GENETIC RELATEDNESS OF EHDV-2 VIRUSES DURING A WIDESPREAD OUTBREAK IN THE UNITED STATES

by

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(Under the Direction of David Stallknecht)

ABSTRACT

In 2012, the United States experienced a particularly widespread outbreak of hemorrhagic disease (HD). Deer (both *Odocoileus virginianus* and *O. hemionus*) in 35 states were affected. Epizootic hemorrhagic disease virus, serotype 2 (EHDV-2) was the predominant virus isolated, representing 66% (135/205) of isolates. The large number of EHDV-2 isolates from a single outbreak provided an ideal opportunity to examine viral relatedness during the outbreak. In phylogenetic analyses of complete gene sequences from the antigen-determining and insect-binding proteins (VP2 and VP7, respectively) of 34 isolates from 21 states, we found subtle genotypes with regional associations in both proteins. This is consistent with presence of multiple strains of EHDV-2 expanding rather than a single virulent strain creating the entire outbreak. VP2 proteins shared 99.0% nucleotide identity; VP7 proteins shared 99.1% nucleotide identity. Very few changes were observed in either protein at the amino acid level.

INDEX WORDS: Epizootic hemorrhagic disease virus, hemorrhagic disease, EHDV-2, molecular epidemiology, phylogenetics

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CHAPTER 1

INTRODUCTION

In 2012, the United States experienced a particularly widespread and severe outbreak of hemorrhagic disease (HD) causing widespread mortality in wild ruminants, particularly in White-tailed deer (*Odocoileus virginianus*). The outbreak spread from coast to coast of the continental US and also had an increase in northern reporting from previous outbreaks (Stallknecht et al. 2015). Nearly two-thirds of viruses isolated from the outbreak were Epizootic Hemorrhagic Disease Virus serotype 2 (EHDV-2), a segmented, double-stranded RNA, non-enveloped, icosahedral virus in family *Reoviridae* and member of the genus *Orbivirus*. EHDV-2 isolates were obtained from 21 different states, from both captive and free-ranging populations. This created an opportunity to use a much larger sample size of a single serotype and complete gene sequences to ask questions as to the relatedness of EHDV-2 viruses during the outbreak.

HD outbreaks are cyclical in nature. They occur seasonally, from late summer to early fall, and time between outbreaks varies depending on a variety of factors ranging from climatic to individual host response (Howerth et al. 2001). The two viruses capable of inducing HD, Bluetongue virus (BTV) and EHDV are both transmitted by biting midges in the genus *Culicoides* (Diptera: Ceratopongonidae). The virus must replicate in the vector to facilitate transmission, increasing complexity of the transmission cycle while adding associated variables into the epidemiological equation (Carpenter et al.

2015). By adding a step in the transmission cycle, additional selection pressure is placed on the virus, essentially creating a "double-filter effect" described by Deyde et al. 2006. These constraints contribute to the notorious sequence conservation found in EHDV compared to other RNA viruses that also lack a proofreading mechanism. Yet, sequence-based methods for discerning epidemiological patterns of EHDV similar to those used on high profile viruses such as Zika or Ebola have not been widely used on EHDV in North America to any conclusive result. Sequence based technologies have improved rapidly since completion of the human genome project in 2001, simultaneously making sequencing more affordable and more widely used in public health (Fallin et al. 2016). Viral epidemics can be recognized and monitored as they progress in serious human diseases, but the methods are often used less frequently in wildlife diseases (Rasmussen and Katze, 2015).

The predominant serotype of the 2012 outbreak, EHDV-2, has been circulating widely in the US since its initial isolation in the mid-20th century (Shope et al. 1960; Nettles and Stallknecht, 1992). EHDV-2 has been the predominant virus isolated from the last four major outbreaks: 1988, 1996, 2007, and 2012 (Stallknecht et al. 2015). Despite evidence of EHDV-2 being present in the United States since 1956, research has yet to determine viral origin of these major outbreaks. Questions remain as to whether one particularly virulent strain is introduced and subsequently spreads to make up the outbreak each year, or whether multiple strains of EHDV-2 are responsible for the high morbidity and mortality events. This knowledge can help support or refute various theories of virus overwintering, contributing to a greater understanding of viral epidemiology. This thesis will analyze the composition of EHDV-2 viruses causing the

2012 HD outbreak using phylogenetic analysis of two key viral proteins, VP2 and VP7, which are the ruminant-binding and insect-binding proteins, respectively. Analysis will utilize full-length gene sequences of both proteins under selection pressure during the transmission cycle.

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CHAPTER 2

LITERATURE REVIEW

Orbiviruses

Members of the family, *Reoviridae*, subfamily, Sedoreovirinae, and genus, *Orbivirus*, are icosahedral, non-enveloped viruses encoded by 10 segments of double-stranded RNA (dsRNA) packaged within a double capsid shell (Mertens et al. 2005). Each genomic segment is associated with a five-fold axis within the nucleocapsid of the virus (Nason et al. 2004). Seven structural proteins (VP1-7) form two concentric capsids containing 4 non-structural proteins (NS1, 2, 3, 3a) and the packaged dsRNA genome. VP2 and VP5 interact to form an outer capsid to the virion, and VP7 covers the inner capsid containing VP1, 3, 4, 6, and the non-structural proteins packaged within the inner capsid (Roy 2008). Viruses in this genus share unique conserved 5' and 3' untranslated regions (UTR's) that can be used to identify species within the genus, such as Epizootic Hemorrhagic Disease Virus (EHDV) and Bluetongue virus (BTV) (Maan et al. 2007a). Across three of the most well known orbiviruses: African Horse Sickness Virus (AHSV), BTV, and EHDV, conserved functional regions exist within certain structural proteins such as VP3, a core structural protein (Roy, 1996). Arthropod vectors, such as biting midges in the genus *Culicoides* (Diptera: Ceratopongonidae), ticks, and mosquitoes transmit orbiviruses to vertebrate hosts worldwide, and this is the only genus within *Reoviridae* that includes arboviruses. Within the conserved group of orbiviruses, many features remain similar, but further degrees of similarity such as key structural functions

are shared between species with shared arthropod vectors i.e. *Culicoides*, mosquitos, or ticks (Jaafar et al. 2014). Culicoides transmit BTV, AHSV, and EHDV; they can be found worldwide except for Antarctica, extreme Polar Regions, New Zealand, Patagonia, and the Hawaiian Islands (Hoff and Trainer, 1981; Meiswinkle et al., 1994).

Orbiviruses affect many species of wild and domestic ruminants (Howerth, 2001). Both EHDV and BTV can cause a similar hemorrhagic fever that is broadly referred to as hemorrhagic disease (HD), but BTV is the prototype virus for the genus *Orbivirus* and primarily affects cattle and sheep (MacLachlan, 1994). EHDV primarily affects free-ranging wildlife, but documented cases of clinical disease in cattle has been documented with the Ibaraki strain (serotype EHDV-2) in Japan, serotype 7 in dairy cattle in Israel, and EHDV-2 in North America (Inaba, 1975; Yadin et al. 2008; Anbalagan et al. 2014; Garrett et al. 2015). This has resulted in the inclusion of both EHDV and BTV on the World Organization for Animal Health's (OIE) reportable disease list (Savini et al. 2011; OIE, 2016).

The current understanding of BTV and EHDV epidemiology is incomplete, especially related to viral maintenance (Wilson et al. 2008). In tropical and subtropical regions, it is possible that these viruses cycle between vectors and ruminant hosts year round. In more temperate areas, where disease caused by BTV or EHDV is more prevalent, disease is typically seasonal (Holbrook, 1996). For disease outbreaks to occur, a variety of epidemiological factors must synergistically occur to facilitate epizootics. First, competent vectors must be present and in sufficient concentration. Secondly, susceptible ruminant hosts must be present and in a sufficiently high density to support transmission during their viremic period (Mellor et al. 2000). Other factors that play into

the cycle of transmission are temperature, wind patterns, geographical features, landscape type, and drought presence, which will be discussed in depth later in this review. Specific serotypes and strains are associated with different geographic areas, such as the BTV-8 outbreak in Europe during 2006 and EHDV-2 (strain Ibaraki) in Japan and Australia (Wilson and Mellor, 2009; Omori et al. 1969).

There are 27 and 7 recognized BTV and EHDV serotypes, respectively (Maan et al. 2015; Anthony et al. 2009 VP2). Many details of EHDV replication mechanisms come from BTV research, as the prototype virus of the genus. In North America, EHDV has historically been the virus of most economic importance due to widespread mortality events of big game species such as *Odocoileus virginianus* (WTD). Thus, the focus of this thesis will be on EHDV, however supporting information from BTV will be used to supplement the understanding of EHDV.

Transmission, replication, and disease pathogenesis in ruminant hosts

The virus transmission cycle occurs when Culicoides feed on a viremic animal. Culicoides are pool feeders; they disrupt the epithelial barrier, secrete saliva rich with proteases and anti-clotting factors into the wound, wait for blood and extracellular fluids to flood in, and then drink (Carpenter et al. 2015). As virions are consumed with blood, the outermost viral capsid (VP2, VP5) is cleaved by proteases from *Culicoides* saliva acting on VP2, leaving the innermost capsid covered by trimers of VP7 proteins to enter the insect vector (Langer et al. 2007; Darpel et al. 2011). Cleavage of the outer capsid facilitates binding of VP7 to the Culicoides midgut cells via the RGF motif (Xu et al. 1997, Tan et al. 2001). There, the virus replicates in the midgut before being released into

secondary organ targets such as the salivary glands, where it can be transmitted to another ruminant host during feeding (Mellor et al. 2000).

Viral replication within the ruminant host first occurs within macrophages and nearby lymph nodes servicing the bite site, in both inoculation of cattle and WTD with BTV and EHDV (MacLachlan et al. 1990; Barrat-Boyes and MacLachlan, 1994; Barratt-Boyes et al. 1995; Quist et al. 1997). During infection, EHDV and BTV infect erythrocytes; virions can be sequestered within infoldings of WTD red blood cells *in vitro*, contributing to cell-association of the virus (Stallknecht et al. 1997). In experimental infections of EHDV, virus can be detected in WTD through day 59 post-infection; however, typical EHDV-2 viremia peaks between day 4-10 and lasts about 14 days (Quist et al. 1997; Stallknecht et al. 1997; Gaydos et al. 2002). Sequestration of virions in erythrocyte membrane invaginations has been hypothesized to provide a protective mechanism that facilitates prolonged viremia and offers protection from co-circulating antibodies in cattle (Barratt-Boyes et al. 1995). This cell-association also carries virus to sites of secondary replication, such as the spleen and lymph nodes (MacLachlan et al. 1990). Virus can also be isolated from peripheral blood mononuclear cells of deer during early infection, from days 4-8, consistent with results from cattle (Whetter et al. 1989; Barratt-Boyes et al. 1992; Stallknecht et al. 1997). EHDV and BTV readily infect endothelial cells; this infection causes severe vascular leakage as a product of endothelial cell infection and subsequent death (Howerth et al. 2001). Another byproduct of endothelium infection is the release of inflammatory cytokines and vasoactive mediators such as circulating interleukin 1 (IL-1) and tumor necrosis factor (TNF-α), which can modify hypothalamic function and result in food aversion and

anorexia (Akermann, 2012). In WTD infected with EHDV-2, IL-1 levels are associated with peak viremia, but in cattle, interferon production occurs before peak viremia, which could play a role in lack of clinical disease (Quist et al. 1997; MacLachlan and Thompson, 1985). Microvascular damage triggers changes in the coagulation cascade, increasing activated partial thromboplastin time and prothrombin time while reducing activity of Factors VIII and XII as early as day 6 (Howerth et al. 1988). This resulting consumptive coagulopathy helps to give HD its common name associated with multiple organ hemorrhagic diathesis, and often, death.

