Segmental configuration and putative origin of the reassortant orbivirus, epizootic hemorrhagic disease virus serotype 6, strain Indiana

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ABSTRACT

In 2006, an exotic reassortant orbivirus, epizootic hemorrhagic disease virus serotype 6 (EHDV-6) [strain (Indiana)], was first detected in the United States. To characterize the reassortment configuration of this virus and to conclusively determine the parental virus of each RNA segment, the complete genome of EHDV-6 (Indiana) was sequenced, in addition to the genomes of representative EHDV-6 and EHDV-2 isolates. Based on genomic comparisons to all other EHDV serotypes, we determined that EHDV-6 (Indiana) originated from a reassortment event between the Australian prototype strain of EHDV-6 (CSIRO 753) and the North American topotype of EHDV-2 (Alberta). Additionally, phylogenetic analysis of all EHDV-6 (Indiana) isolates detected in the United States from 2006 to 2010 suggests that the virus may be undergoing continual reassortment with EHDV-2 (Alberta). In 2010, EHDV-6 (CSIRO 753) was detected in Guadeloupe, demonstrating that the parental virus of the reassortment event is circulating in the Caribbean.

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Introduction

Epizootic hemorrhagic disease virus (EHDV) is an arthropod-borne virus of the genus Orbivirus, family Reoviridae, that is maintained in nature in a transmission cycle involving hemagogous Culicoides midges and ruminant vertebrate hosts (e.g., cattle, deer) (Nettles et al., 1992). Like all orbiviruses, the genome of EHDV is composed of 10 segments of double-stranded RNA that encode seven structural (VP1–VP7) and four nonstructural (NS1–NS3/NS3a) proteins (Mertens et al., 2005). As the genome is segmented, orbiviruses are capable of undergoing reassortment with different serotypes of the same species (serogroup) during co-infection. Recently, based on antigenic and genetic analyses of the two outer capsid proteins (VP2 and VP5), the EHDV serogroup has been proposed to be condensed from ten to seven serotypes (Anthony et al., 2009b). Based on this newly proposed classification scheme, EHDV-318, a virus initially isolated in Bahrain in 1983 and formerly regarded as a separate serotype (Mertens et al., 2005), was demonstrated to be serologically and genetically related to the prototype strain of EHDV-6 isolated in Australia in 1981 (CSIRO 753) (Anthony et al., 2009b). Consequently, the 318 strain was grouped with CSIRO 753 to comprise two related, but genetically distinguishable, strains or topotypes of EHDV-6.

Historically, only two serotypes, EHDV-1 (New Jersey strain) and EHDV-2 (Alberta strain), were endemic to the United States (Chalmers et al., 1964; Shope et al., 1955). However, in 2006, an exotic strain of EHDV-6 was isolated from moribund and dead white-tailed deer (Odocoileus virginianus) in Indiana and Illinois (Allison et al., 2010). Since its initial discovery, the virus has been repeatedly isolated on an annual basis from additional states including Missouri, Kansas, Texas, Michigan, and Arkansas, suggesting that it may now also be endemic in the United States. Preliminary genetic analysis of the virus, designated as EHDV-6 [strain (Indiana)], suggested it was a reassortant derived from an exotic strain of EHDV-6 and endemic EHDV-2 (Alberta) (Allison et al., 2010). The detection of a reassortant EHDV derived from an indigenous serotype and a virus previously never reported in the New World not only provided a unique example of genetic shift among exotic and endemic viruses, but also demonstrated the plasticity of orbiviruses in...
general, not only in terms of the ability of different serotypes to reassort with one another, but also in their ability to enter, and become established in, novel environmental niches previously never occupied.

