Genetic characterization of epizootic hemorrhagic disease virus strains isolated from cattle in Israel

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Epizootic hemorrhagic disease virus (EHDV), a member of the genus Orbivirus not reported previously in Israel, was isolated from Israeli cattle during a ‘bluetongue-like’ disease outbreak in 2006. To ascertain the origin of this new virus, three isolates from the outbreak were fully sequenced and compared with available sequences. Whilst the L2 gene segment clustered with the Australian EHDV serotype 7 (EHDV-7) reference strain, most of the other segments were clustered with EHDV isolates of African/Middle East origin, specifically Bahrain, Nigeria and South Africa. The M6 gene had genetic relatedness to the Australian/Asian strains, but with the limited data available the significance of this relationship is unclear. Only one EHDV-7 L2 sequence was available, and as this gene encodes the serotype-specific epitope, the relationship of these EHDV-7 L2 genes to an Australian EHDV-7 reflects the serotype association, not necessarily the origin. The genetic data indicated that the strains affecting Israel in 2006 may have been related to similar outbreaks that occurred in North Africa in the same year. This finding also supports the hypothesis that EHDV entered Israel during 2006 and was not present there before this outbreak.

INTRODUCTION

In numerous parts of the world, there have been several recent reports of disease outbreaks associated with insect-transmitted viruses, or arboviruses (Doceul et al., 2013; MacLachlan & Guthrie, 2010; Weaver & Reisen, 2010). The 2006 outbreak of a ‘bluetongue-like’ disease in Israeli dairy herds is an example that also had significant economic consequences on the dairy industry (Kedmi et al., 2010b; Yadin et al., 2008). This outbreak was caused by epizootic hemorrhagic disease virus (EHDV), which had not been reported previously in Israel (Kedmi et al., 2010a). Bluetongue virus (BTV) and EHDV are members of the genus Orbivirus in the family Reoviridae, and thus are similar but genetically distinct viruses (Savini et al., 2011). The genomes of these viruses consist of 10 segments of dsRNA that encode 11–12 proteins (Belhouchet et al., 2011; Ratinier et al., 2011). Seven of these proteins are virus structural proteins (VPs) and four are non-structural proteins (NSs). The outer-capsid protein, VP2 (L2 gene segment), is the most variable gene and contains the neutralizing and serotype determination epitopes (Maan et al., 2007). The second outer-capsid protein, VP5 (M6), is also genetically variable and can play some role in serotype determination (Mertens et al., 1989; Singh et al., 2004). Although BTV and EHDV are genetically similar, there are no reports of gene reassortment and thus they are distinct virus serogroups. The complete sequences for the prototype strains of EHDV have been determined (Anthony et al., 2009c). Previously, there were thought to be eight EHDV serotypes, but this study revealed that type 3 was actually type 1 and thus there are now seven serotypes 1, 2 and 4–8 (Anthony et al., 2009c). The sequences of the inner core proteins, VP3 (L3) and VP7 (S7), and the proteins contained inside the core, VP1 (L1), VP4 (M4) and VP6 (S9), as well as the non-structural proteins NS1 (M5), NS2 (S8) and NS3/3a (S10) for the prototype strains have also been determined (Anthony et al., 2009a, c). A fourth non-structural protein has been identified for BTV but not yet demonstrated for EHDV (Belhouchet et al., 2011; Ratinier et al., 2011). Although there have been several genetic characterization studies of the EHDV strains from the USA (Allison et al., 2012; Anbalagan et al., 2014; Anbalagan & Hause, 2014; Cheney et al., 1995, 1996;
Murphy et al., 2006), data are limited outside the USA for EHDV strains (Nara Pereira et al., 2000a, b; Ohashi et al., 2002). The only sequence information for EHDV serotype 7 (EHDV-7) is the prototype strain (Anthony et al., 2009a, b, c) and limited previously published sequence data for the Israel 2006 EHDV-7 strain (Maan et al., 2010b).