In ruminant hosts, BTV and EHDV present much the same way clinically. Experimental infections of heterologous BTV strains in multiple species of sheep and goats found sheep more susceptible to disease than goats but no difference at the breed level or between serotypes BTV-2 and BTV-8. In the same experiment, goats had viremia levels just as high, if not higher, than the diseased sheep (Caporale et al. 2014). Cattle mortality does not typically occur with infection of either BTV or EHDV. Instead, infection with EHDV can cause a drop in milk production, fever, anorexia, swollen conjunctiva, nasal and ocular discharge, lameness, dyspnea, and excessive salivation (Metcalf et al. 1992; Stevens et al. 2015). However, in North America, EHDV serotypes 1 and 2 have not been found to replicate to a high titer in cattle (MacLachlan and Osburn, 2004). In more recent outbreaks, such as 2007 and 2012, confirmed reports of EHDV-2 infected cattle with clinical signs during and in the years following (2008, 2013) severe outbreak periods in free-ranging ruminants suggest cattle play a larger role in outbreak epidemiology (Anbalagan et al. 2014; Garrett et al. 2015; Stevens et al. 2015). HD of wild ruminants exists in the US in three forms: peracute, acute, and chronic. In peracute

HD, death is sudden, within 8-36 hours, and can occur without clinical signs. Clinical signs in the peracute form include high fever, anorexia, weakness, pulmonary edema, along with severe edema of the head, neck, tongue, and conjunctiva. In the acute or "classic" form, individuals live longer and may have signs present in the peracute form accompanied congestion in the heart, rumen, and/or intestines. They may also have ulcerations on the dental pad, tongue, palate, rumen, and omasum. The chronic form is found in individuals surviving disease, marked by hoof rings caused by growth interruption and ulceration of the rumen mucosa (Howerth et al. 2001).

At a cellular level, virus binds to ruminant host cells using outer structural protein, VP2, which facilitates virus endocytosis and therefore is the antigen presenting protein determining serotype (Patel and Roy, 2014). As the virus enters the early endosome, VP2 is digested, exposing VP5, the other outer capsid structural protein. In cryo-EM images of BTV, VP5 undergoes a structural change under increasingly acidic pH that stretches tendrils out, similar to the influenza HA protein, while facilitating release from the late endosome into the cytoplasm (Zhang et al. 2016). The virus replicates within the cytoplasm, and eventually kills the cell in order to release progeny virus. The core is comprised of approximately 780 VP7 protein molecules form trimers that organize themselves into pentameric and hexameric units with channels in between such that 260 "knob-like" protrusions form on the outside of the inner capsid. Disk shaped VP3 molecules associated in dimers form the building blocks for the icosahedral structure of the virus inside the VP7-coated inner core. VP1, 4, 6 assemble themselves within the inner core with VP3, the non-structural proteins, and the dsRNA segments (Roy 2008). During replication and packaging, all viral proteins play a role. NS1 forms

tubules found in peri- or juxtanuclear locations attached to the intermediate filaments of the cellular cytoskeleton (Eaton & Hyatt, 1989). Viral inclusion bodies are often seen near the cell nucleus and thought to be involved in early stages of virion assembly and morphogenesis (Roy, 1996). NS2 associates with viral mRNA and is a major component of granular viral inclusion bodies in virus-infected cells (Thomas et al. 1990; Eaton et al. 1990). NS3/3a are products of the S10 gene; they glycosylate, and are involved with virus exit, thus they play a major role in virulence and are significantly attenuated during passage (Feenstra et al. 2014; Feenstra et al. 2016). Different NS3/NS3a can influence virulence in mammalian cells, yet replication and egress remain constant in insect cells, creating a host-dependent virulence factor caused by a proline residue at position 24 of NS3 (Ftaich et al. 2015).

HD Epidemiology

In addition to possessing similar molecular features, BTV and EHDV share many of the same epidemiological patterns. During outbreaks in North America, disease caused by either BTV or EHDV in white-tailed deer hosts (*Odocoileus virginianus*) is indistinguishable (Howerth et al. 2001). Historically, BTV has mainly caused disease in sheep and cattle throughout Europe, while EHDV has been primarily associated with disease in white-tailed deer in North America. However, important lessons in HD epidemiology can be learned from BTV abroad that can provide additional insight into HD epidemiology in the US, such as vector limits and overwintering. In the US, EHDV serotypes 1 and 2 have been consistently isolated and have circulated widely since the mid 20th century (Nettles and Stallknecht, 1992). In 2006, however, an exotic serotype (EHDV-6) was detected from a white-tailed deer in Indiana (Allison et al. 2010). By

2012, EHDV-6 isolates were consistently detected in 7 states, indicating virus establishment in the US. Sequence analysis determined that this virus emerged in the US from a reassortment event of an Australian-like EHDV-6 outer capsid (VP2 and VP5) with the inner capsid and non-structural proteins of the native and widely circulating EHDV-2 virus (Allison et al. 2012). At this time, EHDV serotypes 1, 2, and 6 are endemic to the US and outbreaks occur in a somewhat predictable manner.

Lessons from Abroad

In the mid-2000's a non-endemic serotype of Bluetongue (BTV-8) was introduced and spread through sheep and cattle in Europe. The prior absence of this particular serotype provided an opportunity for epidemiological investigation of its spread through Europe (Wilson and Mellor 2009). In France, investigations of BTV-1 and BTV-8 spread concluded that meteorological variables and elevation, which impact vector abundance and activity, paired with mammalian host availability, were the most important variables determining spread velocity (Pioz et al. 2012). The importance of meteorological variables, wind for example, to vector activity and virus spread in other parts of Europe has been previously documented in previous decades. Meteorological variables such as wind can successfully spread BTV. It was calculated that very strong winds probably carried infected midges from Cyprus, where an outbreak of BTV was present during October 1977, to Aydin Province, Western Turkey, in October 1977 where the particular serotype of BTV was not previously present, and also in British Columbia during 1987-88 (Sellers and Pedgley, 1985; Sellers and Maarouf, 1991). In addition to wind, environmental temperature has been found to affect activity of the vector and virus

replication in Culicoides (Carpenter et al. 2011). Therefore, changes in temperature due to season change, or geographical barriers with elevation changes like mountain ranges have been found to negatively affect transmittance of BTV by *C. brevitarsis* as was documented in Australia (Bishop et al. 2000). Europe also experienced new incursions of various EHD viruses throughout the Mediterranean Basin, with increased pathogenicity for cattle in serotypes normally considered non-pathogenic for cattle, such as EHDV-6 and EHDV-7. After this, the need for increased EHDV research was raised and EHDV added to the OIE reportable list in 2008 (Savini et al. 2011).

North America

In the United States, only one *Culicoides* species, *C. variipennis sonorensis* is a confirmed vector of EHDV (Foster et al. 1977; Jones et al. 1977). *C. debilipalpis* in the US can also carry BTV (Mullen et al. 1985). The presentation and timing of outbreaks varies widely across space and time. The presence and absence of disease given viral titers seem to create a stratified pattern in the United States, with greater times between epizootic events increasing from Florida to the Northern United States (Howerth et al. 2001). As a result, deer in lower latitudes, including Florida and Texas, exhibit high antibody prevalence for BTV and/or EHDV ranging from 80-100%, few clinical disease reports, and cases of "chronic" HD infection. Therefore, this is considered the "enzootic subclinical region." Chronic HD is considered a "non-active infection, but presents as hoof abnormalities (i.e. growth rings), ruminal scarring, and subsequent winter emaciation. As the vector range extends north with latitude, and elevation particularly on the East coast, the severity of lesions and clinical disease tend to increase with an

associated decrease in antibody prevalence and time between reports of disease. Consequently, the coastal plain region is considered intermediate enzootic, the lower Piedmont is considered transitional enzootic/epizootic, and the upper Piedmont and Appalachian Mountains are considered the epizootic region. Across this enzootic/epizootic gradient, the time between mortality events range between 2-3 years in the coastal region, 2-4 years in the lower Piedmont, and 8-10 years in the upper Piedmont and mountain regions with disease progressing from "chronic" HD to "acute" or "traditional" HD hallmarked by edema, congestion, hemorrhage, and ulcerative lesions, then to "peracute" HD in which death occurs so suddenly that the only gross lesion in edema and is usually found in the most naïve of populations (Couvillion et al. 1981; Howerth et al. 2001; Murphy et al. 2005). The disease does not present as clearly as a gradient from east to west as the stratification of South to North. Recent outbreaks have displayed a far greater span in northern reporting distribution that has not been previously documented (Stallknecht et al. 2015). As a result of the differential spatial-temporal distribution of these viruses and their ability to induce disease in naïve animals, not every year is an 'outbreak' year.

Environmental variables impact disease severity in the United States as they do abroad. A positive association of wetland cover with HD mortality is thought to be a product of the vector's life cycle dependence on wet soils bordering rivers, streams, lakeshores, bogs, saltmarshes and swamps for the larvae stage (Berry et al., 2013). Drought conditions are correlated with outbreak reporting of HD (Stallknecht et al. 2015). Additional predictive variables found to be significant in the southeastern US are temperature, rainfall, wind speed, dew point, normalized difference vegetation index, and

land surface temperature (Xu et al. 2012). As in Europe and Australia, it is possible that geographical boundaries such as mountains or temperature-changing latitudes separating areas of enzootic levels of transmission would impact vector abundance and transmission in the United States. Another stratification, land cover and usage would also impact vector availability and intersection of host availability with density, as found in the Xu et al. model represented by normalized difference vegetation index (2012). Vector competence is an inherited trait within individual Culicoides, controlled by a major locus inherited maternally (Tabachnick, 1991). Consequently, there are several dimensions to the epidemiology of disease and infection of such arboviruses related to viral replication and transmission in both vector and ruminant host.

Overwintering

Another important epidemiological variable in the United States is host immunity. In southeast WTD populations, exposure and maternal antibody dynamics play a significant role in infection and disease (Gaydos et al. 2002). In US regions with infrequent reports of HD, seroprevalence to at least one serotype of EHDV or BTV approaches 100%, which is consistent with yearly exposure and subsequent endemic stability (Stallknecht et al. 1996; Coleman et al. 2001; Flacke et al. 2004). Maternal antibodies passed vertically to fawns can last into the seasonal introduction of EHDV and BTV viruses before waning, providing a mechanism of protecting fawns from disease in spite of viral infection (Gaydos et al. 2002). Many endemic areas in the United States facilitate year round circulation and maintenance of HD infection by providing sufficient host densities and temperatures that facilitate *Culicoides* survival and virus replication

(Howerth et al. 2001; Mayo et al. 2014). Due to the temperature link with vector survival and virus replication, some of the "endemic" areas are located in the warm southeastern US, such as Florida (Wittman et al. 2002). But in cases where outbreaks resume after a "silent" period lasting multiple months and exceeding the typical lifespan of adult midges, the mechanism of virus maintenance becomes paramount (Wilson et al. 2008).