With the unprecedented incursion of multiple bluetongue virus (BTV) serotypes into northern Europe since 2006 (Saegerman et al., 2008; Wilson and Mellor, 2009) and the recognition of EHDV-associated morbidity and mortality in cattle from Réunion Island in 2003 and 2009 (Brédard et al., 2004; Saillée et al., 2011), Morocco, Tunisia, Algeria, and Israel in 2006 (Yadin et al., 2008; http://www.oie.int/eng/info/hebdos/AL564.htm), and western Turkey in 2007 (Temizel et al., 2009), concern regarding the recent geographical expansion of pathogenic orbiviruses has been growing steadily. Historically, other than the Ibaraki strain of EHDV-2 (Omori et al., 1969; Uchinuno et al., 2003), EHDV has not been considered to be a major threat to domestic animal health, whereas the closely-related BTV has been the focus of in-depth research for many years (for a review, see Tabachnick, 1996), primarily due to its pathogenic potential in sheep (and to a lesser extent cattle), in association with trade restrictions on ruminants and associated germplasm from BTV endemic areas. However, with numerous recent EHDV outbreaks involving serotypes previously never recognized as pathogens (i.e., EHDV-6, EHDV-7), coupled with the incurrence of significant economic losses during such outbreaks (Kedmi et al., 2010), EHDV may no longer be regarded as an anecdotal pathogen of cattle and its introduction to new areas should be perceived as it having the potential of becoming a disease agent of clinical and economic importance. Concomitant with the detection and repeated isolation of EHDV-6 (Indiana) in the United States, we instituted a number of research initiatives to comprehensively characterize the virus from a genetic and evolutionary standpoint, determine the susceptibility of a known North American EHDV vector (Culicoides sonorensis) for the reassortant, and to assess the potential risk that the virus may become an emerging pathogen of domestic and wild ruminants in the United States. In this report, we present the genomic sequence of EHDV-6 (Indiana), along with additional representative strains of EHDV-2 and EHDV-6, to more precisely determine the parental viruses responsible for the segmental configuration of the novel reassortant and explore its genetic diversity and evolutionary history within the United States.

Results and discussion

Comparative analysis of EHDV-6 (Indiana) with other EHDV-6 isolates from South Africa (M44/96, strain 318), Australia (AUS 1981/07, strain CSIRO 753), and Bahrain (BAR1983/01, strain 318) revealed that the parental virus of RNA segments 2 and 6 (which encode the outer capsid proteins VP2 and VP5, respectively) in EHDV-6 (Indiana) was very similar to the Australian prototype CSIRO 753 strain, with the two viruses sharing 97–98% nucleotide identities in their VP2 and VP5 sequences and a strong phylogenetic relationship (Tables 1 and 2; Fig. 1). Conversely, the RNA segments encoding the four nonstructural proteins (NS1, NS2, and NS3/NS3a), the three replicative enzymes (VP1, VP4, and VP6), along with the structural proteins that comprise the inner subcore (VP3) and core surface layer (VP7) were derived from EHDV-2 (Alberta) (Tables 1 and 2; Fig. 2). Thus, although EHDV-2 was the majority parental virus, contributing eight of the 10 RNA segments, the reassortant retained the serotype specificity (and hence serotype designation) of EHDV-6, as the outer surface antigens (VP2 and VP5) responsible for the induction of neutralizing antibodies during infection in vertebrate hosts (Iwata et al., 1991, 1992) were retained from EHDV-6 (CSIRO 753).

The genome of EHDV-6 (Indiana) is 19,407 base pairs (bp) in length, with the RNA segments ranging in size from 810 bp for segment 10 (NS3) to 3942 bp for segment 1 (VP1) (Table 1). Based on analysis of the terminal ends of each of the 10 RNA segments in positive-sense orientation, the 5′ and 3′ UTR sequences that are strictly conserved are the hexanucleotide 5′-GUUAAA-3′ and the pentanucleotide 5′-CUUAC-3′, respectively (Fig. 3). The 5′ UTRs ranged in size from eight (VP4) to 32 (NS1) nt, while the 3′ UTRs were, on average, considerably longer, ranging from 22 (VP1) to 115 (NS1) nt (Table 1 and Fig. 3). The 10 RNA segments of the EHDV genome and their associated genes have been described in detail elsewhere (Anthony et al., 2009a, 2009b, 2009c; Cheney et al., 1995, 1996; Iwata et al., 1991, 1992; Jensen and Wilson, 1995; Le Blois et al., 1991; Mecham et al., 2003; Wilson, 1994).