Prior to the 2006 disease outbreaks in northern Africa and the Middle East (Savini et al., 2011), clinical disease in cattle had primarily been associated with the EHDV-2 Ibaraki strains (Ohashi et al., 2002). It is possible that the observed clinical disease associated with an introduced EHDV-7 in Israel (Kedmi et al., 2010b) was a similar situation to the introduction of BTV serotype 8 (BTV-8) into northern Europe. The introduced BTV-8 caused clinical disease in cattle (Saegerman et al., 2008), but whether this was a result of introduction into a naïve cattle population or genetic changes in the virus is unclear. Sequence analysis of BTV-8 introduced into northern Europe revealed that the virus was probably of sub-Saharan African origin (Maan et al., 2008). An isolate of EHDV-7 from the 2006 outbreak in Israel was shown to be infectious but not to cause clinical disease in cattle (Eschbaumer et al., 2012). A similar study demonstrated that an EHDV-7 Israel isolate caused clinical disease in US white-tailed deer (Ruder et al., 2012a). This discrepancy is not surprising because in general white-tailed deer are more susceptible to EHDV than cattle, especially under experimental infection conditions. It was also demonstrated that the primary US vector for BTV, Culicoides sonorensis, is capable of becoming infected and transmitting EHDV-7 (Ruder et al., 2012b). Sequence analyses of a virus strain isolated from the EHDV-7 2006 outbreak have been limited to six of the 10 gene segments (Maan et al., 2010a; Yadin et al., 2008). In this study, a complete genome sequence analysis of three EHDV-7 isolates from the 2006 disease outbreak in Israeli dairy cattle was performed to determine the potential variation among strains and the origin of this introduced virus.

RESULTS

Complete genomes of the three Israeli strains of EHDV-7 were obtained and have been deposited in GenBank (Table 1). The L1 genes from all three Israeli strains (ISR) were 100 % identical to each other and the previously published Israeli 2006 isolate (GenBank accession no. JQ070177), Comparison of the L1 gene of these isolates with the only other previously published Australian prototype serotype 7 isolated in 1981 (GenBank accession no. AM745047) revealed 78 % nucleotide identity. The overall range of sequence identities for all available full-length EHDV L1 sequences was 78.0–94.9 % (Table 2, Fig. 1a). Comparisons in this study used all sequences available, but only representative sequences from the US strains are depicted in the figures. The L1 gene from the Israel isolates was closest to the prototype EHDV-6 from Bahrain isolated in 1983 (GenBank accession no. AM745067) and EHDV-1 isolated in Nigeria in 1967 (GenBank accession no. AM745007) (Table 3). The ISR L1 genes had greater identity to African, Middle Eastern and North American strains than those from Australia and Asia (Table 2). The amino acid identity of the predicted protein sequence for the Israeli serotype 7 VP1 proteins compared with other known VP1 sequences ranged from 90.6 to 99.1 %. The predicted protein sequences had levels of relatedness similar to the nucleic acid sequences.

The EHDV L2 genes from all three Israeli strains were 99.8–99.9 % identical to each other and the previously published 2006 Israeli isolate (GenBank accession no. HM156731) and 75.0 % identical with prototype EHDV-7 (GenBank accession no. AM745048) (Table 3). The overall range of identity for all available full-length EHDV L2 sequences was 43.9–75.0 % (Table 2, Fig. 2a). The Israeli EHDV-7 predicted protein sequences had an amino acid identity range of 99.8–99.9 % with 78.9–79.2 % identity to the predicted VP2 amino acid sequence for the prototype EHDV-7. The amino acid identities of all the EHDV predicted protein sequences compared with the Israeli EHDV-7 VP2 protein ranged from 30.4 to 79.3 %.