A few theories have emerged regarding possible mechanisms of local overwintering. One theory is that of transovarial transmission of virus to progeny, allowing virus to be harbored in eggs and subsequently emerge in adult midges the following spring. This was deemed unlikely in a surveillance study examining *C. sonorensis* trapping during winter months in California after failing to detect BTV in adult male and female midges and further confirmed in laboratory infected and wild caught midges (Mayo et al. 2014; Osborne et al. 2015). Another theory is that a particularly long-lived adult could harbor virus long enough to carry it through winter months. This is problematic, however, because virus replication during the extrinsic incubation period is temperature dependent (Wittman et al. 2002). But, if a long-lived midge re-emerged during a transient period of higher temperature to perpetuate transmission during the inter-seasonal period, overwintering in long-lived midges would be possible. Another theory is that virus latency in ruminant hosts occurs via an unknown mechanism. BTV was re-isolated from an adult North American Elk (*Cervus Canadensis*) 1 and 2 days after Flumethasone injection and 106, 107 days post inoculation, roughly 3 months post-infection (Murray and Trainer, 1970). In another experimental trial, BTV was rescued from IL-2 activated γδ-T-cells, providing a mechanism for an inflammatory response mediated rescue of virus without direct

involvement of vector (Takamatsu et al. 2003). Although the IL-2 mediated virus rescue provides possibility of bypassing vector involvement for virus propagation, the inflammatory proteases and salivary secretions would enhance rescue of latent virus, which was suggested in Luedke et al. upon recovery of BTV from a yearling bull after *Culicoides* bite (1977). Perhaps more likely than vertical transmission or long-lived midges, is a combination of all theories in that the EHDV and BTV viruses may be transmitted and maintained at low levels through a complicated and unknown mechanism utilizing multiple ruminant species or an unknown vector allowing the viruses to persist in the environment, favored by Nevill (1971)(MacLachlan, 2011).

In the absence of local overwintering, sporadic reintroduction by movement of an infected midge or ruminant is the other likely source of EHDV or BTV viruses during an outbreak. As mentioned earlier, it is possible for an infected midge to be carried long distance on the wind. Another example of possible introduction is the movement of infected and subsequent viremic hosts, either naturally or by shipment. For example, if an animal or animals with subclinical infection from a region of endemically circulating viruses were to be moved to an epizootic region with naïve animals and competent vectors, a virus could be introduced to a region and spread through vectors to other competent hosts in which clinical signs would likely appear. Given that most ruminant species are susceptible to infection yet not all of those animals are susceptible to disease, it would seem that viruses would have ample room to reproduce and sustain themselves depending on how conducive the climate conditions are to vector abundance, productivity, and opportunity.

Given the differing temporal spacing of HD epizootics, determining presence of viral genotypes and tracing the molecular epidemiology could be a potential way to examine the source of an outbreak.

Molecular Epidemiology

Tracing the epidemiology of BTV and EHDV outbreaks is most easily accomplished when either an introduction of a new serotype or an isolated outbreak occurs. Currently, there is no single standard way to track the evolution of either BTV or EHDV and no one viral protein has been found to trace lineages of these viruses. Viral proteins from either serogroup are fairly conserved between serotypes within each species. For example, the serotype determining protein, VP2, shares 49.98% nucleotide identity between type strains of serotypes 1 (NJ) and 2 (Alberta), while Japanese EHDV-2 (Ibaraki) and North American EHDV-2 (Alberta) share 71% nucleotide identity. As VP2 is the most variable protein, the other structural and non-structural proteins are even more conserved between serotypes. Another reason for the difficulty of tracking these viruses is the double-filter effect caused by the arthropod vector, of which worldwide vectors are poorly understood (Weaver, 2006; Ruder et al. 2015). In analysis of North American outbreaks, small sample sizes from each outbreak also limit epidemiological conclusions that can be drawn from these data.

Whole genome sequencing is gaining traction in most areas of viral epidemiology given the advancement in technology and the accompanying decrease in price (Radford et al. 2012). Research in BTV has historically been on the forefront of technology. In fact, BTV was the first double-stranded RNA virus to be fully sequenced and the first viral-like particle to be created (Patel and Roy, 2014). Whole genome sequencing of BTV has

allowed for detection of reassortment and detection of vaccine strains by comparing all 10 gene segments within the virus genome. In India, whole genome sequencing was able to detect multiple introductions of "western" BTV topotypes in the form of matching a BTV-2 vaccine strain while also determining that BTV-10 in India matched (>99%) the CA-8 strain from the US (Maan et al. 2015). Not all segments need to be sequenced to determine reassortment of EHDV, however. Reassortment plays a much larger role in BTV diversity than EHDV, and there is no evidence suggesting any one segment reassorts more than others (Nomikou et al. 2015). The complementarity of certain segments within BTV to remain together during reassortment may be a result of RNA-RNA interactions between tailing UTR's of each segment, which was confirmed as possible for BTV and 15 other species of Orbiviruses (Fajardo et al. 2015; Boyce et al. 2016). In North American EHDV, however, evidence for preferential reassortment exists. The North American EHDV-6 virus was created by a reassortment event between an Australian-like EHDV-6 outer capsid (VP2, VP5) and the inner capsid of a native EHDV-2 virus (Allison et al. 2012). Since then, EHDV-6 has continued to reassort with native EHDV-2 viruses (my data). In reassortment between EHDV-1 and EHDV-2, segments for genes VP2, VP5, and VP7 (segments 2, 6, and 7, respectively) remain together as the outermost capsid and coating of the inner capsid, while reassortment between EHDV-2 and EHDV-6 switch the outer capsid proteins VP2 and VP5 from EHDV-6 with the inner core proteins from EHDV-2, including VP7 and all other viral proteins packaged within the core (Anbalagan et al. 2014). Due to the preferential reassortment of EHDV-2 viruses, as few as two viral proteins need to be sequenced to determine reassortment, one from the inner capsid and one from the outer capsid.

Historically, EHDV serotypes 1 and 2 were the main players in North American wildlife mortality events. Although a few BTV serotypes (2, 10, 11, 13, 17) are considered endemic, they have traditionally been involved in local outbreaks, and not the predominant serotypes during widespread mortality events. In 2006, however, EHDV-6 was first detected in Indiana and Illinois and has since been expanding (Allison et al. 2010; Stallknecht et al. 2015). In 1999, an outbreak of EHDV-1 stretching down the east coast from New Jersey to Georgia was examined using partial sequences of two gene targets, NS3 and VP2. From this analysis, it was discerned that the outbreak was from the spread of a single virus strain, but regional clustering during phylogenetic analysis indicated distinctions between eastern and western isolates (Murphy et al. 2006). Examination of EHDV-2 viruses across time yet analyzed the same way yielded a nearly random distribution with respect to time and isolation (Murphy et al. 2005). When the EHDV-2 dataset was re-examined using different statistical methods, a trend towards geographic distance and genetic distance was detected in viruses from the same outbreak, but overall "the results did not allow the rejection of the hypothesis of genotypes being spatially distributed at random" (Biek, 2007). These partial sequence analyses of VP2 were done using roughly 681 bp portion of gene corresponding to a putative epitope region found within BTV (Gould and Eaton, 1990; DeMaula et al. 2000; Murphy et al. 2005).

Early sequence analysis of EHDV-2 VP3 gene sequences (segment 3) found distinct differences between Australian and North American EHDV-2 viruses, indicating presence of global "topotypes" (Cheney et al. 1995). But analysis of 7 different full-length EHDV-2 VP2 sequences over a 13-year timespan found two topotypes within

North America, east and west, lacking any specific pattern of SNP accumulation (Cheney et al. 1996). The difference between finding regional clusters within VP2 sequences of EHDV-2 could be related to the partial sequences used in Murphy et al. 2005 and use of full-length gene sequences in Cheney et al. 1996, but with any statistical analysis, outcomes depend on what question is being asked, the tests performed, and the data used during analysis. Although experimental evidence supporting a putative epitope region located between nucleotides 350 and 1350 of the VP2 open reading frame suggests that important variability would be found within that region of the VP2 gene, Anthony et al. found no specific region of variability within VP2 based on analysis of all seven EHDV serotypes (Pritchard and Gould, 1995; Anthony et al. 2009(a)). Lack of a specific region of variability at the nucleotide level does not mean that structurally important binding residues needed important for binding and antibody neutralization cannot be located in this region. If anything, this disagreement between pure sequence analysis and experimental mutagenesis studies is consistent with the mutational robustness created by phenotypic conservation in the face of genotypic flexibility found within EHDV viruses in North America.

The insect binding protein VP7, and core proteins of North American isolates are fairly conserved within serogroup EHDV (Anthony et al. 2009(b); Anbalagan et al. 2014). However, VP7 has been found to have more of a geographic cluster than VP2 when compared phylogenetically (Ohashi et al. 2002). Reference strains of EHDV-1 and EHDV-2 share 93.7% amino acid identity and favor very strong selection for silent mutations, consistent with structural constraint within the virion and as its role as the insect binding protein (Mecham et al. 2003). Since VP7 is required for virion

construction and is found to be the inner core known to reassort between EHDV serotypes 2 and 6, this gene also makes a good candidate to detect continuing reassortment between North American EHDV serotypes without having to sequence an entire genome (Oldfield et al. 1990). In mutational studies of VP7 and formation of viral-like particles (VLP's), only one lysine residue found at position 255 in BTV VP7 proteins prohibited VLP formation. When mutated to a leucine, VP7 would not form core-like particles with VP3, suggesting steric hindrance that prevents trimer formation or proper folding needed to interact with other VP7 trimers or VP3 proteins (Roy, 1996). While this gene and its translated protein are expected to remain fairly constant within a single serotype, there is evidence that geographical distinctions can be made (Mecham et al. 2003).

The most comparable virus to EHDV is undoubtedly BTV, but lessons can be learned from other related viruses. Orbiviruses belong to the subfamily *Sedoreovirinae* along with the genus Rotavirus, which include the namesake species Rotavirus, a common human pathogen that can cause gastroenteritis. Although long-term mutation rate information is not available on Type III (dsRNA) viruses, some are better understood than others because of their economic importance (Aiewsakun and Katzourakis, 2016). While rotaviruses are not arboviruses, and differ from EHDV in that reassortment plays a large role in creating genetic diversity, some varieties have been studied well using sequence-based epidemiology (Kuzuya et al. 2013). Group A rotaviruses have been shown to reassort in constellations, such that almost all full genome sequences in GenBank have one of two internal gene constellations, either Wa-like strains or DS-1-like strains (McDonald et al. 2009; McDonald et al. 2012). That preferential reassortment

is similar to that which is favored in North American EHDV. In a community study of vaccine pressure, Rotaviruses were found to have stable point mutations through which lineages can be traced (Dennis et al. 2014).

2012 Outbreak

In 2012, the US experienced a widespread outbreak of morbidity and mortality in WTD across most of the continental US due to hemorrhagic disease. In this outbreak, the counties reporting hemorrhagic disease were positively correlated with the presence of the accompanying drought during the seasonal outbreak starting the last week in August (US Drought Monitor: http://droughtmonitor.unl.edu/MapsAndData/MapArchive.aspx). This outbreak was also described as having more northern reporting of disease compared with the previous 10 year period consistent with changing dynamics of infection and disease in the US (Stallknecht et al. 2015). Additionally, unprecedented levels of clinical disease of EHDV were reported in domestic ruminants during 2012, with detections also following in 2013 (Anbalagan and Hause, 2014; Garrett et al. 2015; Stevens et al. 2015). During this outbreak, the Southeastern Cooperative Wildlife Disease Study (SCWDS) isolated 205 EHDV and BTV viruses from across the nation, 6/205 were BTV, 1/205 was a co-infection of BTV and EHDV-2, and 198/205 were EHDV. The dominant serotype during this outbreak was EHDV-2, with 135/205 viruses isolated (66%). EHDV-2 was also the dominant virus present in the last major outbreak in the US, in 2007 (Stallknecht et al. 2015). For the first time, however, EHDV-6 played a large role in the outbreak, representing nearly one-third of isolates. Because the EHDV-2 isolates came from 21 different states spread across the continental US, this provided an opportunity to examine

relatedness of one serotype during a large outbreak with a wide geographic distribution using sequence-based methods.

The role of vectors in changing distribution and evolution of EHDV in the US is currently unknown and remains one of the largest gaps in knowledge. By further understanding overwintering mechanisms and how they shape the evolution of EHDV in the US, biologists can gain a better understanding of how cyclical outbreaks will affect populations and make more informed decisions regarding management.

