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Source: Journal of Wildlife Diseases, 42(3) : 616-624

Published By: Wildlife Disease Association

URL: <https://doi.org/10.7589/0090-3558-42.3.616>

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MOLECULAR CHARACTERIZATION OF EPIZOOTIC HEMORRHAGIC DISEASE VIRUS SEROTYPE 1 ASSOCIATED WITH A 1999 EPIZOOTIC IN WHITE-TAILED DEER IN THE EASTERN UNITED STATES

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ABSTRACT: During the autumn of 1999 (mid-August–late September), an outbreak of hemorrhagic disease in white-tailed deer (*Odocoileus virginianus*) caused by epizootic hemorrhagic disease virus serotype 1 (EHDV-1) occurred along the east coast of the United States from Georgia to New Jersey. An EHDV-1 epizootic of such magnitude had not been described in this region since 1975. To determine the genetic relatedness among the 1999 viruses, as well as among additional EHDV-1 isolates from the eastern and western United States, portions of the S10 and L2 gene segments were sequenced and compared utilizing phylogenetic analyses. Nearly all of the 1999 eastern isolates were identical in nucleotide sequence at one or both loci. Additionally, confirmed cases of EHDV-1 in white-tailed deer occurred in a south (Georgia)-to-north (New Jersey/Virginia) progression over a short period of approximately six weeks. Taken together, these results indicate that this outbreak resulted from the spread of a single viral strain. The phylograms derived from analysis of the entire sample set displayed eastern and western region-specific clusterings (topotypes), as well as an eastern versus western difference in branch lengths, which may reflect the influence of epizootic versus enzootic transmission patterns on viral genetic diversity.

Key words: EHDV, epizootic hemorrhagic disease virus, molecular epidemiology, *Odocoileus virginianus*, *Orbivirus*, viral evolution, white-tailed deer.

INTRODUCTION

Hemorrhagic disease (HD) of wild ungulates in North America is caused by viruses in the epizootic hemorrhagic disease virus (EHDV) and bluetongue virus (BTV) serogroups of the genus *Orbivirus* within the family Reoviridae. The genomes of EHDV and BTV consist of 10 double-stranded RNA segments, encoding four nonstructural (NS1, 2, 3, and 3a) and seven structural (VP 1, 2, 3, 4, 5, 6, and 7) proteins (Knudson and Monath, 1990). Two serotypes of EHDV (EHDV-1 and -2) and five of BTV (BTV-2, -10, -11, -13, -17) are present in the US, and all but BTV-2 have been involved in disease outbreaks among white-tailed deer (*Odocoileus virginianus*) (Stallknecht et al., 2002). The distribution of HD in the US is concordant with the range of the biological vector *Culicoides sonorensis* (Holbrook, 1996). Serological evidence indicates that in much of the southeastern US, where the viruses are endemic, white-

tailed deer are exposed to EHDV and/or BTV annually, or in short two-to-three-year cycles (Stallknecht et al., 2002). Epizootic hemorrhagic disease virus serotype 2 is believed to be the predominant EHDV or BTV to which white-tailed deer are exposed in the southeastern US, as indicated by the annual presence of antibodies to this serotype in Georgia and the frequent isolation of this serotype relative to EHDV-1 and BTV (Stallknecht et al., 1995, 2002).

Prior studies of the molecular epidemiology of EHDV-2 have shown that viruses within this serotype are evolving very slowly and likely exist as a contiguous population throughout the southeastern and western US (Murphy, 2003). Although EHDV-1 was first isolated from New Jersey white-tailed deer in 1955 (Shope et al., 1960), because of the paucity of isolates, no comparable studies have been performed with this serotype. During the autumn of 1999 (mid-August–late September), a large outbreak of