The L3 genes from all three Israeli EHDV strains were 99.9–100 % identical to each other and the previously published Israeli isolate in 2006 (GenBank accession no. JQ070179), and 80.4 % identical with prototype EHDV-7 (GenBank accession no. AM745049). The overall range of nucleotide identity for all available full-length L3 sequences was 79.4–96.8 % (Table 2, Fig. 3a). The closest relationship (96.7-96.8 % identity) was to the EHDV-6 strain M44/96, a South African isolate from 1996 (GenBank accession no. HM636909), followed by 96.5 % identity to the 1983 Bahrain prototype EHDV-6 strain BAR1983 (GenBank accession no. AM745069) (Table 3). The Israeli EHDV-7

**Table 1. GenBank accession numbers for the sequences determined in this study**

<table>
<thead>
<tr>
<th>Virus strain name</th>
<th>Origin</th>
<th>Year</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHD7 ISR 2006-02</td>
<td>Israel</td>
<td>2006</td>
<td>KM391725, KM391745, KM391732, KM391743, KM391727, KM391737, KM391751, KM391753, KM391736, KM391739</td>
</tr>
<tr>
<td>EHD7 ISR 2006-04</td>
<td>Israel</td>
<td>2006</td>
<td>KM391752, KM391735, KM391731, KM391733, KM391729, KM391749, KM391728, KM391726, KM391738, KM391750</td>
</tr>
<tr>
<td>EHD7 ISR 2006-06</td>
<td>Israel</td>
<td>2006</td>
<td>KM391744, KM391730, KM391724, KM391741, KM391746, KM391740, KM391742, KM391734, KM391747, KM391748</td>
</tr>
</tbody>
</table>
predicted protein sequences were 99.9–100 % identical, and shared 96.4 % amino acid identity with the predicted VP3 sequence of the prototype EHDV-7 strain. The identity of the predicted protein sequence for the Israeli EHDV EHDV-7 VP3 protein compared with other EHDV VP3 proteins ranged from 96.1 to 99.9 %. The predicted Israeli EHDV-7 VP3 protein sequences were nearly identical to the Republic of South Africa M44/96 (99.9 %), Bahrain BAH1983 (99.8 %), Nigerian 1967 EHDV-1 (99.7 %) and Nigerian 1968 EHDV-4 (99.7 %) isolates. The next closest related were USA strains (serotypes 1, 2 and 6) (Allison et al., 2012; Anthony et al., 2009a; Cheney et al., 1995; Le Blois et al., 1991; Wilson, 1991) with an amino acid identity range of 98.7–99.1 %. The amino acid identity of the Australian and Japanese strains (Anthony et al., 2009a; Eschbaumer et al., 2012; Iwata et al., 1992; Ohashi et al., 2002) ranged from 96 to 96.8 %.

The M4 genes from all three strains were 100 % identical to each other and to the previously published Israeli isolate in 2006 (GenBank accession no. JQ070180) and 75.3 % identical with the prototype EHDV-7 strain (GenBank accession no. AM745050). The overall range of identity for all available full-length M4 sequences was 74.8–95.8 % (Table 2, Fig. 1b). The sequences of the Israeli EHDV-7 predicted VP4 proteins were 100 % identical and 85.7 % identical with the published prototype EHDV-7 sequence. Overall the Israeli EHDV-7 VP4 sequences were 85.1–98.4 % identical to the sequences of other published EHDV strains. The identity of the predicted protein sequence for the Israeli EHDV-7 VP4 protein to the Australian prototype strain was 85.1 %. The closest relatives were the African strains with an identity range of 97.8–99.5 %. The North American strains were 97.1–98.4 % identical to the Israeli strains.

The M5 genes from all three strains were 99.7–99.8 % identical to each other and the previously published Israeli isolate in 2006 (GenBank accession no. JQ070181) and 78.3 % identical with the prototype EHDV-7 strain (GenBank accession no. AM745051). The overall range of identity of Israeli EHDV-7 sequences to all available full-length M5 sequences was 78.1–98.4 % (Table 2, Fig. 4a). The predicted NS1 protein sequences were 99.3–100 % identical among the Israeli EHDV-7 sequences. The range of identity compared with other published predicted NS1 amino acid sequences was 90.6–99.5 %. The identity of the predicted protein sequence for the Israeli EHDV-7 NS1 protein compared with the Australian prototype strain was 91.7 %. The closest relatives were the African strains with an identity range of 97.8–99.5 %. The North American strains were 97.1–98.4 % identical to the Israeli strains. The Australian and Japanese strains NS1 predicted protein sequences were 91.1–91.3 and 90.9 % identical, respectively.