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CHAPTER 3

GENETIC RELATEDNES OF EPIZOOITC HEMORRHAGIC DISEASE VIRUS SEROTYPE 2 (EHDV-2) ISOLATES FROM THE 2012 OUTBREAK IN THE UNITED STATES[1]

[1] Crum JA, Mead DM, Jackwood MW, Phillips JE, Stallknecht DE. To be submitted to *Journal of Wildlife Diseases*.

Abstract

During summer and early fall of 2012, the United States experienced the largest outbreak of hemorrhagic disease (HD) on record; deer (both *Odocoileus virginianus* and *Odocoileus hemionus*) in 35 states were affected including many northern states where HD typically does not occur. Epizootic hemorrhagic disease virus (EHDV) was the predominant virus isolated, with serotype 2 (EHDV-2) representing 66% (135/205) of all isolated viruses. The large number of EHDV-2 isolates from a single widespread outbreak provided an ideal opportunity to genetically determine if this outbreak resulted from multiple or a single genotype of EHDV-2. It is well known that viruses within the EHDV serogroup are genetically similar, but we hypothesize that subtle genetic distinctions between viruses would exist across the geographic range of the outbreak if multiple EHDV-2 strains were responsible. By sequencing the mammalian binding protein (VP2) gene and the insect vector binding protein (VP7) gene of 34 2012 EHDV-2 isolates from 21 states, viral relatedness and molecular epidemiology of the outbreak were examined. VP2 nucleotide sequences had 99.0% pairwise identity; VP7 nucleotide sequences had 99.1% pairwise identity. Very few changes were observed in either protein at the amino acid level. Despite the high genetic similarity between isolates, subtle nucleotide differences existed. Both VP2 and VP7 gene sequences separated into two distinct clades based on patterns of single nucleotide polymorphisms (SNPs) after phylogenetic analysis. The clades were divided geographically into Mid-Atlantic and Western clades although those divisions were not identical between VP2 and VP7. There

was also an association between the percent sequence identity and geographic distance between isolates ($p<0.001$). Based on these data, we conclude that multiple EHDV-2 strains contributed to this outbreak.

Key Words: Epizootic Hemorrhagic Disease Virus, EHDV-2, Hemorrhagic Disease, phylogenetics

Introduction

Two closely related Orbiviruses in the family *Reoviridae*, Epizootic hemorrhagic disease virus (EHDV) or Bluetongue virus (BTV) cause hemorrhagic disease (HD), a leading cause of morbidity and mortality in white-tailed deer (Odocoileus virginianus) throughout North America. These segmented, double stranded RNA viruses are transmitted by biting midges from the genus Culicoides (Howerth et al. 2001). While both BTV and EHDV affect white-tailed deer, mortality events in the eastern United States are most often associated with EHDV (Stallknecht et al. 2015).

EHDV serotypes 1 and 2 are distributed broadly across the US; a third serotype, EHDV-6, emerged as a reassortant between an exotic EHDV-6 (CSIRO-753 like) and North American EHDV-2 (Alberta) (Allison et al. 2012). EHDV-6 has become endemic in the US since its initial detection in 2006 (Allison et al. 2010; Stallknecht et al. 2015). EHDV-2 is found in Asia, Australia, and North America. EHDV-2, strain Ibaraki, is closely related to the Australian EHDV-2 viruses and cause disease in cattle, whereas North American EHDV-2 viruses are more closely related to EHDV-2 strain Alberta, and primarily affect deer (Inaba 1975; Howerth et al. 2001; Anthony et al. 2009b). North

American EHDV-2 viruses have dominated recent large-scale outbreaks: 1988, 1996, 2007, and 2012 (Stallknecht et al. 2015). During the 2012 outbreak, 205 EHDV and BTV viruses were isolated from animals in 21 states; 135 (66%) of those were identified as EHDV-2. During this same outbreak, previously undocumented levels of clinical disease also were observed in domestic ruminants due to confirmed EHDV infection (Stevens et al. 2015).

Despite a history of HD outbreaks across the US dating back to the mid-19th century, our understanding of the epidemiology of HD is incomplete. This is especially true in relation to viral overwintering mechanisms and outbreak dynamics. HD is seasonal and disease usually is detected during summer through late fall and disappears shortly after periods of colder weather that negatively impact vector abundance and activity (Nettles & Stallknecht, 1992). Currently, there are two theories as to how these viruses are maintained during winter and expand during regional outbreaks. One theory is that a single virus strain is introduced from areas where overwintering conditions are favorable and subsequently spreads during an outbreak. Another theory is that multiple viruses expand from local reservoirs so that multiple strains of virus comprise the outbreak. If the former, one single genotype of virus would be found throughout the outbreak. If the latter theory is correct, subtle genetic diversity, such as a collection of single nucleotide polymorphisms (SNPs) forming distinct genotypes, would be detected. The involvement of multiple viral strains within a single outbreak could result from the movement of infected vectors or animals from enzootic-subclinical regions in the southeastern United States (Sellers et al. 1985; Sellers et al. 1993; Pioz et al. 2012; Koenraadt et al. 2014). Alternatively, multiple viral strains could expand from viruses

that overwinter in vector or host populations within the outbreak area (Murphy et al. 2005; MacLachlan 2011).

Due to the widespread nature of EHDV-2 during the 2012 outbreak, an unprecedented opportunity to examine the relatedness of geographically distinct isolates representing a single serotype during a single epidemic emerged. Because many of the genes comprising EHDV show very little variation, we chose to examine two genes for viral proteins under the most selection pressure in the vector-virus-host system. VP2, the outer capsid protein, is both the mammalian receptor binding protein and antigen determining protein; it experiences selection pressure from the ruminant host (Oldfield et al. 1991; Maan et al. 2007). VP7, the outer protein of the inner capsid, is the insect receptor binding protein (Xu et al. 1997; Tan et al. 2001). Although VP7 is functionally constrained due to protein-protein interactions within the virion, it potentially receives selection pressure from the vector (Tan et al. 2001; Roy, 2008).

Existing studies utilizing sequence analyses have failed to shed light on EHDV overwintering mechanisms. The reasons for this are varied, but generally relate to geographic and temporal scale as well as small sample sizes. Most studies have focused on questions related to virus evolution or the detection of topotypes, and include multiple EHDV serotypes over multiple years. In addition, full-length gene or segment sequences often are not evaluated. A large number of EHDV-2 isolates from the 2012 HD outbreak provided a unique opportunity to focus on a single EHDV serotype during a single year over a wide geographic area in North America. The objective of this study was to determine if a single strain or multiple EHDV-2 strains comprised the outbreak. We hypothesized that genetic diversity would reflect source virus diversity, with minimal

diversity associated with the expansion of an introduced strain and increased diversity and geographic clustering associated with local expansion of endemic foci.

Materials and Methods

Source of viruses

Viruses utilized for this study were isolated from diagnostic samples submitted for BTV and EHDV testing during a large-scale 2012 HD outbreak in the United States (Figure 1; Abdy et al. 1999; Allison et al. 2010). From July to November of 2012, 135 EHDV serotype 2 (EHDV-2) viruses were isolated from white-tailed deer (*n*=124), cattle (*n*=9), mule deer (*n*=1) and an alpaca (*n*=1). Most of the samples were from wild populations, 15% (*n*=19) of white-tailed deer were captive individuals. A geographically representative subset of these viruses, including at least one isolate from each of the 21 states where EHDV-2 was isolated, was selected for sequencing (Table 1; Figure 1). To eliminate the variable of host difference, isolates were chosen from genus *Odocoileus*. To provide an internal control to confirm that viruses circulating in close proximity are genetically conserved we compared sequences from two isolates (CC12-351, CC12-352) from a single county.

Sanger Sequencing of VP2 and VP7

Viral RNA was extracted from low passage cultures using QIAamp® Viral RNA Mini kit (Qiagen, Germany) and frozen at -20°C until use. Viral RNA for VP2 and VP7 were amplified using sequencing primers and SuperScript® III One-Step RT-PCR Platinum® *Taq* High Fidelity (Invitrogen, Life Technologies, Carlsbad, CA). Sequencing primers were designed using alignments of full-length gene sequences and are available upon request. Each 50µL single-tube RT-PCR reaction was assembled according to

SuperScript® protocol, and converted to cDNA at 45°C for 30min followed by 2 min of pre-denaturation at 94°C. PCR amplification immediately followed using 40 cycles of 94°C for 15s, 47°C for 30s, and 68°C for 1 min followed by a final extension cycle of 68°C for 5 min.

Amplicons were separated on a 1.5% Agarose gel stained with EtBr, excised and gel purified using QIAquick® Spin kit (Qiagen, Germantown, MD). Purified cDNA was stored at -20°C until sequenced. BigDye® Terminator v3.1 Sequencing kit reactions were set up according to protocol and purified by Sephadex column (Life Technologies Corp., Carlsbad, CA, USA) Sanger sequencing was performed on an Applied Biosystems 3730xl 96-capillary DNA Analyzer by Georgia Genomics Facility and Macrogen USA (Life Technologies Corp., Carlsbad, California, USA).

Alignments/Phylogenetics

All sequencing reads were trimmed and aligned to references HM636898 and HM636903 for VP2 and VP7 respectively, using Geneious version 8.1.8 (http://www.geneious.com, Kearse et al. 2012). Consensus sequences were trimmed to the open reading frame AUG to TAG for VP2 and AUG to TAC for VP7. Gene sequences from 2012 isolates were aligned using CLUSTALW, MUSCLE, and MAFFT plugins for Geneious® v8.1.8 (Edgar, 2004; Larkin et al. 2007; Katoh and Standley, 2013). ML trees for VP2 were built using 1000 bootstraps and TN93+I model from the representative MUSCLE alignment as determined by best model fit test in MEGA (Kumar et al. 2015). Additional full-length gene sequences for VP2 from GenBank were included for analysis. VP7 maximum likelihood (ML) trees were built in MEGA 5.2.2 using theT92+G+I algorithm as determined by best model fit test in MEGA (ML) and

performed using 1000 bootstraps based on the MUSCLE alignment (Nei and Kumar, 2000; Tamura et al. 2011). As with the large VP2 trees, other full-length VP7 sequences from EHDV serotypes were included in ML tree with 1000 bootstraps. Each protein was analyzed using Tajima's Neutrality Test, test of Homogeneity of Substitution Patterns Between Sequences, ML Estimate of Transition/Transversion Bias in MEGA (Tajima, 1989; Nei and Kumar, 2000; Kumar and Gadagkar, 2001). Additionally, a Mantel matrix comparison test was performed in XLSTAT using the VP2 pairwise percent nucleotide identity matrix and a matching distance matrix created from the central point from each county of isolation (Mantel, 1967; XLSTAT, Addinsoft, Inc., Version 2016.5).

RESULTS

Complete open reading frame sequences of two genes were obtained from 34 different EHDV-2 viruses isolated during the 2012 outbreak. Of the 34 viruses examined, sequences for 19 isolates were unique at the nucleotide level for VP7, and 29 were unique at the nucleotide level for VP2.