The recognition that both VP2 and VP5 (which are the most genetically divergent EHDV proteins) of the prototype reassortant (CC 304-06) were derived from a single serotype (EHDV-6) suggests that their homologous reassortment may have been necessary to form a stable outer capsid, such that a virus containing heterotypic VP2 and VP5 (i.e., from two different serotypes, such as EHDV-2 and EHDV-6) may be structurally and/or functionally debilitated and thus would be less likely to persist in nature (Mertens, 1999). However, based on the structure of the closely-related BTV, heterotypic protein interactions in EHDV-6 (Indiana), such as VP7 interacting with VP2 and VP5 during inner/outer capsid formation (Nason et al., 2004; Zhang et al., 2010), NS3 binding to VP5 or VP2 during viral assembly and egress, respectively (Beaton et al., 2002; Bhattacharya and Roy, 2008), along with heterotypic protein-RNA interactions such as EHDV-2-derived NS2 recognizing the two EHDV-6 RNAs during genome assembly (Lymperopoulos et al., 2006; Roy, 2008), apparently did not compromise the ability of EHDV-6 (Indiana) to survive in nature. However, whether these chimeric protein–protein, protein–RNA, or possibly even RNA–RNA interactions affect the fitness level (e.g., replication, assembly, and transmissibility) of EHDV-6 (Indiana) relative to non-heterotypic viruses is unknown and requires further study. Additionally, whether similarly structured reassortant viruses derived from sympatric endemic serotypes (e.g., VP7 of EHDV-1 and VP2/VP5 of EHDV-2) may also circulate in the United States has not been investigated extensively (Mecham et al., 2003).

Analysis of partial VP2, VP5, and VP7 sequences from 14 EHDV-6 (Indiana) isolates (see Table 3 for list) recovered in the United States

<table>
<thead>
<tr>
<th>Segment*</th>
<th>Gene</th>
<th>5′ UTR (nt)b</th>
<th>ORF (nt)</th>
<th>3′ UTR (nt)</th>
<th>Length (nt)</th>
<th>Length (aa)</th>
<th>MW (kDa)</th>
<th>Parental virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1L)</td>
<td>VP1</td>
<td>11</td>
<td>3909</td>
<td>22</td>
<td>3942</td>
<td>1302</td>
<td>149.7</td>
<td>EHDV-2 (Alberta)</td>
</tr>
<tr>
<td>2 (1L)</td>
<td>VP2</td>
<td>16</td>
<td>2919</td>
<td>36</td>
<td>2971</td>
<td>972</td>
<td>112.6</td>
<td>EHDV-6 (CSIRO 753)</td>
</tr>
<tr>
<td>3 (1L)</td>
<td>VP3</td>
<td>17</td>
<td>2700</td>
<td>51</td>
<td>2768</td>
<td>899</td>
<td>103.1</td>
<td>EHDV-2 (Alberta)</td>
</tr>
<tr>
<td>4 (M4)</td>
<td>VP4</td>
<td>8</td>
<td>1935</td>
<td>40</td>
<td>1983</td>
<td>644</td>
<td>76.0</td>
<td>EHDV-2 (Alberta)</td>
</tr>
<tr>
<td>5 (M5)</td>
<td>NS1</td>
<td>32</td>
<td>1656</td>
<td>115</td>
<td>1803</td>
<td>531</td>
<td>64.6</td>
<td>EHDV-2 (Alberta)</td>
</tr>
<tr>
<td>6 (M6)</td>
<td>VP5</td>
<td>26</td>
<td>1384</td>
<td>30</td>
<td>1642</td>
<td>527</td>
<td>59.1</td>
<td>EHDV-6 (CSIRO 753)</td>
</tr>
<tr>
<td>7 (NS7)</td>
<td>VP7</td>
<td>17</td>
<td>1050</td>
<td>95</td>
<td>1162</td>
<td>349</td>
<td>38.1</td>
<td>EHDV-2 (Alberta)</td>
</tr>
<tr>
<td>8 (S8)</td>
<td>NS2</td>
<td>19</td>
<td>1122</td>
<td>45</td>
<td>1186</td>
<td>373</td>
<td>43.2</td>
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<tr>
<td>9 (S9)</td>
<td>VP6</td>
<td>14</td>
<td>1080</td>
<td>46</td>
<td>1140</td>
<td>359</td>
<td>39.8</td>
<td>EHDV-2 (Alberta)</td>
</tr>
<tr>
<td>10 (S10)</td>
<td>NS3</td>
<td>20</td>
<td>687</td>
<td>103</td>
<td>810</td>
<td>228</td>
<td>25.5</td>
<td>EHDV-2 (Alberta)</td>
</tr>
</tbody>
</table>

* RNA segments are designated as large (L), medium (M), or small (S) based on electrophoretic mobility.

