciprocal cross-protection. Eleven white-tailed deer fawns (*Odocoileus virginianus*) were experimentally infected with EHDV-2 and later challenged with EHDV-1. Two EHDV-2-naïve fawns also were infected with EHDV-1. Deer were monitored via physical examination, complete blood counts, clotting profiles, viral isolation, and serology, and each animal was assigned a quantitative clinical disease severity score based on presence of certain physical and clinical parameters. Infection of naïve controls with EHDV-1 caused severe clinical disease and death of both fawns, whereas deer previously infected with EHDV-2 exhibited no or minimal signs of disease. Thus, infection with EHDV-2 conferred protection against disease caused by subsequent EHDV-1 infection. Although prior EHDV-2 exposure protected deer from severe clinical disease, it did not prevent infection nor viremia indicating they could still act as virus amplifying hosts. These experimental infections suggest that EHDV-1 and 2 may exist in a state of mutual permissiveness.

Key words: Cross-protection, epizootic hemorrhagic disease, epizootic hemorrhagic disease virus, hemorrhagic disease, HD, *Odocoileus virginianus*, white-tailed deer.

INTRODUCTION

Hemorrhagic disease (HD), caused by viruses in the epizootic hemorrhagic disease (EHD) and bluetongue (BLU) virus serogroups (Reoviridae: Orbivirus), is the most important infectious disease of white-tailed deer (*Odocoileus virginianus*) (Nettles and Stallknecht, 1992). Of the two serogroups, EHD viruses are more prevalent in wild deer populations, and of the two EHD virus serotypes known to exist in the United States, EHDV-2 has been isolated most often from wild ruminant mortalities (Nettles et al., 1992).

Deer surviving infection with EHD or BLU viruses develop long-lived neutralizing antibodies (Stallknecht et al., 1991), and the herd immunity conferred by neutralizing antibodies may be one epidemiologic factor responsible for the spatial and temporal distribution of hemorrhagic disease in the southeastern United States (Davidson and Doster, 1997). In southern latitudes, where EHD and BLU virus antibodies are prevalent in deer, widespread infection of deer with EHD and BLU viruses occur frequently and often result in mild or inapparent disease. Antibody prevalence is low in northern latitudes where HD epizootics are infrequent and are characterized by severe clinical disease and mortality (Davidson and Doster, 1997). Temporally, morbidity and mortality associated with HD epizootics do not occur at the same site annually. In southern latitudes, this may be a product of post-epizootic herd immunity (Nettles and Stallknecht, 1992).

Although viruses in the EHD and BLU serogroups cause clinically indistinguish-

able disease, infection of white-tailed deer by a virus from one serogroup does not seem to confer protection against subsequent infection with a virus from the other serogroup (Quist et al., 1997). Deer surviving infection with EHDV-1 or EHDV-2 are protected against homologous virus infection (Shope et al., 1960; Pirtle and Layton, 1961), but nothing is known concerning cross-protection between EHDV-1 and 2. The objective of this work was to experimentally test if infection with EHDV-2 conferred protection against disease caused by subsequent infection with EHDV-1. Field data were used to investigate the reciprocal condition: if initial infection with EHDV-1 confers protection against subsequent EHDV-2 infection.

MATERIALS AND METHODS

Experimental infections

Fourteen white-tailed deer fawns were acquired from the Missouri Department of Conservation and the North Carolina Wildlife Resources Commission. They were moved to an indoor facility at the University of Georgia (Athens, Georgia, USA) and were hand-reared until they were approximately 4–5 mo old. At the onset of the study fawns weighed between 11.4 kg and 31.8 kg (mean=23.9 kg) and were serologically negative for antibodies to EHD and BLU viruses as tested by agar-gel immunodiffusion (AGID; Veterinary Diagnostic Technology, Inc., Wheatridge, Colorado, USA). Fawns also were negative for antibodies to all known North American EHD and BLU virus serotypes as tested by serum neutralization (SN) as previously described (Stallknecht et al., 1995).

In October 1999, 11 deer were infected with EHDV-2 (day 0). Three deer received a sham inoculation and served as controls. Fifty-nine days later (day 59) all 14 deer were infected with EHDV-1. For all inoculations, deer were sedated with approximately 0.5 mg/kg xylazine (Xylazine-100®, Butler Company, Columbus, Ohio, USA) and the hair on the right side of the neck was clipped. For the EHDV-2 inoculation, deer received $10^{6.3}$ median tissue culture infective doses ($TCID_{50}$) (1 ml) subcutaneously (SC) and $10^{6.3}$ $TCID_{50}$ (1 ml) intradermally (ID) divided over multiple sites on the neck (total inoculum=$10^{6.6}$ $TCID_{50}$ EHDV-2). Sham inoculated deer received 1 ml of sham inoculum SC and 1 ml ID divided over multiple sites on the neck. For the EHDV-1 inoculation, deer received $10^{7.3}$ $TCID_{50}$ (1 ml) SC and $10^{7.3}$ $TCID_{50}$ (1 ml) ID divided over multiple sites on the neck (total inoculum=$10^{7.6}$ $TCID_{50}$ EHDV-1). Post-inoculation, sedation was reversed with 2–5 mg yohimbine (Yobine®, Lloyd Laboratories, Shenandoah, Iowa, USA) administered intramuscularly (IM).