The M6 genes from all three strains and the previously published Israeli isolate (GenBank accession no. JQ070182) were 99.9–100 % identical to each other and 82.1–82.4 % identical with the prototype EHDV-7 (GenBank accession no. AM745052). The overall range of identity of Israel
EHDV-7 sequences compared with all available full-length M6 sequences was 60.7–81.9 % (Table 2, Fig. 2b). The closest M6 sequences to the Israeli EHDV-7 isolates were the Australian prototype and the Japanese Ibaraki strains (EHDV-2). The predicted VP5 protein sequences were 99.6–100 % identical among the Israel EHDV-7 strains. The Israeli EHDV-7 strains were 96.6–96.8 % identical to the published prototype strain of EHDV-7. The closest non-EHDV-7 strains were the Australian and Japanese EHDV-2 strains with an identity range of 95.8–97.3 %. The North American strains VP5 sequences were 93.4–93.9 % identical to the Israeli strains. The remaining VP5 predicted protein sequences were 61.4–79.3 % identical.

The S7 genes from all three Israeli EHDV-7 strains were 100 % identical to each other and 80.6 % identical with the prototype EHDV-7 (GenBank accession no. AM745053). The overall range of identity of Israel EHDV-7 sequences compared with all remaining available full-length S7 sequences was 79–82.7 % (Table 2, Fig. 3b). The closest EHDV strains were the Nigerian and Japanese EHDV-2

**Fig. 1.** Neighbour-joining consensus phylogenetic trees using the Jukes–Cantor distance matrix of 1000 bootstrapped iterations for representative EHDV genes encoding inner virus structural proteins. (a) L1 (VP1); (b) M4 (VP4); (c) S9 (VP6). GenBank accession numbers are given in parentheses. Numbers at nodes indicate the percentage of bootstrap support. Bars, nucleotide substitutions per site.
strains both with a nucleotide identity of 82.7% (Table 3). The Israeli EHDV-7 predicted VP7 protein sequences were 97.4% identical to the predicted EHDV prototype EHDV-7 VP7 protein sequence. The closest non-EHDV-7 strains were the Australian and Japanese EHDV-2 strains with an amino acid identity range of 97.1–97.7%. The next closest related VP7 sequences were African source EHDV-6 strains (GenBank accession nos AM745073 and HM636913) at 96.8% predicted amino acid identity. The North American strains VP7 protein sequences were 93.4–96.6% identical to the Israeli strains. The remaining VP7 predicted protein sequences were 90.3–94.3% identical to the Israeli EHDV-7.

The S8 genes from all three strains were 100% identical to each other and 70.2% identical with the prototype EHDV-7 (GenBank accession no. AM745054). The closest related sequences were an EHDV-6 isolate from Bahrain (GenBank accession no. AM745074) with 95.4% nucleotide identity and an EHDV-1 from Nigeria (GenBank accession no. AM745014) with 85.7% identity (Table 3). The overall range of identity compared with Israeli EHDV-7 sequences of the remaining published full-length S8 sequences was 69.2–86.3% (Table 2, Fig. 4). The Israeli EHDV-7 predicted NS2 protein sequences were 92.3% identical to the predicted prototype EHDV-7 NS2 protein sequence. The closest related non-EHDV-7 strains were the Bahrain (EHDV-6) and Nigerian (EHDV-1) strains with amino acid identities of 96 and 88.5%, respectively. The remaining NS2 predicted protein sequences were 70.7–87.1% identical to the Israeli EHDV-7.

The S9 genes from all three strains were 100% identical to each other and 78.1% identical to the prototype EHDV-7 (GenBank accession no. AM745056). The closest related sequences were an EHDV-6 isolate from the Republic of South Africa (GenBank accession no. HM636915) with 98.1% identity, a Nigerian EHDV-4 strain (GenBank accession no. AM745025) with 97.8–98.0% identity and an EHDV-6 strain from Bahrain (GenBank accession no. AM745075) with 96.7–97.2% identity (Table 3). The overall range of identity compared with Israeli EHDV-7 sequences of the remaining published full-length S9 sequences was 54.8–98.1% (Table 2, Fig. 1c). The Israeli EHDV-7 predicted VP6 protein sequences were 99.4–99.7% identical to the predicted prototype EHDV-7 VP6 protein sequence. The closest related non-EHDV-7 strains were the Republic of South Africa (EHDV-6), Bahrain (EHDV-6) and Nigerian (EHDV-4) strains with amino acid identity ranges of 96.7–96.9, 95.5–95.8 and 96.7–97.2%, respectively. The remaining NS2 predicted protein sequences were 70.7–87.1% identical to the Israeli EHDV-7.