Sequences for the mammalian binding protein (VP2) varied considerably more than VP7 sequences within this data set. Average pairwise identity of the 2012 isolates was 99.0%, ranging from 96.61-100%. Average amino acid identity was 99.3%. The open reading frame of the VP2 gene was 2,949 bases long; of those, 2,772 bases (94%) remained fixed; 49 amino acid changes were observed in 983 residues (95% remained fixed). VP2 sequences also formed 2 main clades based on a group of 5 unresolved sequences in an unrooted tree (Figure 2), but there are considerably more polymorphic sites seen in VP7 (Tables 2, 3). At least 11 SNP's distributed across the gene define the mid-Atlantic group, as seen in Table 3. Within the western group (Figure 2), there is a

mixture of isolates from the core of the outbreak (MO, KS) and from potential outliers not connected to the main body of the outbreak (MT, WY, UT, CO). In the Mid-Atlantic clade, however, the branch lengths get much shorter, indicating a higher similarity between viruses in the Southeastern and Mid-Atlantic states (GA, NC, VA, MD, WV, NJ, PA, KY). Located within the clades possessing the Mid-Atlantic genotype, isolates from IN, IA, and KS are also found. In Figure 3, the EHDV-1 (NJ) rooted ML tree of 2012 isolates including Ibaraki and two other EHDV-2 sequences has 487 variable bases in the 2,762 bp ORF alignment. The model of best fit was determined to be TN93 +I +G. The +I parameter, representing the number of invariable sites, used in the trees was 26.38%. In VP2, the transition/transversion ratio is 3.70. When rooted by EHDV-1 (NJ), much of the distinction between clades found in the unrooted tree disappears, illustrating the relative relatedness of the 2012 VP2 gene sequences. In the amino acid sequence tree, no distinction is made between any of the North American EHDV-2 isolates (data not shown). The Mantel test of matrix comparison between the pairwise percent nucleotide identity matrix of VP2 clades and the corresponding geographic distance matrix yielded a statistically significant association ($p<0.001$) between the two matrices. While the association was not strongly linear, the correlation value of -0.67 indicates a negative association between genetic similarity and geographic distance within the two main clades created from the 2012 analysis. Resolution between VP2 sequences from 2012 further decreases when compared to historical isolates and other serotypes with worldwide distribution (Figure 4). Each serotype falls out in distinct clades, visually representing relationships between global distance and temporal clustering. Tajima's Neutrality test on the nucleotide alignment of VP2 produced a *D* statistic of -2.54, a

negative result greater than two standard deviations from a null hypothesis of neutral evolution. This is indicative of recent population expansion after a bottleneck event (Tajima 1989; Nei and Kumar, 2000).

Average pairwise identity of VP7 gene sequences within the group of EHDV-2 isolates from 2012 was 99.1%, ranging from 95.81-100%. At the amino acid level, the isolates were so conserved that only 3 of the 34 VP7 gene sequences were unique. In the open reading frame of 1050 bases, 990 of those bases (94%) remained fixed. Of the 60 bases across the entire reading frame that differed, 58/60 of them were silent mutations. Only two residues were changed across the entire protein sequence. Within the mutated amino acids, both of them were conservative amino acid substitutions (N-275-S and M-349-L). Of the 2012 isolates, two distinct clades are present within the VP7 tree (Figure 5). The clades seem to fall out into two topotypes, based on two single nucleotide polymorphisms (SNP's) at nucleotides 97 and 561 as shown in Table 3. Sub clades are further distinguished by additional SNP's. The Midwestern/Western US clade has a genotype consisting of thymine at nucleotide 97, guanine at nucleotide 285, and cytosine at nucleotide 561, while isolates predominantly from the Mid-Atlantic region of the United States form another clade based on a genotype consisting of cytosine at nucleotide 97 and thymine at nucleotide 561. When additional VP7 gene sequences were added, EHDV-2 still clusters, but American EHDV-6 VP7 sequences are interspersed throughout the Alberta-type, North American EHDV-2 instead of clustering together (Figure 6). Maximum likelihood estimate of transition/transversion bias (R) was found to be 2.57 such that transitional changes that most often are silent mutations, are 2.57 times more likely to occur within VP7. Approximately 62% of the gene (651/1049 bases) is

invariable (+*I*) within this data set. When the best model fit test (MEGA) was performed on the larger VP7 data, the model remained the same, but the proportion of invariable sites (+*I*) dropped to 57.6%

Isolates WDL12-286, CC12-297, CC12-463 do not fall into a resolved clade in either protein. They more closely resemble the historic isolate and reference sequence used for alignment, CC126-00, from Georgia in 2000.

The internal control isolates from the same county (CC12-351,352) were 99.8% identical at the nucleotide level in both proteins. VP2 had 6 nucleotide differences, 2/6 resulted in different amino acids during translation. VP7 had 2 nucleotide differences, but were identical on the amino acid level.

DISCUSSION

The high level of nucleotide and amino acid identity present within both gene sequences was to be expected based on documented sequence conservation of EHDV viruses isolated several years apart (Mecham et al. 2003; Murphy et al. 2005). Although an RNA virus, Orbiviruses are less diverse than other better known RNA viruses and possess the genetic conservation typically found in other arboviruses (Weaver, 2006). Orbiviruses are thought to be slightly more stable due to double-stranded genomes, but are also evolutionarily limited by the vector-host transmission cycle and the double-filter effect found within arboviruses (Deyde et al. 2006). Rotaviruses, (also subfamily *Sedoreovirinae*), have point mutations through which lineages and sub-lineages within serotypes can be traced, similar to what we have seen in the EHDV-2 results of this study even though they are not arboviruses (Desselberger et al. 2001; Dennis et al. 2014). The high level of genetic similarity between gene sequences examined and degree of

robustness is consistent with a period of evolutionary stasis in which the virus is very well adapted to the vector/host system (Lauring et al. 2013).

In the alignments of both VP2 and VP7 nucleotide sequences, several point mutations were associated with the geographical origin of isolates, suggesting regional clustering. With VP2, polymorphisms at 26 nucleotide locations corresponded with division into the two major clades (Table 2, Figure 2). Additional nucleotide diversity within those 26 locations provided more distinct genotypes. For example, guanine instead of adenine at positions 528 and 987, and adenine instead of guanine at 1191 and 1512 separate the tightly clustered mid-Atlantic clade, which includes isolates from GA to NJ, from other nearby isolates in IN, PA, TN and WV. In the full alignment, however, the Franklin county, KY; Lancaster county, NE; Tama county, IA; Blaine county, MT; and Barry county, MI, isolates have many more polymorphic sites than the rest of the 2012 isolates and share more identity with a historical isolate, CC126-00, isolated from a WTD in GA in 2000 (HM636898 and HM636903). In the VP2 alignment, more variable nucleotide sites were found within a distinct region, this region corresponds with the putative epitope binding region between nucleotides 480-1110 of the VP2 gene (Gould & Eaton, 1990; Pritchard & Gould, 1995; DeMaula et al. 2000; Murphy et al. 2005) In Table 3, the summarized alignment of VP7 polymorphisms, only 6 nucleotide locations across the 1,050bp ORF were associated with the tree topology. Isolates from Colorado and Missouri stand out as having guanine and thymine rather than adenine at positions 603 and 1045 respectively. In the VP7 gene sequences, there is no distinction within the ‘eastern’ clades, as seen in the tree, which differs from the VP2 gene sequences.

When examining nucleotide differences, several subtle but distinct regional genotypes emerged within both VP2 and VP7, supporting the idea that multiple strains from endemic foci expand during outbreaks (Wilson et al. 2008). These regional clusters corroborate findings from similar North American analyses using temporally and geographically distributed EHDV-2 sequences (Cheney et al. 1996; Murphy et al. 2005). While it is possible that multiple introduction events could have provided the multiple viral strains seen during this outbreak, the slight difference found in the eastern clade that creates somewhat of an intermediate genotype suggests evolution within the greater eastern clade that would be consistent with long term evolution and circulation. Certain geographical and geological factors such as landscape cover, changes in elevation, and temperature changes have been found to define boundaries to BTV spread in Europe and Australia (Bishop et al. 2000). The greater eastern clade does not stop at the Appalachian Mountains, however; the eastern clade extends west through the Appalachian Highlands to the largely agricultural land throughout the Midwest and Interior Plains, with a border somewhere near the Ohio River.

Other aspects of the data reinforce the intricate nature of the vector-virus-host system and warn against oversimplifying conclusions from this data. First, the two genes examined do not match exact geographic patterns suggesting that they undergo differing selection pressures. There are also outliers within the rough geographic clusters of genetically similar, if not identical, isolates, which created the variety in examining the Mantel distribution between genetic and geographic distance. This can be caused by a variety of things. For example, many species of wild and domestic ruminants can develop sufficient viremia for transmission. Therefore, interstate movement of infected domestic

animals (including captive deer) or vectors cannot be ruled out as a possible method of EHDV-2 introduction (Sellers and Pedgley, 1985). Sequence analysis of paired historical isolates from the same locations should provide future insight into local maintenance of EHDV-2 strains. In BTV, reassortment plays a much larger role in intratypic diversity through reassortment that historically has not been documented in EHDV (He et al. 2010; Nomikou et al. 2015 Boyce et al. 2016). Since at least 2006, EHDV serotypes 2 and 6 can, and have, undergone reassortment, thus providing another method for the two genes to have differing inheritance patterns (Allison et al. 2012). As 33% of all EHDV isolations were EHDV-6 in 2012, examination of VP2 and VP7 proteins in those isolates would also help to elucidate those reassortment mechanisms in North America.

Putting these 2012 data in context of other serotypes and a larger temporal scale in the large VP2 and VP7 trees, little resolution within the various serotypes can be seen. In the large VP2 tree, a trend towards temporal association (year of isolation) was seen on a very small scale within EHDV-2 viruses, but a few sequences in 2012 shared higher nucleotide identity with the historic isolate CC126-00, from a WTD in GA during 2000 (Figure 4*)*. These viruses were primarily located on the outskirts of the main body of the outbreak as reported in Figure 1, but the Tama, IA isolate CC12-463 fell well within the outbreak. Another outlier sequence, from Barry, MI, appeared within different clades in both VP2 and VP7 trees. Michigan experienced their first large outbreak of EHDV-6 in 2012; this could suggest another introduction of diversity within the EHDV-2 viruses. The potential trend of temporal relatedness based on year of isolation lacked sufficient sample size to infer temporal relatedness outside of the 2012 samples. These data also failed to find any pattern relating to date of isolation and genetics on a monthly or weekly

scale, such as being able to trace the passage of virus across the outbreak. Higher variation in VP2 gene sequences was to be expected, as was the phenotypic conservation of VP7 (Anthony et al. 2009a,b; Maan et al. 2007; Mecham et al. 2003). The strongly negative Tajima's Neutrality Test statistic of VP2 also suggest that the differences found between clades of VP2 are from different viral origins. The negative $D=-2.54$ corresponds with a lower than expected diversity given the number of variable sites.

VP7 has the largest difference in nucleotide identity and amino acid identity of all viral proteins in both EHDV and BTV, such that high levels of synonymous mutations should be expected (Anthony et al. 2009a). This protein is also fairly conserved between serotypes, and is commonly used as a target for EHDV and BTV differentiation. Despite expecting high conservation, there was variation in the VP7 gene sequences from the same county in the center of the outbreak, Green county, MO. Given the high level of conservation between geographically distinct isolate sequences, this was surprising and perhaps indicative of a greater degree of variation in a smaller community that is not shown in a larger scale genetic survey of an outbreak. One of the two isolates, CC12-351, was sequenced by another group using NexGen sequencing, but matched our sequence, resolving any doubt about contamination or methodology (Wilson et al. 2016). While little variation is observed in VP7, it is important to note presence of topotypes and the inability to distinguish North American EHDV-6 VP7 genes from EHDV-2 VP7 genes in the large tree (Figure 6). This is suggestive of continuing reassortment between North American EHDV-6 viruses and the more diverse group of co-circulating EHDV-2 viruses, although EHDV reassortment is far less frequent than within BTV serotypes (He et al.2010; Boyce et al. 2016).