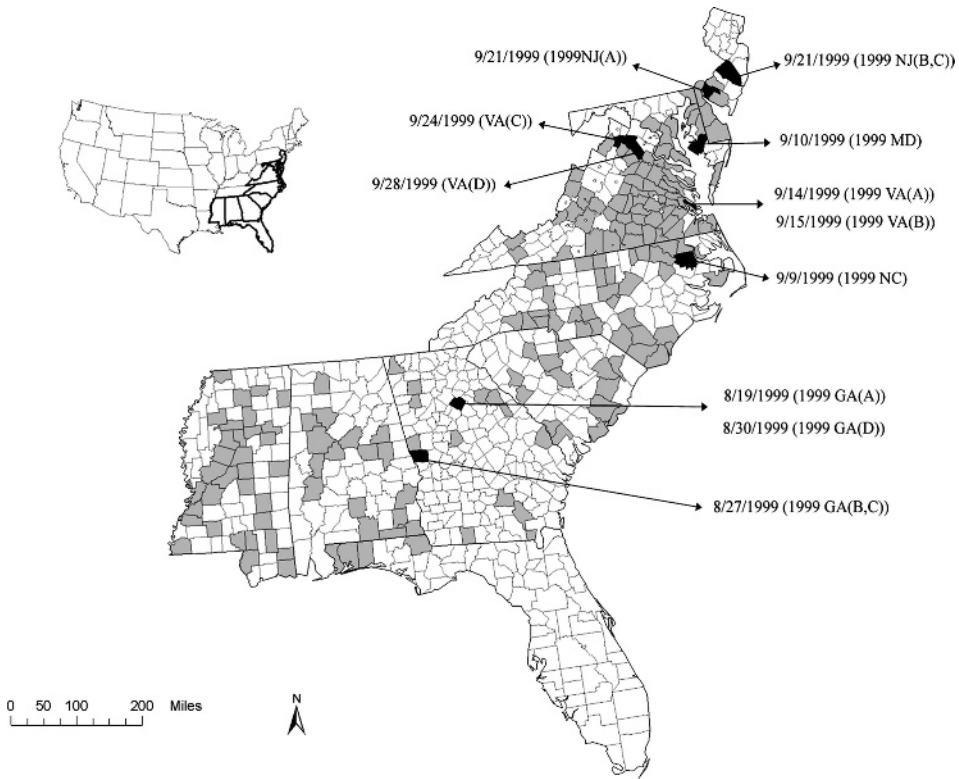


FIGURE 1. County-level map of region involved in the 1999 EHDV-1-related HD outbreak. Gray areas represent counties from which HD activity (die-offs of deer and/or presentation of acute and chronic lesions indicative of HD) was reported in a survey of state wildlife officials as developed and administered by the Southeastern Cooperative Wildlife Disease Study (Nettles et al., 1992). Black areas represent counties of origin for 1999 isolates in the present study.

hemorrhagic disease in white-tailed deer caused by EHDV-1 occurred in a wide band in the eastern US, extending from Georgia to New Jersey (Fig. 1). This outbreak not only afforded a unique opportunity to explore the genetic relatedness among EHDV-1 isolates within a regional outbreak, but also enabled us to compare recent and older isolates to assess long-term genetic change.

The objectives of this study were to (1) determine the level of genetic variation among EHDV-1 isolates from a single regional outbreak (1999) and (2) determine the genetic relatedness of these viruses with previous and subsequent EHDV-1 isolates made from the eastern US (1955, 1991, and 1996) and western US (2000 and 2001), respectively. To this

end, portions of the S10 and L2 gene segments of EHDV-1 isolates made from white-tailed deer were sequenced and utilized in phylogenetic analyses.

MATERIALS AND METHODS

Virus isolation, propagation, and PCR amplification of target genes

All viruses (Table 1) were isolated in cow pulmonary artery endothelial (CPAE) or baby hamster kidney (BHK₂₁) cells as previously described (Murphy et al., 2005) and passaged fewer than four times. Viruses from Texas were isolated from the blood of clinically normal white-tailed deer fawns; all other viruses were isolated from moribund and deceased white-tailed deer or moribund mule deer (*Odocoileus hemionus*). Infected BHK₂₁ cells in 75 cm² tissue-culture flasks were used for total RNA extraction using RNeasy (Tel-

TABLE 1. Sources of epizootic hemorrhagic disease virus serotype 1 isolates.