The EHDV-2 inoculum was prepared from an EHDV-2 isolate obtained from a white-tailed deer lymph node cultured on baby hamster kidney cells (BHK_{21} cells) (American Type Culture Collection, Rockville, Maryland, USA). The original isolate was obtained from a white-tailed deer submitted to the Southeastern Cooperative Wildlife Disease Study (SCWDS) as a diagnostic case (CC87–90) from Clarke County, Georgia. A white-tailed deer fawn, serologically negative for antibodies to EHD and BLU viruses by AGID, was used to prepare the inoculum. The deer was inoculated with 3 ml (1.5 ml SC and 1.5 ml ID divided over multiple sites on the neck) of a sonicated BHK_{21} cell suspension containing $10^{6.9}$ $TCID_{50}$ EHDV-2. Coinciding with the first febrile episode on day 5 post-infection, the deer was anesthetized with 4.4 mg/kg of tiletamine and zolazepam (Telazol®, Fort Dodge Animal Health, Fort Dodge, Iowa) IM, and 340 ml of blood was collected in sodium citrate. Platelet rich plasma was removed, and blood cells were washed and resuspended twice with Dulbecco's phosphate-buffered saline (DPBS). This was sonicated and frozen at −70 C. The viral titer of this inoculum was $10^{6.3}$ $TCID_{50}$ per ml as determined by endpoint titration. The serotype was confirmed as EHDV-2 by virus neutralization as previously described (Quist et al., 1997).

The sham inoculum was prepared by taking 30 ml of whole blood from the same animal prior to EHDV-2 infection. This blood was prepared exactly the same as the true inoculum and no virus was detected in the sham inoculum by virus isolation attempts.

The EHDV-1 inoculum was prepared from an EHDV-1 isolate obtained from a white-tailed deer ethylenediaminetetracetic acid (EDTA)-blood sample cultured on BHK_{21} cells. It was obtained from a white-tailed deer submitted in August 1999 (submission A0–6652) to the Athens Diagnostic Laboratory, University of Georgia, Athens, Georgia from Walton County, Georgia. Flasks monolayered with BHK_{21} cells were inoculated with 10 μl of EHDV-1 suspension, and infected cells and supernatant were harvested 4 days later at 80% cytopathic effect. The flasks were scraped and the contents were mixed and centrifuged at 720×G for 10 min. The pellet was resuspended in DPBS, sonicated, and recentrifuged at

720×G for 10 min. Aliquots of the supernatant were frozen at −70 C. The viral titer of this inoculum was $10^{7.3}$ $TCID_{50}$ per ml as determined by endpoint titration. The serotype was confirmed as EHDV-1 by virus neutralization.

Deer were manually restrained and sampled at approximately the same time every 2 days beginning 4 days prior to EHDV-2 infection (day −4) and continuing through day 14. They were then sampled once weekly until 4 days prior to EHDV-1 infection (day 55) at which time sampling every 2 days was resumed until 14 days after EHDV-1 infection (day 73). On day 73, all surviving deer were euthanized with an overdose of euthanasia solution (Beuthanasia®-D Special, Schering-Plough Animal Health Corporation, Union, New Jersey, USA). Necropsies were performed on all deer within 1 hr following euthanasia or immediately upon being found dead.

When sampled, deer received a visual examination, body temperature was measured rectally with a digital thermometer, and blood was drawn via jugular venipuncture. Blood in K_3 EDTA was used for complete blood counts (CBC), platelet counts, plasma protein measurement, and fibrinogen measurement. Blood in sodium citrate was used for blood coagulation analyses and viral isolation. Serum from blood collected in additive-free sterile tubes was used for serology. Complete blood counts were performed on a Baker System 9000 automated cell counter (Baker Instrument Corporation, Allentown, Pennsylvania, USA). White blood cell count, red blood cell count, and hematocrit were evaluated. The white blood cell differential counts and platelet counts were performed manually. Plasma protein was calculated using a hand-held refractometer. Fibrinogen was measured by heating the plasma to 56 C for 3 min, remeasuring plasma protein post-heating, and recording the difference between the two measurements. Plasma was frozen at −70 C for later evaluation of activated partial thromboplastin time (APTT), prothrombin time (PT), and evidence of fibrin degradation. All samples from each deer were run en-block using an Amelung KC4A™ Micro Coagulation Analyzer (Sigma, St. Louis, Missouri, USA) to evaluate changes in APTT and PT over the course of the experiment. Deer were tested for fibrin degradation using the D-di Test® (Diagnostica Stago, Parsippany, New Jersey, USA) as described by the manufacturer.