Accumulation of SNPs forming distinct genotypes with regional clusters is consistent with local virus maintenance and emergence from endemic foci during advantageous vector, virus, host, and environmental conditions. While only two genes were sequenced, complete sequences detected additional subtleties that would have been missing if partial sequences were used. These two proteins' locations in separate pieces of the viral capsid also allowed us to detect potential reassortment of the currently expanding EHDV-6 North American strain. Extra sequencing of additional historic EHDV-2 isolates and recent EHDV-6 isolates from the same location would elucidate those mechanisms further. In conclusion, presence of distinct regional genotypes within both genes examined is consistent with multiple strains of EHDV-2 viruses comprising the 2012 outbreak.

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Table 1. List of isolates chosen for sequencing. All samples were from wild populations, except for individual marked with *. Date listed is date of positive virus isolation.

Species	Date	Isolate	County	State
O. virginianus	2012	23592	Lawrence	OH
O. virginianus	8/2/12	CC12-277	Dickinson	KS
O. virginianus	8/2/12	CC12-283	Franklin	KY
O. virginianus	8/9/12	CC12-288	Wilkes	NC
O. hemionus	8/9/12	CC12-290	San Juan	UT
O. virginianus	8/9/12	CC12-297	Blaine	MT
O. virginianus	8/9/12	CC12-304	Coffey	KS
O. virginianus	8/16/12	CC12-332	Howard	MO
O. virginianus	8/21/12	CC12-337	Caldwell	NC
O. virginianus	8/24/12	CC12-351	Greene	MO
O. virginianus	8/24/12	CC12-352	Greene	MO
O. virginianus	8/24/12	CC12-353	Sullivan	IN
O. virginianus	8/28/12	CC12-362	Cape May	NJ
O. virginianus	8/28/12	WDL-12-286	Barry	MI
O. virginianus	8/30/12	CC12-376	Hawkins	TN
O. virginianus	9/6/12	CC12-390	Pottawatamie	IA
O. virginianus	9/7/12	CC12-399	McCreary	KY
O. virginianus	9/11/12	CC12-408	Morgan	CO
O. virginianus	9/11/12	CC12-413	Middlesex	NJ
O. virginianus	9/18/12	CC12-454	Cumberland	PA
O. virginianus	9/18/12	CC12-460	Dorchester	MD
O. virginianus	9/20/12	CC12-463	Tama	IA
O. virginianus	9/20/12	CC12-477	Ritchie	WV
O. virginianus	9/20/12	CC12-497	Lancaster	NE
O. virginianus	9/20/12	CC12-500	Crawford	PA
O. virginianus	9/21/12	CC12-507	Amherst	VA
O. virginianus	9/27/12	CC12-530	Wyoming	WV
O. virginianus	9/27/12	CC12-534	Bedford	VA
O. virginianus	9/27/12	CC12-537	Frederick	MD
O. virginianus	9/27/12	Donnie Ronnie*	Clarke	GA
O. virginianus	10/11/12	CC12-562A	Crook	WY
O. virginianus	10/12/12	CC12-567	Allegheny	MD
O. virginianus	10/23/12	CC12-587c	Ingham	MI
O. virginianus	11/6/12	175707*	Pulaski	AR

Table 2. Summary table of VP2 polymorphisms at selected locations corresponding with resolution in unrooted tree of VP2 isolates. Unless otherwise noted, positions are assumed consensus. The locations without a consensus base listed correspond with a degenerate base code, indicating that one nucleotide does not dominate that location.

	114	232	478	513	528	618	746	857	963	987	1077	1191	1367	1512	1566	1701	1833	1923	1956	1986	2403	2428	2505	2523	2529	2655
CONSENSUS						**C**	**A**	**A**	**G**						**C**		**A**		**G**	**G**	**T**	**T**	**T**	**C**	**T**	
CC12-283/Franklin/KY/8-2	G	A	G	T	A					A	C	G	C	G		A		T								C
CC12-497/Lancaster/NE/9-20	G	A	G	T	A					A	C	G	C	G		A		T								C
CC12-463/Tama/IA/9-20	G	A	G	T	A					A	C	G	C	G		A		T								C
CC12-297/Blaine/MT/8-9	G	A	G	T	A					A	C	G	C	G		A		T								C
WDL12-286/Barry/MI/8-28	G	A	G	T	A					A	C	G	C	G		A		T								C
CC12-352/Greene/MO/8-24	A	A	G	T	A	T	G	G	A	A	C	G	C	A	T	A	G	T	A	A	C		C	T		C
CC12-408/Morgan/CO/9-11	A	A	G	T	A	T	G	G	A	A	C	G	C	A	T	A	G	T	A	A	C		C	T		C
CC12-332/Howard/MO/8-16	A	A	G	T	A	T	G	G	A	A	C	G	C	A	T	A	G	T	A	A	C		C	T		C
CC12-351/Greene/MO/8-24	A	A	G	T	A	T	G	G	A	A	C	G	C	A	T	A	G	T	A	A	C		C	T		C
CC12-290/SanJuan/UT/8-9	G	A	G	T	A	T	G	G	A	A	C	G	C	A	T	A	G	T	A		C	C	C			T
CC12-587c/Ingham/MI/10-23	G	A	G	T	A	T	G	G	A	A	C	G	C	A	T	A	G	T	A		C	C	C		C	C
CC12-304/Coffee/KS/8-9	G	A	G	T	A	T	G	G	A	A	C	G	C	A	T	A		T	A		C	C	C		C	C
CC12-562a/Crook/WY/10-11	G	A	G	T	A	T	G	G	A	A	C	G	C	A	T	A	G	T	A		C	C	C		C	C
17507/Pulaski/AR/11-6	A	A	G	C	A					A	C	G	C	G		A		T								C
CC12-376/Hawkins/TN/8-30	A	G	C	C	A					A	T	G	T	G		G		C								T
CC12-390/Pottawatamie/IA/9-6	A	G	C	C	A					A	T	G	T	G		G		C								T
CC12-277/Dickinson/KS/8-2	A	G	C	C	A					A	T	G	T	G		G		C								T
CC12-353/Sullivan/IN/8-24	A	G	C	C	A					A	T	G	T	G		G		C								T
CC12-477/Ritchie/WV/9-20	A	G	C	C	G					A	T	A	T	G		G		C								T
CC12-500/Crawford/PA/9-20	A	G	C	C	G					A	T	A	T	G		G		C								T
CC12-399/McCreary/KY/9-7	A	G	C	C	G					G	T	A	T	A		G		C								T
CC12-567/Allegheny/MD/10-12	A	G	C	C	G					G	T	A	T	G		G		C								T
CC12-537/Frederick/MD/9-27	A	G	C	C	G					G	T	A	T	G		G		C								T
CC12-413/Middlesex/NJ/9-11	A	G	C	C	G					G	T	A	T	G		G		C								T
23592/Lawrence/OH/	A	G	C	C	G					G	T	A	T	G		G		C								T
CC12-288/Wilkes/NC/8-9	A	G	C	C	G					G	T	A	T	G		G		C								T
CC12-362/CapeMay/NJ/8-28	A	G	C	C	G					G	T	A	T	G		G		C								T
CC12-507Amherst/VA/9-21	A	G	C	C	G					G	T	A	T	G		G		C								T
CC12-530/Wyoming/WV/9-27	A	G	C	C	G					G	T	A	T	G		G		C								T
CC12-534/Bedford/VA/9-27	A	G	C	C	G					G	T	A	T	G		G		C								T
CC12-337/Caldwell/NC/8-21	A	G	C	C	G					G	T	A	T	G		G		C								T
CC12-454/Cumberland/MD/9-18	A	G	C	C	G					G	T	A	T	G		G		C								T
CC12-460/Dorchester/MD/9-18	A	G	C	C	G					G	T	A	T	G		G		C								T
DonnieRonnie/Clarke/GA/9-27	A	G	C	C	G					G	T	A	T	G		G		C								T

Table 3. Summary table of VP7 polymorphisms at selected locations corresponding with resolution in tree of VP7 isolates. Unless otherwise written, nucleotides are that of the consensus.

	97	414	555	561	579	591	603	1045
CONSENSUS		**C**	**T**		**T**	**A**	**A**	**A**
CC12-297/Blaine/MT/8-9	T			T		G		
CC12-463/Tama/IA/9-20	T			T		G		
WDL12-286/Barry/MI/8-28	T			T		G		
17507/Pulaski/AR/11-6	T			T				
CC12-562a/Crook/WY/10-11	T			C				
CC12-304/Coffey/KS/8-9	T			C				
CC12-290/SanJuan/UT/8-9	T			C	C			
CC12-283/Franklin/KY/8-2	T			C	C			
CC12-497Lancaster/NE/9-20	T			C	C			
CC12-587c/Ingham/MI/10-23	T			C	C			
CC12-408/Morgan/CO/9-11	T	T		C	C		G	T
CC12-332/Howard/MO/8-16	T	T		C	C		G	T
CC12-351/Greene/MO/8-24	T	T		C	C		G	T
CC12-352/Greene/MO/8-24	T	T		C	C		G	T
CC12-567/Allegheny/MD/10-12	C			T				
23592/Lawrence/OH/	C			T				
CC12-454/Cumberland/PA/9-18	C			T				
CC12-288/Wilkes/NC/8-9	C			T				
CC12-337/Caldwell/NC/8-21	C			T				
CC12-362/CapeMay/NJ/8-28	C			T				
CC12-399/McCreary/KY/9-7	C			T				
CC12-413/Middlesex/NJ/9-11	C			T				
CC12-460/Dorchester/MD/9-18	C			T				
CC12-477/Ritchie/WV/9-20	C			T				
CC12-500/Crawford/PA/9-20	C			T				
CC12-530/Wyoming/WV/9-27	C			T				
CC12-534/Bedford/VA/9-27	C			T				
DonnieRonnie/Clarke/GA/9-27	C			T				
CC12-507Amherst/VA/9-21	C			T				
CC12-537/Frederick/MD/9/27	C			T				
CC12-390/Pottawatamie/IA/9-6	C		C	T				
CC12-277/Dickinson/KS/8-2	C		C	T				
CC12-353/Sullivan/IN/8-24	C		C	T				
CC12-376/Hawkins/TN/8-30	C		C	T				