Sample name ^a	Origin	Date of isolation
1955 NJ	New Jersey	1955
1991 TN	Lewis County, Tennessee	1991
1996 TN	Jefferson County, Tennessee	29 August 1996
1999 GA(A)	Walton County, Georgia	19 August 1999
1999 GA(B)	Harris County, Georgia	27 August 1999
1999 GA(C)	Harris County, Georgia	27 August 1999
1999 GA(D)	Walton County, Georgia	30 August 1999
1999 NC	Bertie County, North Carolina	9 September 1999
1999 MD	Dorchester County, Maryland	10 September 1999
1999 VA(A)	York County, Virginia	14 September 1999
1999 VA(B)	York County, Virginia	15 September 1999
1999 NJ(A)	Salem County, New Jersey	21 September 1999
1999 NJ(B)	Burlington County, New Jersey	21 September 1999
1999 NJ(C)	Burlington County, New Jersey	21 September 1999
1999 VA(C)	Warren County, Virginia	24 September 1999
1999 VA(D)	Farquier County, Virginia	28 September 1999
2000 TX(A)	Kerr County, Texas	27 November 2000
2000 TX(B)	Kerr County, Texas	27 November 2000
2000 TX(C)	Kerr County, Texas	27 November 2000
2000 TX(D)	Kerr County, Texas	27 November 2000
2001 ID	Washington County, Idaho	2 October 2001
2001 WY	Carbon County, Wyoming	11 September 2001

^a All viruses were isolated from white-tailed deer with the exception of 2001 ID and 2001 WY, which were isolated from mule deer.

Test Incorporated, Friendswood, Texas, USA), according to the manufacturer's protocol.

Portions of the S10 and L2 gene segments (which encode the NS3 and VP2 proteins, respectively) were RT-PCR amplified as previously described (Murphy et al., 2005), utilizing gene-specific primers (NS3+2/NS3-805 or VP2+352-1/VP2-1349-1; Table 2). The region of the L2 gene sequenced corresponds to some of the putative neutralization epitopes in the cognate gene of BTV-10 (Gould and Eaton, 1990; DeMaula et al., 2000). These genes were chosen for several reasons. The S10 gene encodes the NS3/3a protein, which has been demonstrated to be involved in BTV egress from mammalian host cells (Roy, 1996). It is presumed that the NS3 protein has minimal exposure to the host immune system and, therefore, would not be subject to this selection modality. In contrast, the L2 gene product, VP2, is a virion surface protein that forms the epitope recognized by neutralizing antibodies in mammalian hosts infected with BTV (Huismans and Erasmus, 1981) and therefore would be predicted to be highly variable because of the selective pressure driven by the host antibody response. Previous phylogenetic studies of BTV and EHDV isolates have demonstrated geographic

relationships (topotypes) either within a single serotype or among several serotypes, depending upon the gene used for analysis, as well as the geographic origin of the isolates (i.e., inter-versus intracontinental) (Cheney et al., 1995, 1996; Pierce et al., 1998; Bonneau et al., 1999). The S10 and L2 genes have been utilized previously to compare genetic variation among EHDV-2 isolates within the US, and results from these studies suggest a very low level of genetic change over 23 years (Murphy et al., 2005).

Direct sequencing

Gel-purified S10 and L2 gene PCR products (729 base pairs and 594 base pairs in length, respectively) were sequenced on the forward and reverse strands at the Molecular Genetics Instrumentation Facility, University of Georgia (Athens, Georgia, USA) using dye-deoxy terminator nucleotides (Big Dye, Applied Biosystems, Foster City, California, USA) and an ABI 3100 Capillary Sequencer (Applied Biosystems). A panel of internal and flanking primers (Table 2) was used in multiple sequencing reactions to generate overlapping contiguous fragments and a subsequent consensus sequence, corresponding to bases 21-765 (S10) and 531-1124 (L2) of the

TABLE 2. Oligonucleotide primers used in RT-PCR amplification and sequencing of epizootic hemorrhagic disease virus serotype 1 isolates. NS3 corresponds to the S10 gene segment, VP2 corresponds to the L2 gene segment.