Using BHK_{21} cells, cattle pulmonary artery endothelial (CPAE) cells (American Type Culture Collection) and techniques previously described (Quist et al., 1997), blood samples collected in acid citrate were used for virus isolation, endpoint titration, and virus identification. When virus was isolated from blood, but could not be titrated (virus titers less than $10^{2.3}$ $TCID_{50}$) titers were considered to be zero for purposes of calculating and graphing geometric mean virus titers by infection group. Serum was tested for precipitating antibodies to EHD and BLU virus serogroups by AGID. All samples that were positive for antibodies to EHD or BLU virus serogroup were tested by SN tests.

Clinical disease severity scores (CDSS) were calculated for each deer for EHDV-2 infection and for EHDV-1 challenge. Using data from all deer infected, individual scatter plots were created for each infection for each of the following measured parameters: plasma protein, rectal temperature, APTT, PT, platelet count, and lymphocyte count. Deer were given one point for having at least one outlier in a parameter based on visual appraisal of the scatter plots. Deer also were given one point if they had evidence of fibrin degradation in serum at anytime during the infection and one point for each of the following clinical signs that were exhibited: depression, erythema of lightly haired areas, and salivation or oral lesions. The total number of points assigned to a deer during an infection was taken as that deer's CDSS. A minimum score of 0 indicated no signs of disease and a maximum score of 10 indicated most severe clinical disease.

Clinical disease severity scores were graphed for each serotype for three total means: the mean score for the 11 fawns infected with EHDV-2 (experimental days 0–14), the mean score for 10 of the same fawns when challenged with EHDV-1 (experimental days 59–73), and the mean score for two fawns only infected with EHDV-1 (experimental days 59–73). Mean CDSSs also were compared between fawns obtained from Missouri (MO, USA; $n=8$) and North Carolina (NC, USA; $n=3$) that were infected with EHDV-2 (experimental days 0–14).

Statistical analysis

Data were analyzed as two separate trials. First the effects of origin of deer on response to EHDV-2 infection for days 0–14 were evaluated. Clinical disease severity scores of eight infected Missouri fawns and three infected North Carolina fawns were compared by *t*-test, using Proc TTEST (SAS®, version 8e, SAS Institute Inc., Cary, North Carolina) and the Satterthwaite adjustment to account for unequal variances. Effects of origin of these 11 deer on viral titers (following logarith-