The S10 genes from all three strains were 100% identical to each other and 78.1% identical to the prototype EHDV-7 (GenBank accession no. AM745056). The closest related

<table>
<thead>
<tr>
<th>Genome segment (gene)</th>
<th>No. of strains aligned</th>
<th>EHD strains most closely related to ISR-EHD7s</th>
<th>GenBank accession no.</th>
<th>Gene identity</th>
<th>Predicted protein identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (VP1)</td>
<td>21</td>
<td>EHD6-RSA1996/M44-96</td>
<td>HM636907</td>
<td>94.9</td>
<td>99.1</td>
</tr>
<tr>
<td></td>
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<td>AM745067</td>
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<td>98.7</td>
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<tr>
<td></td>
<td></td>
<td>EHD1-NIG1986/lba22619</td>
<td>AM745007</td>
<td>93.5</td>
<td>98.8</td>
</tr>
<tr>
<td>2 (VP2)</td>
<td>45</td>
<td>EHD7-AUS1981/CSIRO 775</td>
<td>AM745048</td>
<td>75.0</td>
<td>79.7–79.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EHD2-JPN/KSB-14-E-97</td>
<td>AB078628</td>
<td>74.5–74.6</td>
<td>79.1–79.3</td>
</tr>
<tr>
<td>3 (VP3)</td>
<td>31</td>
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<td>HM636909</td>
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<td>99.8–99.9</td>
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<td>AM745069</td>
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<td>99.7–99.8</td>
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<td>98.4</td>
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</tr>
<tr>
<td></td>
<td></td>
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<td>98.9–99.3</td>
</tr>
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<td>6 (VP5)</td>
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<td>AM745048</td>
<td>82.4</td>
<td>96.8</td>
</tr>
<tr>
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<td>AM745083</td>
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<td>96.6–96.7</td>
<td>95.5–95.8</td>
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<td>100</td>
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<td>AM745076</td>
<td>95.2</td>
<td>100</td>
</tr>
</tbody>
</table>

Note that some of the strains were sequenced by multiple laboratories and were included in the total number of strains aligned.

Table 3. Summary of EHDV strains sharing the highest percentage sequence identity with the Israeli EHDV-7 strains
sequences were EHDV-6 strains from South Africa (GenBank accession no. HM636916), Tunisia (GenBank accession no. KC986826) and Bahrain (GenBank accession no. AM745076) with 95.6, 95.5 and 95.2 % nucleotide identity, respectively (Table 3). The overall range of identity to the Israeli EHDV-7 sequences of the remaining published full-length S10 sequences was 76.2–89.4 % (Table 2, Fig. 4c). The Israeli EHDV-7 predicted NS3 protein sequences were 100 % identical to each other and 89.9 % identical to the predicted NS3 protein sequence of the prototype strain of EHDV-7. The EHDV-6 strains from Bahrain and Nigeria were also 100 % identical to the predicted Israel EHDV-7 NS3 protein sequence. The remaining NS3 predicted protein sequences were 86.8–97.4 % identical to the Israeli EHDV-7 isolates.