b The position of the UTRs corresponds to the genomic RNA in positive-sense orientation.
from 2006 to 2010 indicated that they were all reassortants derived from EHDV-6 (VP2 and VP5) and EHDV-2 (VP7). For VP2 and VP5, all North American EHDV-6 isolates formed a distinct and well-supported clade with CSIRO 753, which was separate from African and Bahrainese EHDV-6, as well as other serotypes (Fig. 1). Thus, heterologous reassortment of the RNA segments encoding the outer capsid was not observed in any of the viruses isolated in the USA, further suggesting that co-segregation of both segments from one parental serotype may be a prerequisite for forming a stable virus capable of surviving and persisting in nature. Contrary to the outer capsid proteins, all EHDV-6 (Indiana) isolates clusters independently of EHDV-6 and most closely with EHDV-2 (Alberta) in the VP7 tree (Fig. 2). Interestingly, the 14 EHDV-6 (Indiana) isolates did not form a single cluster in the VP7 phylogeny, but rather were split into five separate clusters based primarily on spatial and/or temporal associations, although most of these clusters did not receive strong bootstrap support. This phylogenetic pattern is compatible with a continual reassortment of VP7 between EHDV-6 (Indiana) and EHDV-2 (Alberta) as the former lineage spreads throughout the United States, yet maintaining a stable association between the outer capsid proteins. Thus, this unique configuration of the outer capsid provides a rare perspective for the analysis of virus reassortment (i.e., in the other eight segments) as it persists over time, assuming a single introduction of the virus into the USA. However, one important consequence of the marked lack of phylogenetic resolution in VP7 among the EHDV-6 (Indiana) sequences, and specifically with their relationship to EHDV-2 (Alberta), is that determining the exact origin and evolution of EHDV-6 (Indiana) is impossible to resolve on these data alone. Finally, although a small cluster of EHDV-6 strains from South Africa, Bahrain and Reunion Island (see below) fall within the EHDV-2 (Alberta) and EHDV-6 (Indiana) strains in the main VP7 tree, they occupy a more divergent position tree in the phylogenetic analysis of the main clade of EHDV-2/6 viruses (as is also seen in the VP2 and VP3 trees), suggesting that their clustering with the North American viruses is an artifact caused by the analysis of highly divergent sequences.

Other than the initial isolation of the CSIRO 753 strain of EHDV-6 in northern Australia in 1981 (St. George et al., 1983), its discovery (as a reassortant) in the United States in 2006 was previously the only other description of this strain of EHDV-6 outside of Australia. However, in 2010, EHDV-6 was detected again in the Western Hemisphere, this time in sentinel cattle from Guadeloupe, located in the Leeward Island group of the Lesser Antilles in the Caribbean Sea. Analysis of the VP2, VP3, VP5, and VP7 genes demonstrated that this virus was not EHDV-6 (Indiana), but rather the non-reassortant parental CSIRO 753 strain. Although the Guadeloupe virus (isolate 5124) was sampled from close geographical proximity to the United States, and represented an ideal candidate to be the immediate parental virus of the outer capsid proteins of EHDV-6 (Indiana), phylogenetic analysis of both VP2 and VP5 showed that isolate 5124 and EHDV-6 (Indiana) are no more closely related to one another than to the prototype Australian virus (Fig. 1).

Although EHDV-2 (Alberta) has currently yet to be authoritatively reported outside of Canada or the United States, there is serological evidence that this serotype may be endemic in Latin America and/or the Caribbean (Gumm et al., 1984; Homan et al., 1985), such that the reassortment event could have occurred over a wide geographic area. Conversely, if EHDV-2 (Alberta) is localized to North America, this would mean that CSIRO 753 underwent reassortment with endemic EHDV-2 after its introduction into the United States and is either still circulating clandestinely or (as might be suggested by the repeated isolation of only the reassortant EHDV-6) subsequently went extinct in the United States. However, until more extensive
Fig. 1. Maximum likelihood (amino acid) phylogenetic trees of the EHDV outer capsid proteins, comprising (a) 29 VP2 sequences and (b) 28 VP5 sequences. All EHDV-6 (Indiana) sequences are highlighted in bold italic. All branch lengths are drawn to a scale of amino acid substitutions per site, and values of the approximate likelihood ratio test are shown for key nodes. The tree is mid-pointed rooted for purposes of clarity only. Viruses are listed as serotype/strain/isolate/location/year.
oribivirus surveillance is conducted (i.e., in the Caribbean region and Central/South America), it is likely that the evolutionary pathway of the emergence of EHDV-6 (Indiana) in the United States will remain obscure. The time frame of when EHDV-6 (Indiana) or the parental EHDV-6 (CSIRO 753) entered into the United States is also unknown. However, because the virus has been recovered for consecutive years (i.e., 2006–2010), coupled with the absence of its detection prior to 2006 despite active ongoing orbivirus surveillance in wild ruminants, suggests that its introduction may have occurred relatively recently. Although putative mechanisms of entry of the virus into the United States could entail the introduction of exotic game ruminants for ranching/breeding facilities, illegal use of an unlicensed foreign-derived vaccine, or inadvertent contamination of therapeutic or vaccine formulations with the virus (Rabenau et al., 1993), the detection of the parental CSIRO 753 strain of EHDV-6 in Guadeloupe may suggest that the virus entered into the United States through a wind-borne introduction of Culicoides, or via the movement of viremic cattle, from Central/South America.