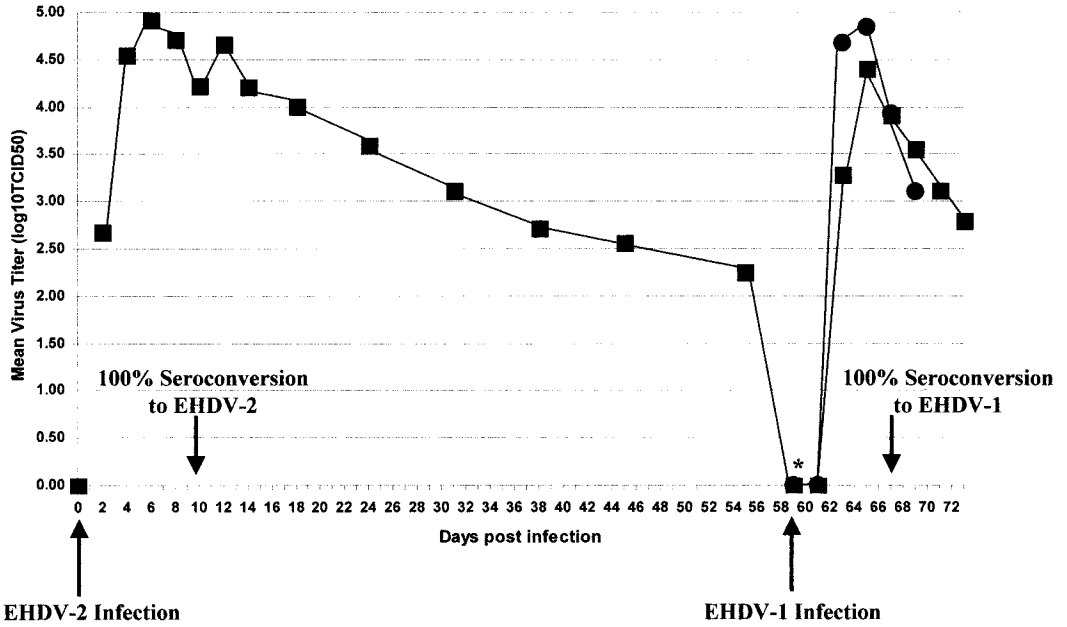

FIGURE 1. Mean geometric virus titer by day postinfection (=fawns initially infected with EHDV-2 on day 0 [n=11], then challenged with EHDV-1 on day 59 [n=10]; ●=control fawns that were not infected with EHDV-2, but only infected with EHDV-1 on day 59 [n=2]). The 100% seroconversion to EHDV-2 and EHDV-1 labels refer only to fawns initially infected with EHDV-2 (n=11) then challenged with EHDV-1 (n=10). *On days 59 and 61 virus was detected in blood of two deer (MO 3 and NC 23) but could only be titrated as less than $10^{2.3}$ $TCID_{50}$ per ml and were graphed as zero.

mic transformation), clinical chemistry parameters, and antibody titer developments (days 8–14, following $\text{logarithmic}_{\text{base2}}$ transformation) were evaluated by analysis of variance, using Proc GLM (SAS®) in a repeated measurement split plot in time model that included origin, deer (origin) as main plot error, day post-experimental infection, and day×origin interaction.

To evaluate response to EHDV-1 infection on days 59 through 73, deer were grouped into three exposure categories: fawns not previously exposed to EHDV-2 (two fawns; one from MO, one from NC), previously EHDV-2 infected MO fawns (seven fawns), and previously EHDV-2 infected NC fawns (three fawns). Analyses of variance were done using Proc GLM (SAS®). Effects on clinical disease severity scores were evaluated by one way analysis of variance, with effects of previous EHDV-2 infection and effects of origin tested by orthogonal contrasts. Effects of previous infection and of origin on viral titers (following logarithmic transformation), clinical chemistry parameters, and antibody titer development (days 59–73, following $\text{logarithmic}_{\text{base2}}$ transformation) were also evaluated by orthogonal contrasts following analysis of variance of a repeated measurement split plot in time model that included exposure, deer (exposure) as main plot error, day post experimental infection, and day×exposure interaction. Means and standard deviations are presented in the text (mean±SD).

Two fawns, one control (MO 11) and one EHDV-2 infected fawn (MO 2), were challenged with EHDV-1 on day 59, but were not included in the statistical analysis for the EHDV-1 challenge trial, the EHDV-1 viremia graph (days 59–73; Fig. 1), nor the EHDV-1 (challenge) and the EHDV-1 (naïve) CDSS graphs (Fig. 2).

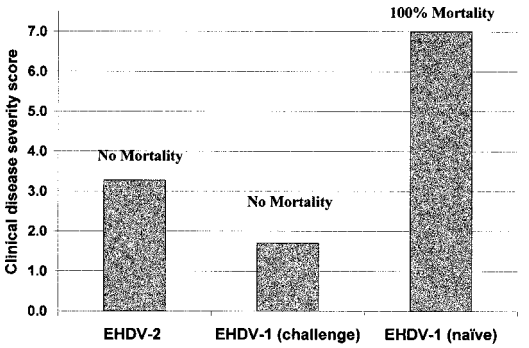

FIGURE 2. Mean clinical disease severity scores by virus infection: EHDV-2=fawns infected with EHDV-2 (days 0–14; n=11, CDSS=3.3±2.3); EHDV-1 (challenge)=fawns previously exposed to EHDV-2 then challenged with EHDV-1 (days 59–73; n=10, CDSS=1.7±1.8); EHDV-1 (naïve)=fawns infected only with EHDV-1 (days 59–73; n=2; CDSS=7±0).

RESULTS

Experimental EHDV-2 infection

Five of 11 (45%) fawns had a detectable EHDV-2 viremia by day 2, all were viremic by day 4, and peak mean viremia was seen on day 6 (Fig. 1). Mean viremia for the 11 deer peaked at $10^{4.9}$ $TCID_{50}$ per ml with peak viremias for each fawn ranging from $10^{3.1}$ to$10^{6.1}$ $TCID_{50}$ per ml. Mean viremias decreased until day 59 at which time EHDV-2 could only be isolated from two deer (MO 3 and NC 23) at titers of less than$10^{2.3}$ $TCID_{50}$ per ml. Precipitating and EHDV-2 SN antibodies were detectable in all fawns by postinfection day 10 (Fig. 1). Mean SN antibodies peaked on day 14 then declined and leveled out for the remainder of the experiment.