DISCUSSION

The strains circulating at the time of the 2006 EHDV-7 outbreak appear to be very genetically similar. Phylogenetic analysis of the genes encoding the inner structural proteins VP1, VP4 and VP6 was most similar to strains of African origin (Fig. 1). These genes encode part of the virus replication machinery (Anthony et al., 2009a) and are generally conserved among these viruses. The genes encoding the outer-capsid proteins (VP2 and VP5) were the most variable Orbivirus genes, which is consistent with what was found with Israeli strains. The L2 gene (VP2) was only 75 % identical to the prototype EHDV-7 Australian strain and just slightly less identical on the nucleic acid level to EHDV-2 from Japan. Similarly, M6 (VP5) was closely related to Australian and Asian origin viruses but formed a completely separate clade (Fig. 2). The data are consistent with the EHDV-7 ISR strains being a result of reassortment of Australia/Asian origin viruses. The phylogenetic relationship of L2 has been shown previously to correlate with serotype first and then geographical origin (Cheney et al., 1996). It is clear that the EHDV-2 and EHDV-7 ISR strains have some degree of similarity in both the L2 (VP2) and M6 (VP5) genes. Also, there was a noted separation of the EHDV-2 genes from North America versus those of Australian/Asian origin. The relationship noted here may also represent similar relationship between EHDV-7 from two separate geographical origins. There was insufficient data available to rule out either hypothesis.

The phylogenetic analysis of the inner core proteins again suggests a relationship with African origin (Fig. 3). The L3 (VP3) protein has been used to identify geographical genetic types (topotypes) for the related BTVs (Pritchard et al., 1995, 2004). As the VP7 has been identified as an attachment protein for the insect vector (Xu et al., 1997), it was hypothesized that the S7 gene would also demonstrate topotypes. Whilst phylogenetic studies of the S7 gene from BTVs did indicate tendencies, they have not demonstrated a clear separation of genetic topotypes (Bonneau et al., 2000; Wilson et al., 2000). The S7 (VP7) gene was shown to be highly conserved among geographically distinct North American EHDV strains (Anbalagan et al., 2014; Mecham et al., 2003). This gene was also highly conserved among the Israeli strains and formed an isolated clade but by percentage identity was most closely related to the Australian/Asian strains. Interestingly, the Israeli EHDV-7 S7 gene sequences seemed to form a clade in between those of Australian/Asian and American/African origin (Fig. 3b).

The non-structural genes were all highly conserved, as has been reported previously (Jensen et al., 1994; Jensen & Wilson, 1995; Murphy et al., 2005; Wilson, 1994a, b). The sequences of the non-structural genes of the Israeli EHDV-7 strains were nearly identical. The phylogenetic analysis again suggested that these genes were of African origin.

The strains affecting Israel in 2006 may have been related to similar outbreaks that occurred in North Africa during the same year. The genetic data suggest an African and Mediterranean origin, which supports this hypothesis. Serological evidence gathered during the outbreak investigation in 2006 suggests that EHDV was not widely abundant in Israel prior to this epidemic (Kedmi et al., 2011). Whilst the route of virus introduction into Israel remains unknown, outbreak spread within the country is probably related to wind-borne dispersal of infected Culicoides, the known vector of this virus (Kedmi et al., 2010a, b). The recent reports of EHDV activity including the emergence of a new serotype of EHDV in Reunion Island (Cêtre-Sossah et al., 2014) and the outbreak in Israel (Kedmi et al., 2010a, 2011) indicate that more studies are needed to understand the role of EHDV in animal health.

METHODS

**Virus and RNA purification.** The three EHDV-7 strains were originally isolated at the Kimron Veterinary Institute during the 2006 outbreak and were subsequently identified as EHDV-7 at the Institute for Animal Health, Pirbright Laboratory (Pirbright, Surrey, UK). The ISR 2006/02 strain was isolated by one pass in an embryonated chicken egg and then one pass in Culicoides KC cells, and was then passed five times in baby hamster kidney (BHK) cells prior to our receiving them. ISR 2006/04 was isolated with one pass in KC cells and then five passes in BHK cells prior to our receiving them. ISR 2006/06 was isolated by one pass in embryonated chicken egg (ECE) and one KC passage and then passed five times in BHK cells prior to our receiving them. All were isolated from the Jordan Valley in Israel where the outbreak occurred. In order to propagate virus for RNA extraction, BHK-21 cells at 80–100 % confluency were infected at...
<1 m.o.i. Infected cells were scraped from roller bottles at 95% cytopathic effect and pelleted by centrifugation, and the RNA purified from the cell pellets using Trizol LS as described by the manufacturer (LifeTechnologies). The dsRNA was further purified as described previously (Wilson, 1990) and the purity assessed by agarose gel electrophoresis and UV absorbance.