Primer name	Primer sequence
NS3+2	5'-TTAAAAAGAGGTTGGCATC-3'
NS3+201	5'-ACGGGCGCAACTATGGCACA-3'
NS3-620	5'-TGCATCTTTCTTCATTATTTTC-3'
NS3-262	5'-AACGCCTCCGCATACGAAGC-3'
NS3+601	5'-AAATAATGAAGAAAGATGC-3'
NS3-805	5'-GTGTGTCGAGGATGGCATA-3'
VP2+352-1	5'-CGAGGCCCATAGAAAGTT-3'
VP2+387-1	5'-GTGGACAGATACTTCTACAT-3'
VP2+635-1	5'-GATCCAAAGATTATAAATA-3'
VP2-1155-1	5'-CGATATACGCATCCAAGTT-3'
VP2-1349-1	5'-CATTCTTTGCTGAAATGAT-3'

published sequences (S10, GenBank accession number L29023 [Jensen and Wilson, 1995]; L2, GenBank accession number D10767 [Iwata et al., 1992]). Nucleotide sequences determined in this study are available in GenBank, accession numbers DQ899833-DQ899874.

Neighbor-joining sequence analysis

Chromatograms were viewed and contiguous fragments (contigs) built using the Sequencher program (Gene Codes Corporation, Ann Arbor, Michigan, USA). Contigs were formatted with the ToPir program of the Wisconsin package (GCG, version 8.0 software, Accelrys, San Diego, California, USA) and aligned with the Clustal X program (Thompson et al., 1994) using default parameters. The Modeltest program, version 3.06 (Posada and Crandall, 1998), was utilized to estimate models of nucleotide substitution for each locus. Pairwise distances between isolates were calculated using a maximum likelihood approach as implemented in the PAUP* program, version 4.0, beta 10 (Swofford, 2002), to correct for multiple substitutions. The Neighbor-Joining search algorithm in PAUP* was utilized for generating a phylogram and bootstrapped consensus tree (100 replicates) for each gene segment. Trees were outgroup rooted using the cognate gene region of BTV-13 (S10 gene, Genbank accession number AFO44712 [Pierce et al., 1998]) and the Ibaraki (Japanese) strain of EHDV-2 (L2 gene, GenBank accession number ABO30735 [Ohashi et al., 2002]). The overall number of nucleotide differences among isolates at each locus was calculated using the MEGA program (Kumar et al., 2001). MEGA was also utilized to translate the open reading frame of

each isolate, as well as compare the number of amino acid substitutions between viruses.

RESULTS

Nucleotide sequence variation was very low at both the S10 and L2 loci with an average of 98.1% identity (range: 94.9–100%) and 98.8% identity (range: 96.8–100%), respectively. Translation of the open reading frame of each gene revealed a high level of protein conservation at both loci, with an average of 99.0% (range: 97.1–100%) and 98.3% (range: 93.3–100%) identity at the S10 and L2 loci, respectively.

For the analyses of the S10 locus dataset, a maximum likelihood model of nucleotide substitution was selected, and model parameters estimated utilizing a hierarchical likelihood ratio test method as implemented in the Modeltest 3.06 and PAUP* software programs. The outgroup taxon (BTV-13) was removed from the model selection and parameter estimation process because its inclusion in the estimation decreased the log likelihood score of the best-fit model selected for the ingroup and thus is assumed to evolve via a different mechanism from the ingroup taxa. The estimated parameters include a transition-to-transversion ratio of 20.49, and frequencies of each nucleotide (A=0.3231, C=0.1866, G=0.2602, and T=0.2301). The rate of substitution

among sites was estimated to be equal, and no sites were considered invariant.