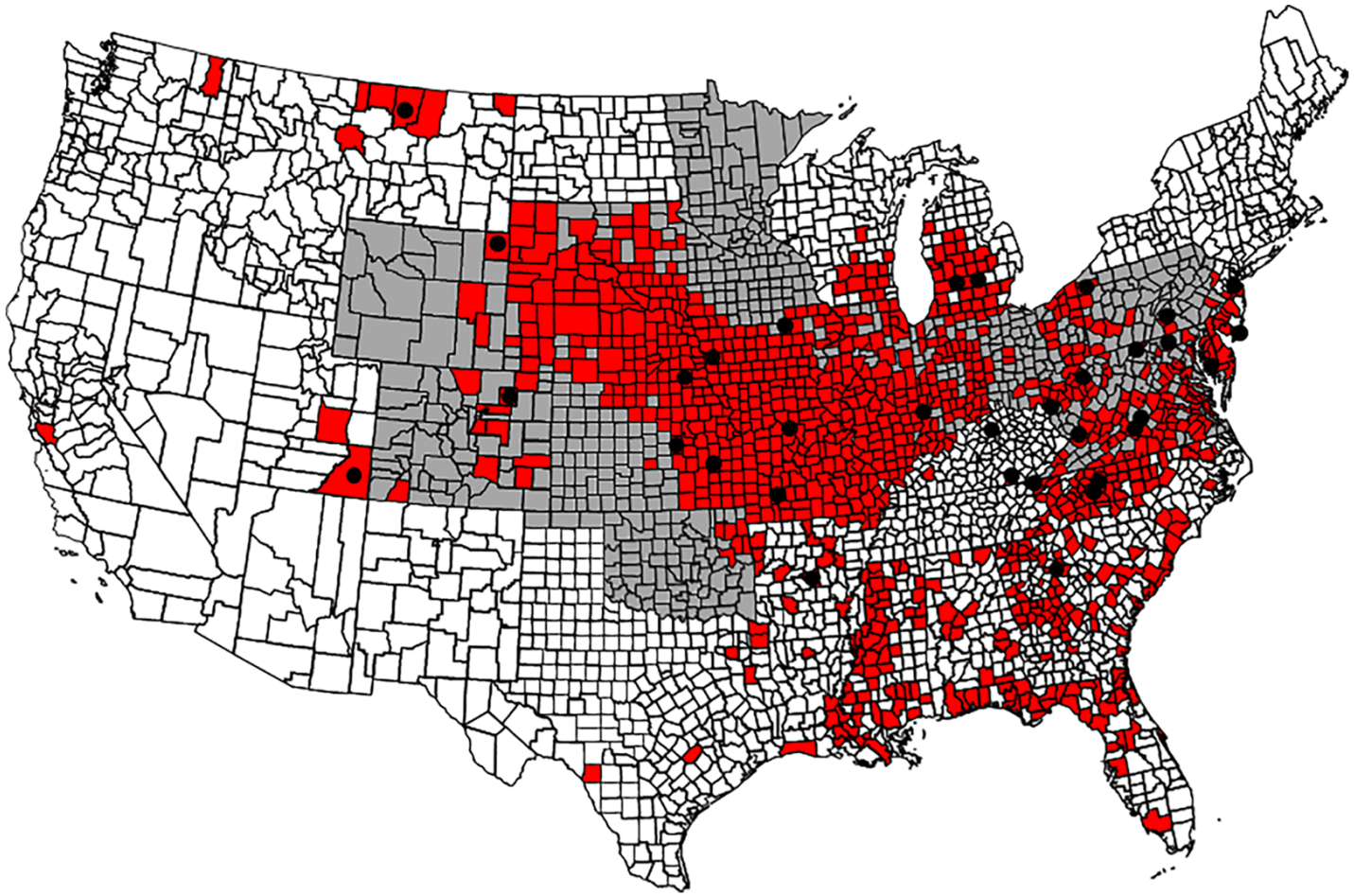

Figure 1. Map of reported 2012 HD morbidity and mortality shown in red (SCWDS 2012 HD Morbidity and Mortality Report). States also shaded in gray had confirmed reports of EHDV cases in domestic cattle or bison during 2012 (Stevens et al. 2015). Location of EHDV-2 isolates chosen for sequencing are shown by dots approximate to county level.

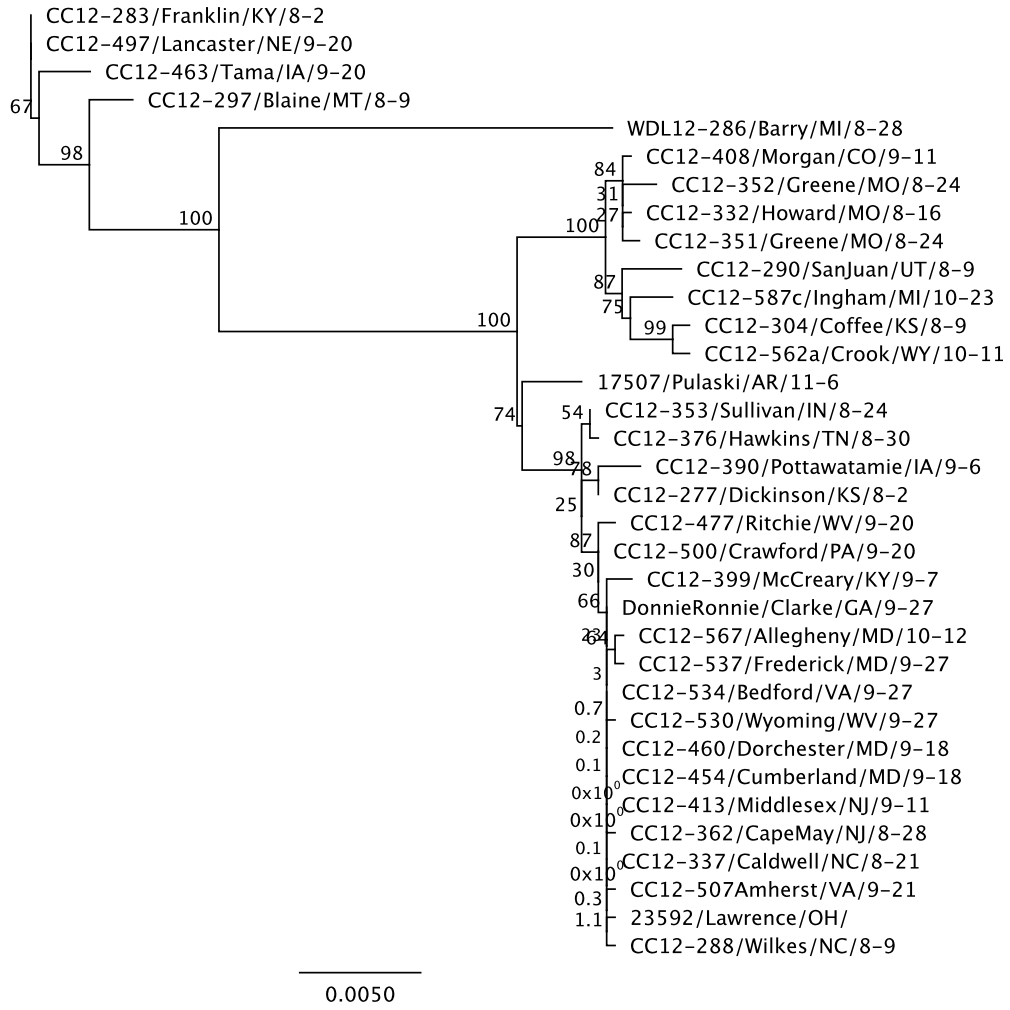

Figure 2. VP2 tree of 2012 Isolates. Unrooted PHYML tree, 1000 bootstraps with bootstrap support labeled. Isolate name followed by the county and state of origin, then month and day of isolation in 2012.

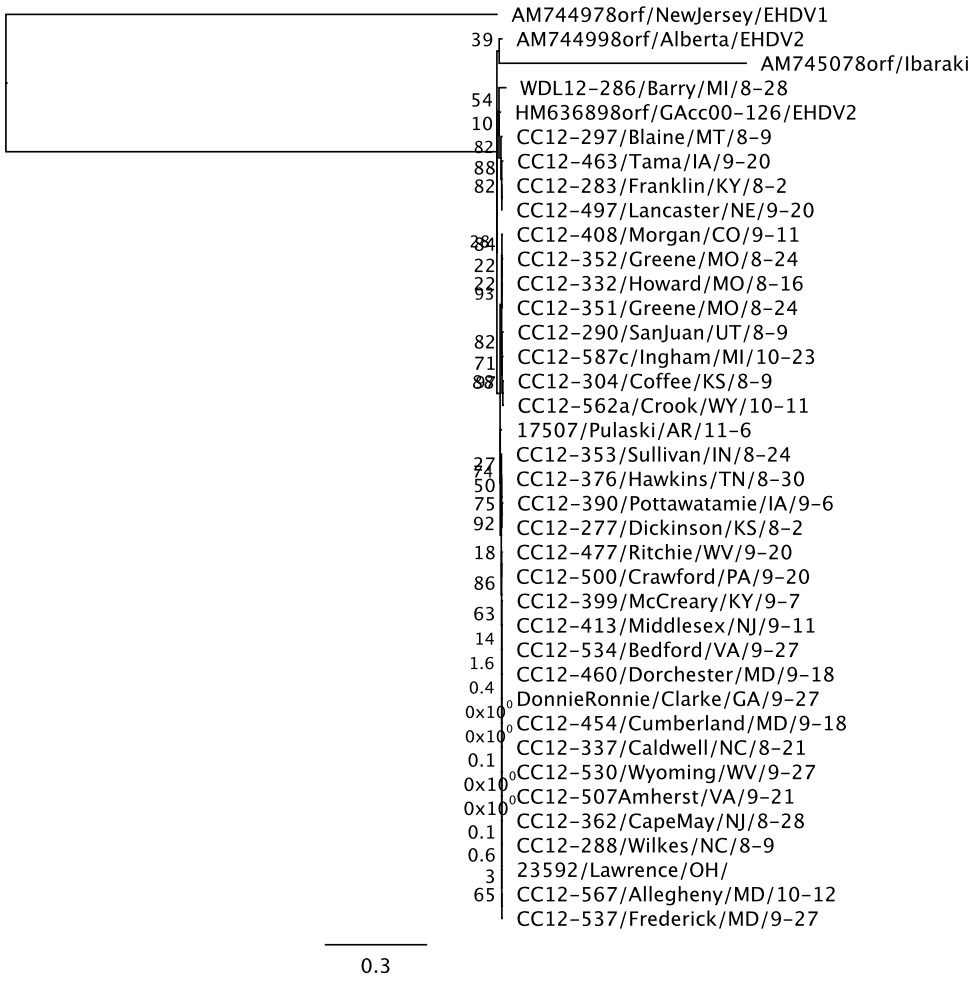

Figure 3. VP2 nucleotide PHYML tree of 2012 isolates with AM744978 (EHDV-1, strain NJ) as outgroup, AM74507 (EHDV-2, strain Ibaraki), AM74498 (EHDV-2, strain Alberta), and HM636898 (EHDV-2, CC126-00, from Georgia in 2000) included as references. Node labels are bootstrap support out of 1000 bootstrap replicates.

