

# Detection of a novel reassortant epizootic hemorrhagic disease virus (EHDV) in the USA containing RNA segments derived from both exotic (EHDV-6) and endemic (EHDV-2) serotypes

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Epizootic hemorrhagic disease virus (EHDV) is a *Culicoides*-transmitted orbivirus that infects domestic and wild ruminants and is provisionally thought to be distributed throughout Africa, North America, Australia, East Asia and the Middle East. Historically, of the seven proposed serotypes of EHDV, only EHDV-1 and EHDV-2 have been reported from North America. In 2006, EHDV isolates were recovered from moribund or dead white-tailed deer (*Odocoileus virginianus*) in Indiana and Illinois that could not be identified as either EHDV-1 or EHDV-2 by virus neutralization tests or by serotype-specific RT-PCR. Additional serological and genetic testing identified the isolates as EHDV-6, a serotype that, although originally described from Australia, has recently been recognized as an emerging pathogen of cattle in Morocco, Algeria and Turkey. In 2007 and 2008, EHDV-6 was isolated again from white-tailed deer, this time in Missouri, Kansas and Texas, suggesting that the virus is capable of overwintering and that it may become, or already is, endemic in a geographically widespread region of the USA. Genetic characterization of the virus indicates that it is a reassortant, such that the outer capsid proteins determining serotype specificity (VP2 and VP5) are derived from exotic EHDV-6, whilst the remaining structural and non-structural proteins are apparently obtained from indigenous EHDV-2 (Alberta).

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## INTRODUCTION

Epizootic hemorrhagic disease virus (EHDV) is a serogroup of the genus *Orbivirus*, family *Reoviridae*, that infects domestic and free-ranging ruminants. EHDV is arthropod-

The GenBank/EMBL/DDBJ accession numbers for the sequences found in this study are GQ387643 (NS1), GQ387644 (NS3), GQ387634 (VP1), GQ385939 (VP2), GQ387637 (VP3), GQ387638 (VP5) and GQ387641 (VP7) [all EHDV-6 (Indiana)] and GQ387635 (EHDV-1 VP1), GQ387636 (EHDV-2 VP1) and GQ387640 (EHDV-2 VP5).

A supplementary table showing the EHDV serogroup- and serotype-specific primers used is available with the online version of this paper.

borne and is maintained in nature in a ruminant–*Culicoides* biting midge cycle. According to current International Committee on the Taxonomy of Viruses (ICTV) designations, there are ten recognized serotypes/strains of EHDV: serotypes 1–8, EHDV-318 and Ibaraki virus (IBAV) (Mertens *et al.*, 2005). However, based on recent genetic and phylogenetic analyses of the outer capsid proteins responsible for serotype specificity (VP2 and VP5) for all prototype strains, the current classification of the EHDV serogroup has been proposed to be condensed into seven serotypes, with the inclusion of EHDV-3 into EHDV-1, EHDV-318 into EHDV-6, and IBAV into EHDV-2 (Anthony *et al.*, 2009).

In North America, two serotypes, EHDV-1 (New Jersey strain), first authoritatively described by Shope *et al.* (1955) during a large-scale die-off of white-tailed deer in the north-eastern USA, and EHDV-2 (Alberta strain), originally isolated in southern Alberta, Canada, in 1962 (Chalmers *et al.*, 1964) and later characterized as being serologically distinct from EHDV-1 (Barber & Jochim, 1975), are known to be endemic. Three serotypes have been recognized in Africa: EHDV-3 (proposed to be EHDV-1) and EHDV-4, which were initially isolated from *Culicoides* spp. in Nigeria in 1967 and 1968, respectively (Lee *et al.*, 1974), and EHDV-318 (proposed to be EHDV-6), which was first isolated from cattle in Bahrain in 1983 and subsequently described from sentinel calves in the Sudan (Mohammed & Mellor, 1990; Rabenau *et al.*, 1993; Anthony *et al.*, 2009). EHDV serotypes 5, 7 and 8, along with the prototype strain of EHDV-6 (CSIRO 753), were initially isolated from sentinel cattle herds in Queensland and the Northern Territory of Australia during 1977–1982 (St George *et al.*, 1983). Additionally, topotypes of EHDV-1 and EHDV-2 are present in Australia, which, although genetically divergent from analogous North American serotypes, share common neutralization epitopes (Gould & Pritchard, 1991; Weir *et al.*, 1997). IBAV, which is now recognized, as has previously been suggested, as a topotype of EHDV-2, has been associated with periodic outbreaks in cattle in East Asia since its first description during a large-scale epizootic in Japan involving 40 000 cattle in 1959 (Omori *et al.*, 1969; Bak *et al.*, 1983; Liao *et al.*, 1996; Uchinuno *et al.*, 2003; Anthony *et al.*, 2009).