Fawns had variable degrees of clinical disease between days 6 and 14 and all fawns survived. The extremes of clinical illness were mild depression versus severe depression with fever, hypoproteinemia and submandibular edema, congestion of mucous membranes, lymphopenia, and prolongation of APTT and PT. Most deer had signs between these two extremes. Clinical disease severity scores ranged from 1–7 with a mean CDSS of 3.3±2.33 (Fig. 2).

Clinical disease was more severe in the eight fawns from MO (CDSS: 4.1±2.17) than in the three fawns from NC (CDSS: 1±0) (P=0.005). Fawns from MO exhibited higher temperatures (P=0.002), lower lymphocyte counts (P<0.001), and greater prolongation in PT (P=0.005).

Virus was not isolated from any of the three sham-inoculated, contact-control fawns. A control fawn (MO 11) seroconverted to EHDV-2 on day 31.

Experimental EHDV-1 challenge

Epizootic hemorrhagic disease virus, serotype 1 SN antibodies were not detected in any of the fawns prior to or at the time of EHDV-1 infection (day 59). Two fawns (MO 2 and MO 11) were removed from the EHDV-1 analysis as previously described. The mean EHDV-2 SN antibody titer for the 10 fawns previously exposed to EHDV-2 was 1:263 at the time of EHDV-1 challenge (day 59). All 10 fawns developed an EHDV-1 viremia 4 days after being infected (day 63) with mean viremia peaking 4 days later ($10^{4.4}$ $TCID_{50}$ per ml; day 67) (Fig. 1). Peak viremias for these 10 fawns ranged from $<10^{2.3}$ to$10^{5.6}$ $TCID_{50}$ per ml. Epizootic hemorrhagic disease virus, serotype 1 was the only virus isolated. The 10 fawns previously infected with EHDV-2 developed detectable EHDV-1 SN antibodies by 8 days after infection (day 67) (Fig. 1).

All 10 fawns previously exposed to EHDV-2 survived the EHDV-1 challenge infection. The fawn with the highest EHDV-2 SN antibody titer (MO 17; 1:1,280) on the day of EHDV-1 challenge (day 59) was the only fawn to develop severe clinical disease (CDSS=6). Interestingly, this fawn (MO 17) only developed very mild clinical disease (CDSS=1) from EHDV-2 infection. Of the other nine fawns exposed to EHDV-2 then challenged with EHDV-1, three had no signs of clinical disease (CDSS=0), five had mild clinical disease (CDSS=1 or 2), and

one had moderate clinical disease (CDSS=3). The mean clinical disease severity score for the 10 fawns previously exposed to EHDV-2 was 1.7±1.8 (Fig 2).

The two naïve fawns (not previously infected with EHDV-2) were both viremic 4 days after infection (day 63) and had a peak mean viremia ($10^{4.9}$ $TCID_{50}$ per ml) 2 days later (day 65) (Fig. 1). Only one of these fawns (MO 1) developed SN antibodies to EHDV-1 prior to death (day 69). In both animals, EHDV-1 infection caused severe clinical disease (CDSS=7±0) and death (Fig. 2). By 8 days postinfection (day 67), both deer had developed severe depression, fever, hypoproteinemia, congestion of mucous membranes, lymphopenia, thrombocytopenia, and APTT prolongation. They became moribund on days 69 and 70 and were humanely euthanized according to criteria established to prevent unacceptable pain and suffering (University of Georgia IACUC #A990105, Animal Welfare Assurance #A3437-01). Postmortem lesions were consistent with HD.

As evaluated from days 59 to 69, EHDV-1 viremia did not differ between the two naïve fawns and the 10 fawns previously exposed to EHDV-2 (P=0.81; Fig. 1). The mean clinical disease severity score for the 10 fawns previously exposed to EHDV-2 infection when challenged with EHDV-1 (Fig. 2) was lower (P=0.004) than the mean score in the two naïve fawns (Fig. 2) in which EHDV-1 caused 100% mortality. In the 10 fawns previously exposed to EHDV-2, state of origin had no effect on clinical disease severity scores for the EHDV-1 infection (P=0.68). During EHDV-1 challenge, the 2 naïve fawns had higher temperatures (P=0.04), lower lymphocyte counts (P=0.03), lower plasma protein levels (P<0.001), greater prolongation of the APTT (P<0.001), greater prolongation of the PT (P<0.001), and higher fibrinogen levels (P=0.01) than 10 fawns that were previously exposed to EHDV-2.