**Whole-genome amplification and sequencing.** Whole-genome amplification was done essentially as described previously (Potgieter et al., 2009). Briefly, the restriction sites were removed from the 'anchor primer', PC3-T7restrict (5′-p-GTTCAGCCTGACCACGTT-AATACGACTCACTATATTTTTATAGTGAGTCGTATTA-OH-3′) to facilitate sequencing, followed by high-performance liquid chromatography.

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Neighbour-joining consensus phylogenetic trees using the Jukes–Cantor distance model of 1000 bootstrapped iterations for the EHDV genes encoding core virus structural proteins. (a) L3 (VP3); (b) S7 (VP7). GenBank accession numbers are given in parentheses. Numbers at nodes indicate the percentage of bootstrap support. Bars, nucleotide substitutions per site.

![Fig. 4](https://example.com/fig4.png)

**Fig. 4.** Neighbour-joining consensus phylogenetic trees using the Jukes–Cantor distance model of 1000 bootstrapped iterations for the EHDV genes encoding virus non-structural proteins. (a) M5 (NS1); (b) S8 (NS2); (c) S10 (NS3/NS3a). Numbers at nodes indicate the percentage of bootstrap support. Bars, nucleotide substitutions per site.
purification. PC3-T7restrict (600 ng) was ligated to dsRNA (500 ng) in 50 mM HEPES/NaOH (pH 8.0), 18 mM MgCl₂, 0.01 % BSA, 1 mM ATP, 3 mM DTT, 10 % DMSO and 20 % PEG 6000 with 90 U of T4 RNA ligase (TaKaRa, Clontech) in a final volume of 90 μL. Ligation was performed at 37 °C for ~16 h. Ligated dsRNA was purified using a QIAquick gel extraction kit (Qiagen) according to the manufacturer’s protocol, without the gel dissolution steps and eluting in 30 μL nuclease-free water. A portion of the cleaned ligated product (8 μL) was combined with 25 pmol PC2restrict primer (5′-AGCTGTTGTCAGCGTGGAAC-3′) and 1 μl 300 mM methyl mercury hydroxide and allowed to denature for 10 min. The mixture was placed on ice and 1.5 μl 1 M β-mercaptoethanol (Sigma-Aldrich) was added to neutralize the reaction for 1 min. The cDNA was then generated using cloned avian myeloblastosis virus reverse transcriptase (LifeTechnologies), and the remaining RNA was neutralized by 0.1 M NaOH for 30 min at 65 °C and then annealed with 0.1 M Tris/HCl and 0.1 M HCl at 65 °C for 90 min. Amplification of the cDNA was performed using 25 pmol primer PC2restrict using TaKaRa Ex Taq (Clontech) as described previously (Potgieter et al., 2009). The amplification products were confirmed by agarose gel electrophoresis and purified using a QIAquick PCR purification kit (Qiagen).

Approximately 1 μg cDNA was barcoded and used to generate a sequencing library using an Ion Xpress Plus Fragment Library kit and an Ion Xpress Barcode Adapters kit following the manufacturer’s recommendations (LifeTechnologies). Fragmented libraries were quantified using an Ion Library Quantification kit following the manufacturer’s protocol. Template generation was performed on an Ion OneTouch instrument and enriched on an Ion OneTouch ES using an Ion OneTouch 200 Template kit v2 DL following the manufacturer’s recommendations (LifeTechnologies). Enriched template was sequenced with an Ion PGM sequencing 300 kit on a 314 chip following the manufacturer’s recommendations. The .sff files were imported to Geneious 6.0 (Biomatters) for contig creation. Partial contigs were assembled and BLASTed to determine reference sequences that were then used for reference assemblies. Sequence alignment was performed with CLUSTAL W and consensus phylogenetic trees reconstructed using neighbour-joining analysis. Bootstrap confidence values were generated using 1000 replicates (Biomatters).

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