Although all ruminants appear to be susceptible to EHDV infection, the clinical course of disease differs substantially depending upon the species infected. In a generalized scheme, domestic ruminants (i.e. cattle, sheep and goats) infected with EHDV tend to develop inapparent to mild infections, with the latter regressing quickly without serious complications (MacLachlan & Osburn, 2004). The exception to inapparent EHDV infection in cattle is IBAV, in which mortality rates as high as 10% have been documented during field outbreaks, and clinical disease has been reproduced experimentally in calves (Omori *et al.*, 1969). However, in recent years, the recognition of EHDV-associated morbidity and mortality in cattle from many parts of the world has increased dramatically. EHDV-7 was recently implicated in cattle outbreaks in Israel and, although mortality rates were low (<1%), morbidity rates ranged from 5–80%, with clinical signs reported in 105 different herds (Yadin *et al.*, 2008). Additionally, morbidity and mortality associated with EHDV infection in cattle was reported from Réunion Island in 2003 (serotype not disclosed) (Bréard *et al.*, 2004), Morocco and Algeria in 2006 (reported as EHDV-318/EHDV-9; reclassified as EHDV-6) (<http://www.zkea.com/promed/archive/2006-12/17017.html>; Yadin *et al.*, 2008; Anthony *et al.*, 2009) and western Turkey in 2007 (EHDV-6) (Temizel *et al.*, 2009), suggesting that the geographical distribution and/or the

recognition of clinical disease associated with EHDV infection (primarily serotype 6) is increasing.

During September and October 2006 in Indiana and Illinois, six virus isolates were recovered from moribund or dead white-tailed deer and identified by RT-PCR as EHDV using serogroup-specific primers to the NS1 gene; however, these isolates could not be neutralized with EHDV-1- or EHDV-2-specific antisera, nor could RNA be amplified with EHDV-1- or EHDV-2-specific primers directed against VP2, the gene predominantly responsible for serotype specificity. Subsequent virus neutralization tests with exotic EHDV antisera, along with RT-PCR using primers directed against the VP2 gene of exotic EHDV serotypes, identified the viruses as EHDV-6, a serotype that, prior to 2006, had formerly only been described from Australia. In 2007, EHDV-6 was detected again in the mid-western USA, this time in Missouri, suggesting that the virus may be capable of overwintering in northern temperate areas (39.97° N). In 2008, EHDV-6 was recovered from Kansas and Texas, demonstrating that the virus is geographically widespread and suggesting that it may (or already has) become endemic in the USA. Moreover, the newly identified isolates all appeared to be derived from the same reassortment event, such that the two surface antigens (VP2 and VP5) were obtained from EHDV-6, whilst multiple non-structural (NS1 and NS3) and structural (VP1, VP3 and VP7) genes were apparently derived from EHDV-2 (Alberta). As the virus has retained the serotype specificity of EHDV-6, we tentatively propose the name EHDV-6 [Indiana (strain)] for the EHDV-6/EHDV-2 reassortant.

## RESULTS

### Virus serotyping

The six 2006 isolates, identified as EHDV using serogroup-specific primers directed against the NS1 gene, were not neutralized by antisera to EHDV or bluetongue virus (BTV) serotypes currently considered indigenous to the USA (EHDV serotypes 1 and 2; BTV serotypes 2, 10, 11, 13 and 17). Isolates were then sent to the National Veterinary Services Laboratory (NVSL) for further virus neutralization testing using antisera to exotic EHDV serotypes. A significant reduction in cytopathology was observed in wells inoculated with the 2006 viruses and EHDV-6 antiserum (CSIRO 753); antisera to EHDV serotypes 3 (1), 4, 5, 7 and 8 did not neutralize the 2006 viruses. By virus neutralization, the viruses were therefore preliminarily serotyped as EHDV-6. The isolates were then sent to the Institute for Animal Health (IAH) for confirmatory testing and were screened with serotype-specific primers directed against the VP2 gene of all EHDV serotypes. RT-PCR products were amplified from RNA derived from the 2006 isolates using VP2-specific primers directed against EHDV-6 (primers not shown); no amplicons were generated using VP2 primers specific to other EHDV serotypes. Based on

virus neutralization and VP2-specific RT-PCR, the 2006 isolates were determined to contain an L2 RNA segment derived from EHDV-6.