The one contact-control fawn that seroconverted to EHDV-2 31 days into the experiment (MO 11) and was removed from the statistical analysis became viremic 4 days after being infected with EHDV-1 and had a peak viremia of $10^{3.3}$ $TCID_{50}$ per ml 2 days later. It developed EHDV-1 SN antibodies 2 days later (day 67). This fawn developed only minimal signs of disease (CDSS=1) and survived the infection.

One experimental fawn (MO 2) was removed from the statistical analysis because it lacerated its right front carpus on day 57, necessitating sedation, repeated bandaging, and antibiotic therapy. When challenged with EHDV-1 (day 59), this fawn had an EHDV-2 SN titer of 1:640. Viremia was detectable on days 63, 65, and 67 at $<10^{2.3}$ $TCID_{50}$ per ml and EHDV-1 SN antibodies were detected on day 65. This fawn developed mild clinical disease (CDSS=2) and survived infection.

DISCUSSION

Prior infection with EHDV-2 conferred protection against disease caused by subsequent infection with EHDV-1, as indicated by a much lower mean CDSS in fawns previously exposed to EHDV-2 than in naïve fawns challenged with EHDV-1. It is interesting that the mean clinical disease severity score for the EHDV-1 challenge was also lower than the score for the original EHDV-2 infection (Fig. 2), despite use of an EHDV-1 challenge inoculation almost a log higher than the original EHDV-2 challenge ($10^{7.6}$ $TCID_{50}$ EHDV-1 vs. $10^{6.6}$ $TCID_{50}$ EHDV-2) and the use of a viral strain that appeared to be more virulent based on 100% mortality in naïve fawns. The mechanism by which cross-protection lessens clinical disease severity is unknown, but it is probably not related to reducing viremia. Prior infection by EHDV-2 did not cause a decrease in subsequent EHDV-1 viremias as was observed with cross-protection between the closely related Cache Valley virus and Potosi virus in white-tailed deer, where deer immune to one virus exhibit lower and shorter viremias when infected with the other virus

(Blackmore and Grimstad, 1998). Although prior infection by EHDV-2 lessened clinical disease caused by subsequent EHDV-1 infection, it did not prevent EHDV-1 infection and viremia. This is an important finding because it suggests that the cross-protection does not prevent previously infected deer from acting as virus amplifying hosts for a second viral serotype.

The mechanism by which prior exposure to EHDV-2 protects deer against clinical disease caused by subsequent EHDV-1 challenge is unknown. High serum neutralizing antibody titers to EHDV-2 were not associated with decreased clinical disease severity in fawns when challenged with EHDV-1. In fact, of all the fawns previously exposed to EHDV-2 the fawn with the highest EHDV-2 SN antibody titer (MO 17; 1:1,280) developed the most severe disease suggesting that cross-protection may be conferred by cell mediated immunity. Cellular immunity is believed to be responsible for the partial cross protection that may occur between serotypes of other Orbiviruses within the family Reoviridae. Sheep vaccinated with a recombinant virus containing the major core protein VP7 of BLU-1 were partially protected against clinical disease when challenged with a virulent BLU-3, but were not protected from BLU-3 viremia (Wade-Evans et al., 1996). None of the sheep vaccinated with BLU-1 VP7 developed BLU-1 neutralizing antibodies suggesting partial protection from clinical disease was mediated through a cellular immune response (Wade-Evans et al., 1996). Similarly, BALB/c mice immunized with VP7 crystals of African horse sickness virus (AHSV), serotype 9 were protected from clinical disease when challenged with a virulent ASHV-7 (Wade-Evans et al., 1997). Titers to VP7 in mice protected from heterologous challenge did not correlate with protection and passive antibody transfer from immunized mice failed to protect them from challenge with ASHV-7, suggesting cell-mediated immunity was responsible for the cross-protection (Wade-Evans et al., 1997).

We do not know how the control contact MO 11 became infected with EHDV-2. It is possible that it was infected via oral consumption of virus shed in the feces or oral secretions of infected deer. Epizootic hemorrhagic disease virus serotype 1 can be detected in the feces and oral secretions from a relatively large proportion of deer experimentally infected with EHDV-1 (Gaydos et al., 2002a) and it is possible that this also may occur with EHDV-2 infection. Ditchfield et al. (1964) reported isolation of EHDV-2 from feces from deer orally inoculated with EHDV-2. The orally infected deer were febrile on the fifth to seventh postinoculation day, but other signs of clinical disease were not apparent. Although deer were visually monitored daily, body temperatures were not taken during the time when MO 11 seroconverted, thus a febrile episode could have been missed. Even though fawns were housed indoors in a *Culicoides*-free building where biting arthropods were not observed, we cannot definitively rule out the possibility of arthropod transmission.