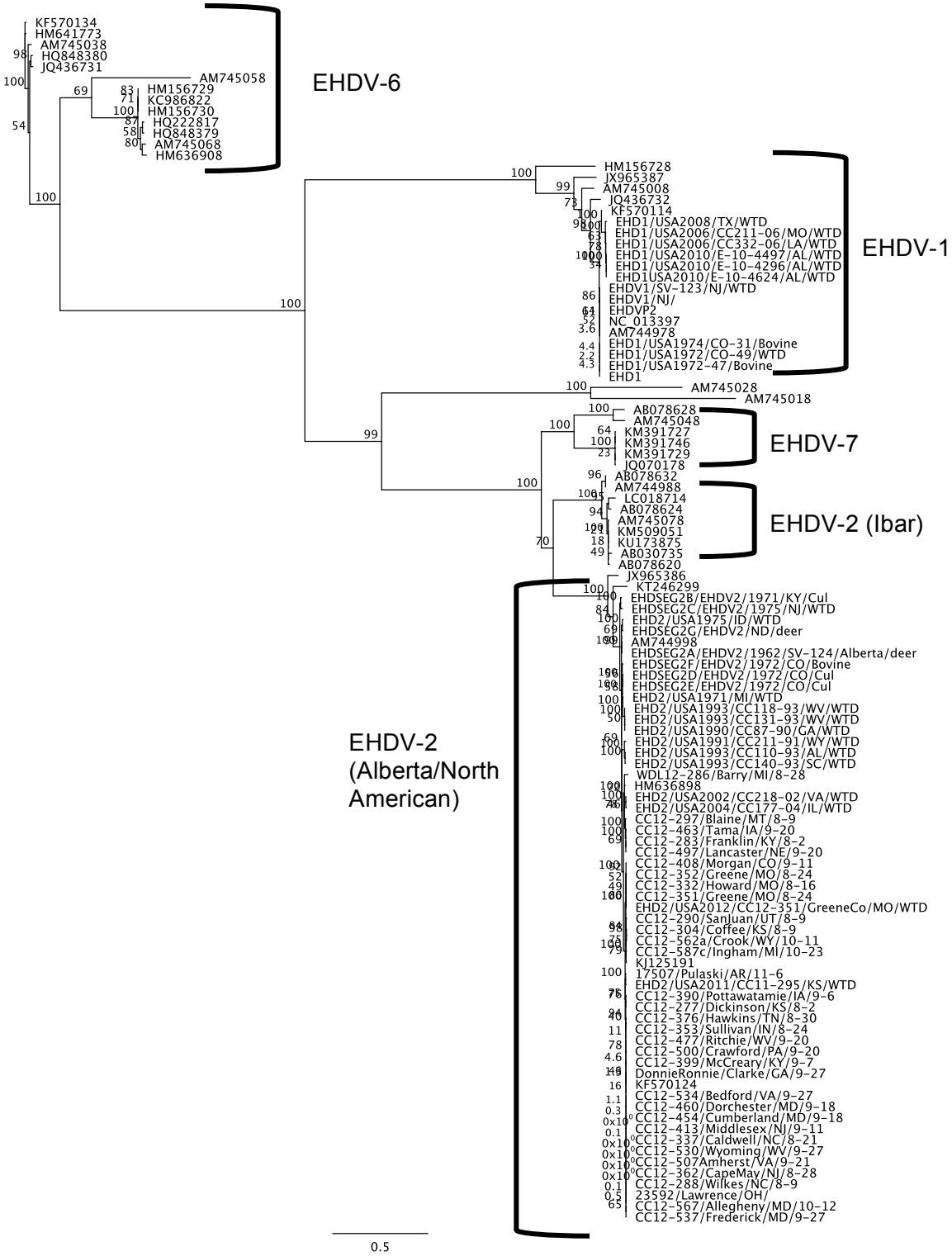

Figure 4. Unrooted VP2 nucleotide tree using 75 additional full-length VP2 gene sequences from GenBank. PHYML tree (1000 bootstrap replicates).

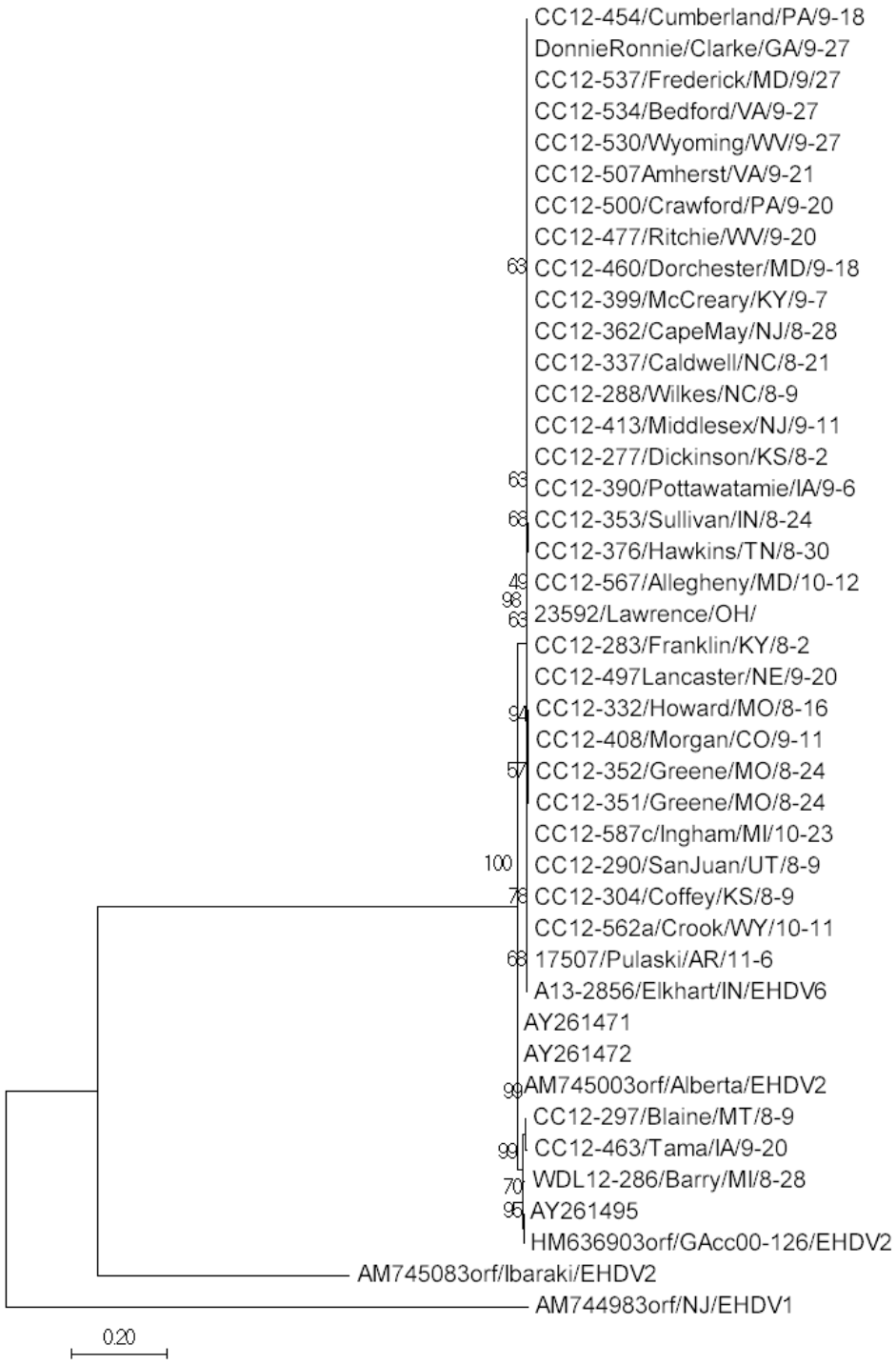

Figure 5. VP7 tree, 1000 bootstraps T93+G+I. Rooted by AM744983 (EHDV-1, strain NJ). AM745083 (EHDV-2, strain Ibaraki), HM636903, AM745003 (EHDV-2, strain Alberta), AY261471, and AY262172 also used as references.

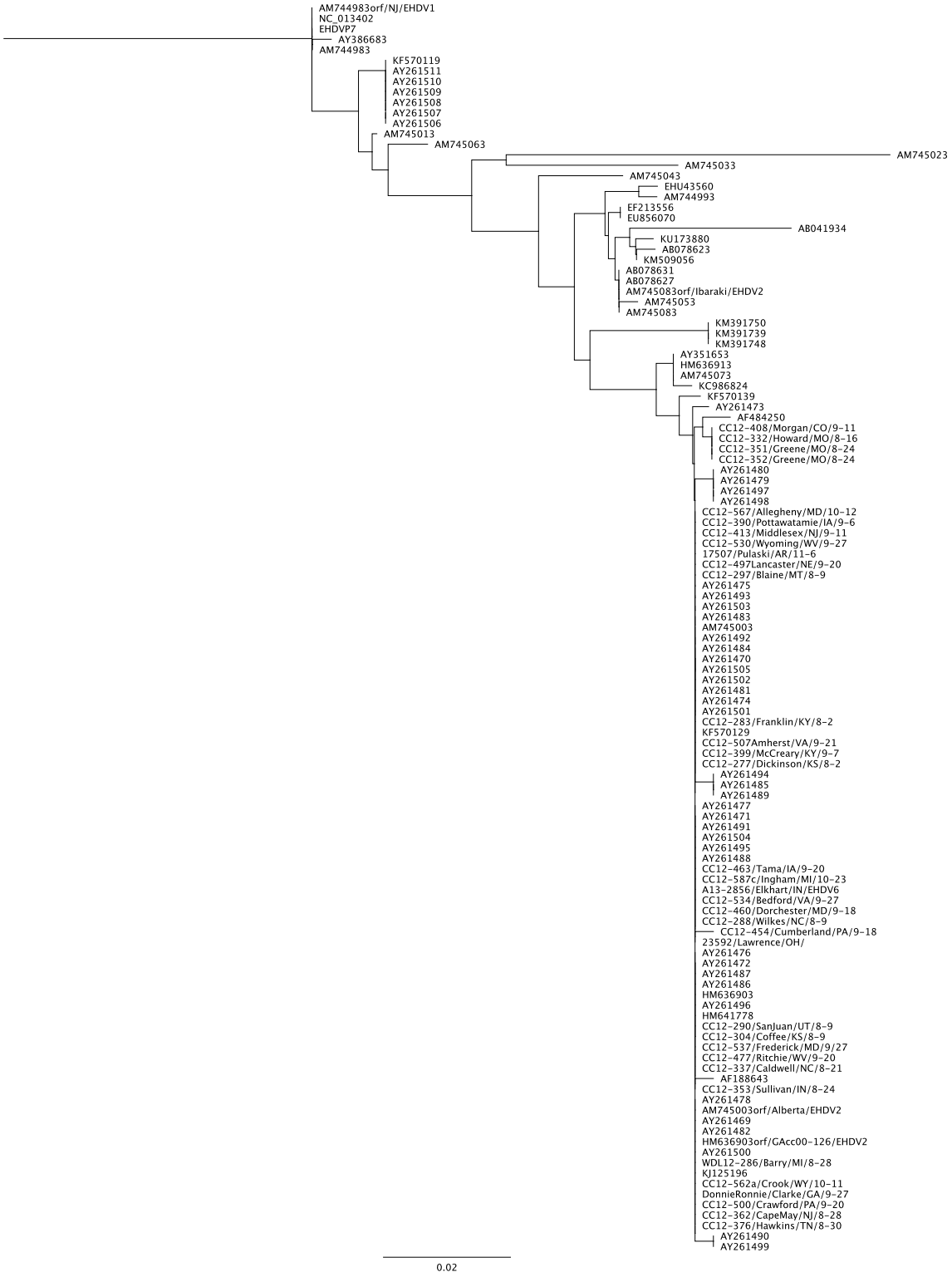

Figure 6. VP7 ML tree, 1000 bootstraps with gene sequences from all serotypes across globe for reference.

CHAPTER 4

SUMMARY AND CONCLUSIONS

Overall, the North American EHDV-2 virus genes analyzed from the 2012 HD outbreak were closely related. However, subtle distinctions between isolates in the form of SNPs created two major genotypes in each protein. When plotted on a map, those genotypes appear to be regionally associated into east and west. VP2 even has somewhat of an intermediate genotype with key bases found in each genotype that bridge the two 'clusters', as seen in Table 2. Two distinct clades were formed based on VP7, also falling into eastern and western topotypes. While these "regional" genotypes did not match exactly between proteins, this does not indicate lack of a pattern, but an insight into diversity of the inner and outer capsid genes of the most widely distributed EHDV serotype in North America.

Based on previous phylogenetic analyses, the difference in percent nucleotide identity between proteins was expected to be greater, however the percent of bases remaining fixed in either protein was 94%. Of the remaining 6% of polymorphic bases making up either protein, 49/983 amino acids varied in VP2, the antigen-determining protein, compared with 2/350 amino acids that varied in VP7. These statistics more accurately represent the mutational robustness of either the mammalian-binding protein (VP2) or the insect-binding protein (VP7).

Although the lack of genetic diversity in the viral population examined was initially frustrating, it provided answers to the question of what viruses composed the 2012 EHDV-2 outbreak in North America. Multiple genotypes composed the viruses isolated from dead or moribund individuals, but the mechanism of viral origin with regards to overwintering remains unknown. Future analysis of paired historical samples to determine whether viruses are evolving in local populations would be beneficial. Additional examination of virulence factors such as sequencing of UTR's and non-structural proteins would also be relevant given reported increase in bovine clinical disease. Obtaining a viral structure of EHDV would also further analysis on current sequences by enabling models to determine if mutations make a phenotypic difference beyond the translational amino acid change.