Fig. 2. Maximum likelihood (nucleotide) phylogenetic tree of 76 core surface protein (VP7) sequences of EHDV. All EHDV-6 (Indiana) sequences are highlighted in bold italic. The time frame of when EHDV-6 (Indiana) or the parental EHDV-6 (2006–2010), coupled with the absence of its detection prior to 2006 despite active ongoing orbivirus surveillance in wild ruminants, suggests that its introduction may have occurred relatively recently. Although putative mechanisms of entry of the virus into the United States could entail the introduction of exotic game ruminants for ranching/breeding facilities, illegal use of an unlicensed foreign-derived vaccine, or inadvertent contamination of therapeutic or vaccine formulations with the virus (Rabenau et al., 1993), the detection of the parental CSIRO 753 strain of EHDV-6 in Guadeloupe may suggest that the virus entered into the United States through a wind-borne introduction of Culicoides, or via the movement of viremic cattle, from Central/South America.

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Fig. 3. 5′ and 3′ UTRs of each of the 10 segments of the genome of EHDV-6 (Indiana), with segments 2 and 6 (highlighted in bold italic) being derived from the exotic parental EHDV-6 (CSIRO 753), while the remaining eight segments being obtained from endemic EHDV-2 (Alberta). Start and stop codons for each ORF are highlighted.
In the northwest African and Turkish EHDV-6 outbreaks of 2006–2007, the virus isolated from clinically ill cattle was demonstrated to be closely related genetically to strain 318 reported from Bahrain (BAR1983/01), rather than the Australian prototype (http://www.expasy.ch/tools/protparam.html) (Gasteiger et al., 2003). Genomic and phylogenetic analysis of the 1996 South African EHDV-6 isolate in this study (M44-96) also demonstrated that it was closely related to EHDV-6 from Bahrain (Table 2; Figs. 1 and 2). Similar to the Reunion Island, Moroccan, Tunisian, Algerian, and Turkish cases, the South African virus was also associated with cattle morbidity (Barnard et al., 1998). However, previous isolations of strain 318 from Sudan have not been associated with overt clinical signs and represented fortuitous isolates from sentinel calves (Mohammed and Mellor, 1990). Although the reasons for the different pathogenities of strain 318 are speculative, introduction of the virus into non-endemic regions that lack any herd immunity (e.g., Turkey), increased vector abundance/virus transmission during aberrant climatic conditions in endemic areas (e.g., South Africa) (Barnard et al., 1998), endemic circulation coupled with a lack of a comprehensive disease surveillance infrastructure (e.g., Sudan) or other putative factors (e.g., host genotype) may possibly contribute to the reported discrepancies in cattle virulence. Nevertheless, the isolation of strain 318 in lower sub-Saharan Africa suggests that this topotype of EHDV-6 may be widely distributed throughout the African region (e.g., Sudan, Morocco, Tunisia, Algeria, South Africa, Reunion Island), in addition to the Arabian Peninsula and the Middle East (e.g., Bahrain, Oman, Turkey) (Al-Busaidy and Mellor, 1991).

In contrast to strain 318, CSIRO 753 has not been incriminated in any outbreaks in cattle. Previously, experimental infections with CSIRO 753 demonstrated that although cattle became viremic, clinical disease was not observed (St. George et al., 1983; Uren, 1986). Similarly, the detection of CSIRO 753 in Guadeloupe was a result of standard sentinel testing and did not involve the recognition of any clinical signs in the infected cattle. In parallel with these observations, EHDV-6 (Indiana) has yet to be associated with clinical disease in cattle in the United States, although it is pathogenic to white-tailed deer as demonstrated in experimental trials (unpublished results), in addition to its field isolation from deer exhibiting severe hemorrhagic disease. Whether EHDV-6 (Indiana) may become a concern to the cattle industry in the United States is currently unknown. Nevertheless, as acknowledged by Gibbs et al. (2008), the economic impacts entailed by the introduction of BTV into Europe and EHDV into the Mediterranean region should act as a portent that the recent detection of multiple exotic serotypes of BTV and EHDV in the United States could have the potential for similar consequences. As such, the proactive characterization of these exotic orbiviruses will likely provide a preliminary assessment of the potential risk that they may pose to domestic and wild ruminant populations in the United States.