The difference in clinical disease seen between NC and MO deer infected with EHDV-2 may represent differences in innate immunity to EHD virus infection based on geographic origin of deer; which has been hypothesized (Nettles and Stallknecht, 1992) and demonstrated experimentally (Gaydos et al., 2002b). Geographic patterns for hemorrhagic disease among white-tailed deer in NC differ between the eastern and western halves of the state, where HD activity is more frequent, herd immunity is higher, and epizootics usually cause milder disease in the eastern half of the state (Davidson and Doster, 1997). Assuming all three NC deer originated from the eastern half of the state, innate resistance could potentially explain why these deer were more resistant to EHDV-2 infection than were MO deer, which originated from an area that experiences HD patterns similar to the

western half of NC. Unfortunately, we do not know from where in NC these experimental deer originated.

Field observations made by three of the authors (W. R. D., D. E. S, and J. K. G.) from natural EHD infections at Cowan's Ford Wildlife Refuge (35°22′N, 80°58′W) in Mecklenburg County, NC suggest that the reciprocal of our experimental design may occur. Specifically, that EHDV-1 infection followed by EHDV-2 infection also may result in decreased disease severity during the second infection but not prevent viremia. In the summer and fall of 1999, a hemorrhagic disease epizootic, caused primarily by EHDV-1, was responsible for deer deaths from New Jersey south to Georgia and west to Louisiana (USA; SCWDS, unpublished data). In September and October of 1999, more than 20 dead deer were reported in Mecklenburg County and a follow-up survey of hunter-harvested deer from Cowan's Ford Wildlife Refuge detected monospecific EHDV-1 SN antibodies in 66% of the deer sampled and SN antibodies to both EHDV-1 and 2 in the other 33% sampled (n=9). The following year (August 2000), five apparently healthy deer were collected from Cowan's Ford as part of a routine herd-health examination. On closer examination, one deer exhibited postmortem lesions consistent with mild HD. Epizootic hemorrhagic disease virus, serotype 2 was isolated from EDTA-blood collected from this and two other deer, confirming EHDV-2 viremia in 60% of the deer examined. All five deer had SN antibodies to EHDV-1, suggesting that prior EHDV-1 exposure may have lessened clinical disease associated with subsequent EHDV-2 infection, but did not prevent viremia. The possibility that the EHDV-2 virus isolated from deer at Cowan's Ford in 2000 was a low-virulence strain of EHDV-2 cannot be ruled out. In 2000, deer mortality confirmed by EHDV-2 virus isolation, was documented in Georgia, (Buckingham, Dawson, Habersham, Rockdale, Troup, and White counties), North Carolina (Iredell and Randloph counties), and South Carolina (Greenwood county) (SCWDS, unpubl. data), but it is unknown if the virus isolated in Mecklenburg County, North Carolina in 2000 was the same virus.

Cross protection between EHD viruses may be an important factor in the varying temporal and spatial distributions of disease caused by EHD viruses in the southeastern United States. In southern latitudes where antibody prevalence and virus serotype diversity are high, morbidity and mortality associated with HD epizootics do not occur at the same sites annually. The existence of cross-protection between these viruses suggests that EHDV-1 and EHDV-2 could maintain sympatric cycles of infection and replication with clinical disease and epizootics becoming apparent only at random intervals where herd immunity to both viruses decreased below some epizootic threshold.

The presence of cross-serotype immunity between EHDV-2 and EHDV-1 and the potential existence of reciprocal immunity between EHDV-1 and EHDV-2 is interesting from a viral evolutionary perspective. The idea that cross-immunity protects against clinical disease but does not decrease viremia suggests that these viruses may exist in a state of mutual permissiveness rather than in a state of competition.

ACKNOWLEDGMENTS

The authors thank B. Anderson, D. Berndt, J. Corn, B. Hanson, J. Huffman, D. Kavanaugh, L. Lewis, R. Longe, P. Luttrell, M. McCreary, J. Smith, M. Stevenson, and A. Yellin for help feeding and restraining fawns. We also thank J. Beringer, the Missouri Department of Conservation, H. Hall, and the North Carolina Wildlife Resources Commission for providing research fawns and G. D. Marshall and the Mecklenburg County Parks and Recreation Department for assistance collecting samples. We appreciate helpful input on study design from V. F. Nettles, J. R. Fischer, and S. Poet and review of the manuscript by D. Mead, S. Little, and two anonymous reviewers. This project was supported through sponsorship from fish and wildlife agencies in Alabama, Arkansas,

Florida, Georgia, Kentucky, Louisiana, Maryland, Mississippi, Missouri, North Carolina, Puerto Rico, South Carolina, Tennessee, Virginia, and West Virginia. Funds were provided by the Federal Aid to Wildlife Restoration Act (50 Stat. 917) and through Grant Agreement 14-45-0009-94-906, National Biological Service, U.S. Department of the Interior.