In the S10 phylogram (Fig. 2), isolates segregate into two distinct clades. The eastern clade contains the 1955 prototype EHDV-1 strain, originally isolated from a New Jersey white-tailed deer in 1955, as well as a relatively conserved (maximum number of nucleotide changes between samples = 7) grouping of isolates collected in the eastern US between 1991 and 1999. The western clade contains isolates collected in the western US between 2000 and 2001 and has a maximum of 10 nucleotide substitutions between isolates. Within the eastern clade, there is further division of isolates. The largest subclade contains the majority of the 1999 isolates, most of which are identical. Also within the eastern clade are three smaller clades, containing a 1996 TN isolate, a 1991 TN isolate, and two 1999 Georgia isolates. None of the isolates in the S10 western clade are identical, even though four of the isolates (TX(A)–(D)) are from the same herd, county, and year.

For the analysis of the L2 locus, a maximum likelihood model of nucleotide substitution was applied with the following parameters: a transition-to-transversion ratio of 12.41 and unequal frequencies of each nucleotide ($A=0.3231$, $C=0.1866$, $G=0.2602$, and $T=0.2301$). The rate of substitutions across sites was estimated to follow a gamma distribution with a shape parameter of 0.73, and the proportion of invariant sites was estimated to be 0.62. All parameters were estimated utilizing Modeltest 3.06 and PAUP*, with the outgroup taxon (Ibaraki) removed.

The L2 phylogram (Fig. 3) displays nearly identical region-specific groupings as found in the S10 phylogram. Several of the 1999 isolates are identical at this locus, as are two of the western isolates. In both phylograms the branch lengths among the western isolates are more heterogeneous than the branch lengths connecting the eastern isolates. This indicates eastern and western region-

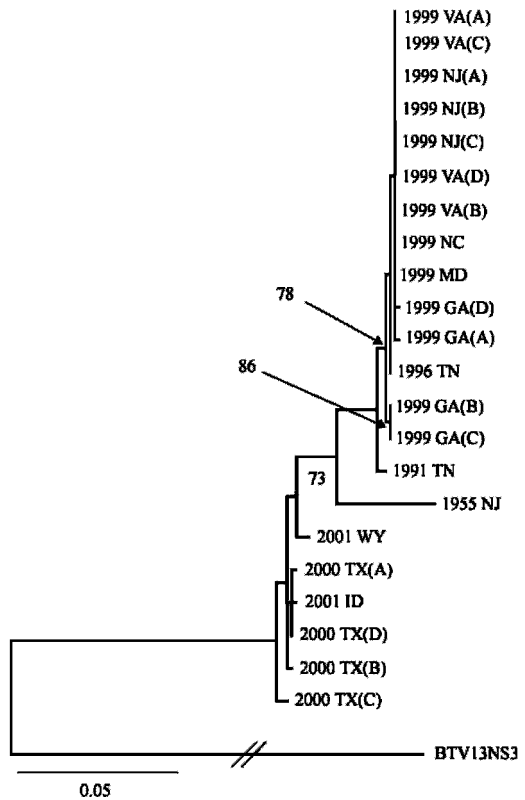


FIGURE 2. Neighbor-joining phylogram generated from analysis of a portion of the S10 gene segment from isolates of EHDV-1. Significant bootstrap values (greater than 70%) are displayed. One hundred bootstrap replicates were performed. Scale bar represents substitutions/site. Branch length to the BT13 (outgroup) taxon is 2.8 substitutions/site.

specific differences in the level of genetic variability.