### Materials and methods

All viruses were isolated, propagated, and identified as previously described (Allison et al., 2010). For genomic analysis, cDNA was amplified from dsRNA using an oligo-ligation, sequence-independent protocol followed by pyrophosphate-based CS20/FLX sequencing as detailed previously (Potgieter et al., 2009). The complete genomes of two EHDV-6 isolates, one from South Africa (M44-96) and one from the United States (CC 304-06), as well as an EHDV-2 isolate from the United States (CC 126-00), were sequenced (Table 3). Partial sequences (numbering based on the prototype CC 304-06) of VP2 (582nt; nts 412–993), VP3 (463nt; nts 448–910), and VP7 (537nt; nts 341–877) genes of other EHDV-6 isolates recovered in the United States from 2006 to 2010 were analyzed to determine their reassortant status. Additionally, partial sequences of VP3, VP5, and VP7 from a 2010 EHDV-6 isolate (5124) from Guadeloupe (Table 3), along with existing VP2 sequence from 5124 (GenBank ID HQ848380), were analyzed to determine its relationship to EHDV-6 (Indiana) and other EHDV-6 isolates. Primers are available upon request. The nucleotide and protein pairwise identities of EHDV-6 (Indiana) to other EHDV-6 and EHDV-2 isolates were determined by CLUSTALW alignments using the EMBL-EBI server (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Chenna et al., 2003). The predicted molecular weights of the EHDV proteins were determined using the ProtParam tool on the ExPaSy server (http://www.expasy.ch/tools/protparam.html) (Gasteiger et al., 2003).

We undertook a variety of phylogenetic analyses to determine the evolutionary relationships of the EHDV-6 (Indiana) isolates compared to other representative EHDV serotypes. For the larger VP7 data set, we first estimated a maximum likelihood (ML) tree using 76 sequences, 1163 nt in length, and incorporating taxa from EHDV-1, 2, 4, 5, 6, 7 and 8. This analysis utilized the ML method available in PAUP* (Swofford, 2003) and employed TBR branch-swapping. The best-fit model of nucleotide substitution (GTR + I + Γ) was determined using MODELTEST (Posada and Crandall, 1998; parameters available on request). Because of the substantial genetic diversity among these sequences, we performed an additional ML phylogenetic analysis using the procedure described above on a subset of 57 EHDV-2 (Alberta) and EHDV-6 (Indiana) isolates that provided more resolution on the origins of EHDV-6 (Indiana). For both of these data sets, the support for individual nodes on the tree was estimated using a bootstrap resampling procedure in PAUP*, utilizing 1000

### Table 3

List of EHDV isolates and genes analyzed in the study.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Serotype</th>
<th>Strain</th>
<th>Date</th>
<th>Host</th>
<th>Location</th>
<th>Sequence GenBank ID</th>
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<tbody>
<tr>
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<td>EHDV-6</td>
<td>Indiana</td>
<td>2006</td>
<td>O. virginianus</td>
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<td>Genomic Unused</td>
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<td>VP2, VP5, VP7</td>
</tr>
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<td>06-29537</td>
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<td>VP2, VP5, VP7</td>
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<td>O. virginianus</td>
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America or the Caribbean (e.g., Hoar et al., 2004; Sellers and Maarouf, 1989).
replicate neighbor-joining trees and the ML substitution models inferred for each data set. In the case of the smaller VP2 (29 taxa) and VP5 (28 taxa) data sets, the sequences in question were so divergent that our phylogenetic analysis utilized amino acid rather than nucleotide sequences (alignment lengths of 1017 and 547 amino acid residues for VP2 and VP5, respectively). Accordingly, both VP2 and VP5 phylogenies were inferred using the ML method available in PhyML (Goubook and Gascuel, 2003). In both cases, we utilized the WAG + G model of amino acid substitution and employed SPR branch-swapping, and nodal support was assessed using the approximate likelihood ratio test available in PhyML.

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