LITERATURE CITED

BLACKMORE, C. G. M., AND P. R. GRIMSTAD. 1998. Cache Valley and Potosi viruses (Bunyaviridae) in white-tailed deer (*Odocoileus virginianus*): Experimental infections and antibody prevalence in natural populations. American Journal of Tropical Medicine and Hygiene 59: 704–709.

COUVILLION, C. E., V. F. NETTLES, AND W. R. DAVIDSON. 1981. Hemorrhagic disease among white-tailed deer in the southeast from 1971 through 1980. Proceedings of the 85th annual meeting of the United States Animal Health Association, St. Louis, Missouri, pp. 522–537.

DAVIDSON, W. R., AND G. L. DOSTER. 1997. Health characteristics and white-tailed deer population density in the southeastern United States. *In* The science of overabundance: Deer ecology and population management, W. J. McShea, H. B. Underwood and J. H. Rappole (eds.). Smithsonian Institution Press, Washington, D.C., pp. 164–184.

DITCHFIELD, J., J. G. DEBBIE, AND L. H. KARSTAD. 1964. The virus of epizootic hemorrhagic disease of deer. Transactions of the North American Wildlife Conference 29: 196–199.

GAYDOS, J. K., A. B. ALLISON, B. A. HANSON, AND A. S. YELLIN. 2002a. Oral and fecal shedding of epizootic hemorrhagic disease virus, serotype 1 from experimentally infected white-tailed deer. Journal of Wildlife Diseases 38: 166–168.

———, W. R. DAVIDSON, F. ELVINGER, D. G. MEAD, E. W. HOWERTH, AND D. E. STALLKNECHT. 2002b. Innate resistance to epizootic hemorrhagic disease in white-tailed deer. Journal of Wildlife Diseases 38: 713–719.

NETTLES, V. F., AND D. E. STALLKECHT. 1992. History and progress in the study of hemorrhagic disease or deer. Transactions of the North American Wildlife and Natural Resources Conference 57: 499–516.

———, S. A. HYLTON, D. E. STALLKECHT, AND W. R. DAVIDSON. 1992. Epidemiology of epizootic hemorrhagic disease virus in wildlife in the USA. *In* Bluetongue, African horse sickness and related orbiviruses, T. E. Walton and B.I. Osburn (eds.). Proceedings of the Second International Symposium, CRC Press, Boca Raton, Florida, pp. 238–248.

PIRTLE, E. C., AND J. M. LAYTON. 1961. Epizootic hemorrhagic disease in white-tailed deer- Characteristics of the South Dakota strain of virus. American Journal of Veterinary Research 22: 104–108.

QUIST, C. F., E. W. HOWERTH, D. E. STALLKNECHT, J. BROWN, T. PISELL, AND V. N. NETTLES. 1997. Host defense responses associated with experimental hemorrhagic disease in white-tailed deer. Journal of Wildlife Diseases 33: 584–599.

SHOPE, R. E., L. G. MACNAMARA, AND R. MANGOLD. 1960. A virus-induced epizootic hemorrhagic disease of the Virginia white-tailed deer (*Odocoileus virginianus*). Journal of Experimental Medicine 111: 155–170.

STALLKNECHT, D. E., J. L. BLUE, E. A. ROLLER III, V. F. NETTLES, W. R. DAVIDSON, AND J. E. PEARSON. 1991. Precipitating antibodies to epizootic hemorrhagic disease and bluetongue viruses in white-tailed deer in the southeastern United States. Journal of Wildlife Diseases 27: 238–247

———, V. F. NETTLES, E. A. ROLLOR III, AND E. W. HOWERTH. 1995. Epizootic hemorrhagic disease virus and bluetongue virus serotype distribution in white-tailed deer in Georgia. Journal of Wildlife Diseases 31: 331–338.

WADE-EVANS, A. M., C. H. ROMERO, P. MELLOR, H. TAKAMATSU, J. ANDERSON, J. THEVASAGAYAM, M. J. FLEMING, P. P. C. MERTENS, AND D. N. BLACK. 1996. Expression of the major core structural protein (VP7) of bluetongue virus, by a recombinant capripox virus, provides partial protection of sheep against a virulent heterotypic bluetongue virus challenge. Virology 220: 227–231.

———, L. PULLEN, C. HAMBLIN, R. O'HARA, J. N. BURROUGHS, AND P. P. C. MERTENS. 1997. African horse sickness virus VP7 sub-unit vaccine protects mice against a lethal, heterologous serotype challenge. Journal of General Virology 78: 1611–1616.

Received for publication 17 July 2001.