DISCUSSION

The 1999 EHDV-1 epizootic in white-tailed deer was the largest outbreak of HD related to this serotype since 1975, both in regards to number of cases reported as well as geographic range (McConnell et al., 1977; Pearson et al., 1992). Over the course of approximately six weeks, cases were confirmed from progressively more northern latitudes, beginning in Georgia in mid-August and ending in New Jersey and Virginia in late September (Fig. 1). Given the rapid movement and relatively short time course of the outbreak, as well

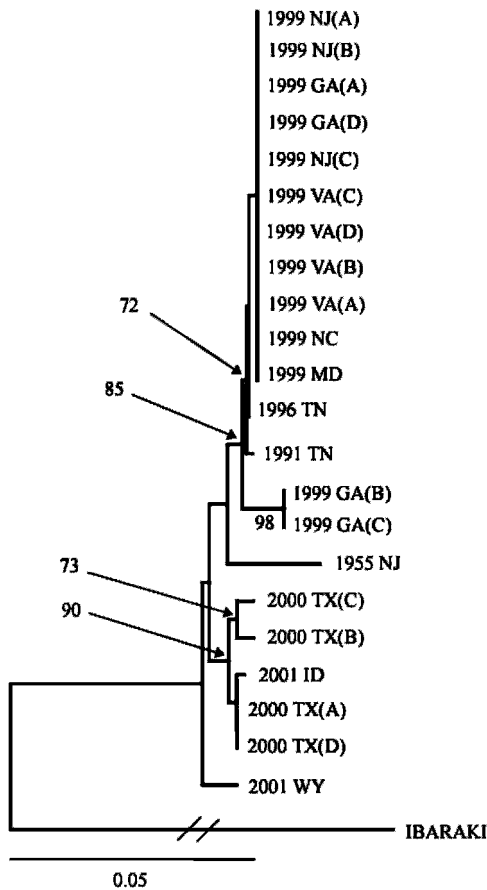


FIGURE 3. Neighbor-joining phylogram generated from analysis of a portion of the L2 gene segment from isolates of EHDV-1. Significant bootstrap values (greater than 70%) are displayed. One hundred bootstrap replicates were performed. Scale bar represents substitutions/site. Branch length to the Ibaraki (outgroup) taxon is 0.27 substitutions/site.

as the high degree of genetic relatedness among the 1999 isolates, it seems likely that the majority of viruses involved in this epizootic originated from a common source and may represent a single strain. However, two isolates, 1999 GA(B) and 1999 GA(C), did not group with the other 1999 isolates in either the S10 or L2 phylograms. Since these isolates originated from the westernmost and southernmost edges of the known EHDV-1 outbreak area, it is possible that they emerged from a separate source. Additionally, 1999 GA(A) and 1999 GA(D) were identical at

the L2 locus but differed by two nucleotides from each other, and one nucleotide from the other 1999 isolates within the same subclade of the S10 phylogram. The white-tailed deer from which the 1999 GA(A) and 1999 GA(D) viruses were isolated were penmates; therefore, it is hypothesized that the genetic differences represent either random drift or reassortment events with other EHDV-1 viruses that may have been cocirculating in the area.

As with the 1999 EHDV-1 viruses, a high degree of genetic relatedness among EHDV-2 isolates from white-tailed deer in the US has also been reported (Murphy et al., 2005). With EHDV-2, there is an apparent lack of a regional segregation (topotyping) in phylograms based on both the S10 and L2 gene sequences (Murphy, 2003). White-tailed deer in the southeastern US are exposed to EHDV-2 in two-to-three-year cycles (Stallknecht et al., 2002), and it is possible that these frequent HD events in a nearly contiguous deer host population would create a situation in which EHDV-2 could “travel” between the eastern and western US over a virtual animal “bridge.” This may result in the formation of a single virus population.

In contrast, EHDV-1 appears to exist in distinct regional populations representing the eastern and western US, as evidenced by the topotyping effect seen in both the S10 and L2 phylograms. Unlike EHDV-2, outbreaks of HD caused by EHDV-1 have been described only twice, in 1955 (Shope et al., 1960) and 1975 (McConnell et al., 1977), both occurring in New Jersey. However, serologic and virus isolation evidence indicates that white-tailed deer in the southeastern US are occasionally exposed to this serotype, especially in the coastal plain areas, and in Texas, where annual enzootic activity involving multiple EHDV and BTV occurs (Stallknecht et al., 1996, 2002). It is possible that the regional variation detected among the EHDV-1 isolates is related to the lack of frequent

epizootic activity involving this serotype; this is in direct contrast to the regular cycles of transmission observed with EHDV-2. Therefore, unlike EHDV-2, EHDV-1 may be more restricted to localized enzootic areas and in the absence of frequent epizootic events is unable to traverse over long geographic spans.