### RT-PCR and phylogenetic analysis

RT-PCR directed against multiple RNA segments was performed on the EHDV isolates. RT-PCR products were amplified using serogroup-specific primers against NS1, NS3, VP1, VP3 and VP7 (see Supplementary Table S1, available in JGV Online). CLUSTAL alignment (VP1) or BLAST analysis (NS1, NS3, VP3 and VP7) of the RT-PCR products indicated that the viruses were very similar in nucleotide and deduced amino acid sequences to North American serotypes, particularly EHDV-2 (Fig. 1a, c; Table 1). However, serotype-specific primers (both primary and nested) directed against the VP2 gene of EHDV-1 or EHDV-2 (Supplementary Table S1) did not result in detectable amplicons when using RNA from the 2006 isolates as template. Additionally, two sets of degenerate VP5-specific primers that detected both EHDV-1 and EHDV-2 (Supplementary Table S1), did not amplify any of the isolates, suggesting that both of the outer capsid proteins (VP2 and VP5) of the reassortants were derived from EHDV-6.

Random cloning of the L2 segment of clinical case (CC) isolate 304-06 (hereafter referred to as 304-06) resulted in the generation of two overlapping clones. The contig generated by these two clones was 1014 nt, resulting in a deduced open reading frame of 338 aa. CLUSTAL alignment of the 304-06 nucleotide contig with the cognate VP2 sequences of EHDV-1 and EHDV-2 indicated identities of 29 and 30 %, respectively. Protein BLAST analysis of the translated contig revealed that it contained a limited amino acid identity to EHDV serotypes currently present in GenBank: 40 % identity to EHDV-1, 38 % identity to Australian and North American EHDV-2 and 37 % identity to Japanese EHDV-2 (IBAV). CLUSTAL alignment of the VP2 338 aa contig from 304-06 versus the cognate region of available VP2 sequences of other serotypes [EHDV-1, EHDV-2 (including North American, Australian and Japanese topotypes) and EHDV-6 (South African strain M44/96)] revealed a number of amino acid motifs ( $\geq 3$  consecutive amino acids) [<sup>1</sup>TCYDL<sup>5</sup>, <sup>156</sup>NIR<sup>158</sup>, <sup>183</sup>RAIW<sup>186</sup>, <sup>232</sup>CDR<sup>234</sup>, <sup>256</sup>YKW<sup>258</sup>, <sup>334</sup>DAY(V/I)D<sup>338</sup>] that appeared to be conserved among the serotypes.

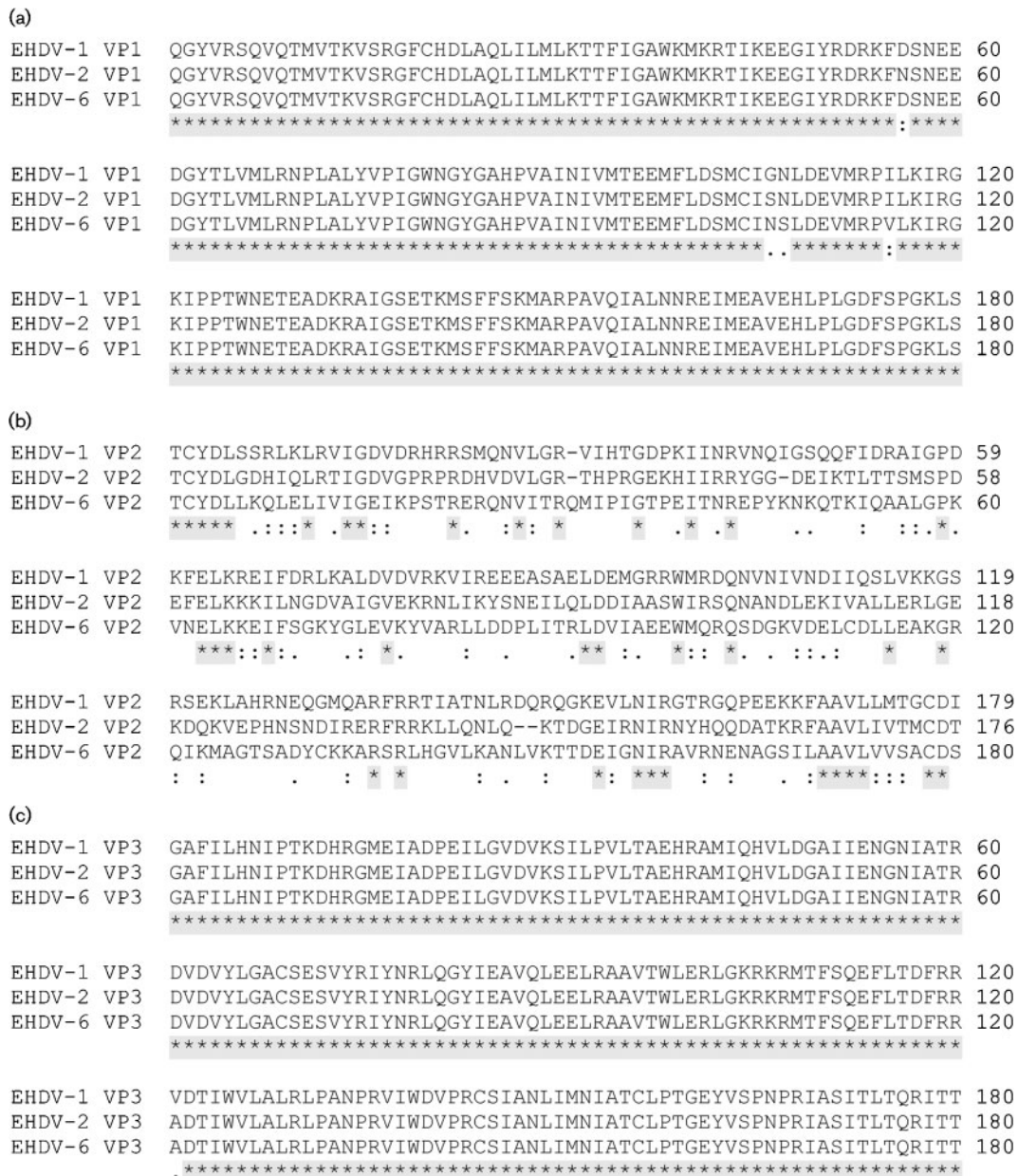
Alignment of the VP2 nucleotide and amino acid contig from 304-06 with a 1996 bovine EHDV-6 isolate from South Africa (M44/96) revealed identities of 73 and 79 %, respectively, providing comparative genetic evidence to corroborate the serological data that EHDV-6 was the parental virus of the L2 RNA segment (Table 1). Additionally, nucleotide and amino acid comparisons of the complete coding sequence of VP5 between 304-06 and M44/96 indicated identities of 80 and 94 %, respectively, confirming that both of the outer capsid proteins (VP2 and VP5) of 304-06 were derived from EHDV-6. In compar-