The phylograms of both the S10 and L2 loci also demonstrate a variation in branch length among the eastern versus western EHDV-1 isolates. This suggests a different level of nucleotide sequence heterogeneity among the viruses from these two regions. Nearly all of the 1999 isolates were identical at both loci, even though they originated from different geographic locations at different time points. The paucity of nucleotide sequence variation during an epizootic is not surprising, in that peak viremia (and therefore the peak transmission period) in the white-tailed deer host likely would occur several days before the emergence of an antibody response that could exert selective pressure upon these viruses (Quist et al., 1997; Gaydos et al., 2002a). This high level of genetic conservation also has been described among isolates collected from diseased deer during epizootics of EHDV-2-related HD in the eastern and western US (Murphy, 2003; Murphy et al., 2005). The observation of a higher level of genetic variation among viruses cocirculating in an enzootic transmission pattern (Texas isolates) in contrast to the genetic conservation observed among isolates from an epizootic transmission pattern (1999 eastern isolates) is not unique to EHDV-1. A similar phenomenon has been described for other arboviruses including Venezuelan equine encephalitis (family *Togaviridae*, genus *Alphavirus*), wherein the substitution rate among isolates associated with epizootics up to 35 years apart was 20-fold lower than that demonstrated for a closely related enzootic subtype (Powers et al., 1997).

In contrast to the conservation observed among viruses in the eastern subclades, the branches connecting the isolates in the

western subclades of both the S10 and L2 phylograms are of variable length. This suggests a higher level of genetic variation among the western isolates. Although based on only a small number of western samples ($n=6$), this finding may be related to the broader geographic area represented by the western isolates, and the fact that these isolates represent areas in which both enzootic (Texas) and epizootic (Idaho, Wyoming) transmission occur. All of the 2000 Texas isolates originated from white-tailed deer at the same research facility in a hyperenzootic region (Stallknecht et al., 1996), and it is evident that several strains of EHDV-1 were cocirculating among these animals. Additionally, the 2000 Texas isolates were made from clinically normal fawns, and three of these fawns were viremic in the presence of serum-neutralizing antibodies to this serotype (Gaydos et al., 2002b). The simultaneous presence of virus and antibodies could provide an opportunity for antibody selection and continuous generation of viral variants in white-tailed deer from enzootic foci. It is interesting that the two 1999 EHDV-1 isolates from Georgia (1999 GA(B) and 1999 GA(C)), which did not group with the other 1999 isolates in either the S10 or L2 phylograms, also originated from a location immediately adjacent to the coastal plain, an area of recognized enzootic activity (Stallknecht et al., 1995).

The 1999 outbreak provides an example of epizootic transmission, with rapid dissemination, host mortality, and very little viral genetic variation. The genetic similarity between the 1999 eastern viruses and other eastern isolates from previous years is consistent with the presence of known enzootic foci of EHDV-1 as exist in the coastal plain of the eastern US (Stallknecht et al., 1995). Viruses within these geographic foci may be genetically distinct from those in western endemic foci, as is present in Texas (Stallknecht et al., 1996), since a strong topotyping effect was seen among eastern and western EHDV-1 isolates. Although a topotype

effect also was observed among EHDV-2 viruses before 1990, it was not demonstrable among more recent (1990–2001) isolates (Murphy, 2003). This difference in phylogenetic pattern demonstrates the need for additional studies to further delineate the molecular epidemiology of EHDV-1.

ACKNOWLEDGMENTS

The authors are grateful to personnel of the Southeastern Cooperative Wildlife Disease Study for laboratory assistance. This work 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, US Department of the Interior.

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Received for publication 21 July 2003.