ison, EHDV-1 and EHDV-2 VP5 amino acid sequences shared 65 and 75 % identity, respectively, with 304-06 (Table 1). Phylogenetic analysis of both of the outer capsid proteins (VP2 and VP5) demonstrated that 304-06 and M44/96 formed a distinct clade (with 100 % bootstrap support) separate from the other EHDV serotypes/topotypes (Fig. 2a, b).

In contrast, the VP7 sequence from 304-06 (975 nt) shared 99 % nucleotide identity with EHDV-2, whilst displaying an 89 % nucleotide identity to EHDV-6 M44/96, confirming that the parental virus of the S7 RNA segment was EHDV-2 (Table 1). Phylogenetic analysis of the VP7 amino acid sequences for available EHDV serotypes grouped 304-06 with an EHDV-2 Alberta isolate from the USA (Fig. 2c). Interestingly, the EHDV-6 (Indiana) (304-06) and EHDV-2 (Alberta) (211-91) VP7 sequences from the USA clustered with the EHDV-6 isolate from South Africa (M44/96) to form a distinct clade, separate from EHDV-2 topotypes from Australia and Asia (Fig. 2c). Sequence comparison of additional structural genes (VP1 and VP3) from 304-06 versus endemic serotypes and EHDV-6 also indicated that EHDV-2 was the likely parental virus of their corresponding RNA segments (Table 1).

Comparison of partial sequences of two non-structural genes (NS1 and NS3) from 304-06 with the prototype strains of all EHDV serotypes demonstrated that 304-06 shared the highest nucleotide identity (97 %) in both genes with EHDV-2 SV-124, isolated in Alberta in 1962 (Table 2). Additionally, the partial NS3 sequence (448 nt) for 304-06 shared an even higher nucleotide identity (99.8 %) when compared with more recent EHDV-2 isolates from mid-western and central USA (Murphy *et al.*, 2005), verifying that the S10 segment of 304-06 is derived from EHDV-2. When comparing the amino acid identities of NS1 and NS3 of 304-06 with other EHDV serotypes, the only serotype to display >90 % identity in both proteins, other than endemic EHDV-1 and EHDV-2, was EHDV-6 M44/96 (97 % in both NS1 and NS3) (Table 2).

Based on sequence data obtained from partial cloning of the L2 RNA segment from 304-06, two sets of primers were developed for diagnostic use in order to amplify the VP2 gene of the reassortant viruses from clinical samples (designated EHDV-6Ind-VP2; see Supplementary Table S1). EHDV-6 (Indiana) isolates from 2007–2008 were identified using these primer sets. The primer set EHDV-6Ind-VP2-1242/-1506 amplifies a 265 bp fragment, whilst the second internal set, EHDV-6Ind-VP2-1280i/1479i, are nested primers that amplify a 200 bp fragment; hence, these primers are useful in detecting RNA directly from tissue if virus isolation cannot be performed on clinical samples. Partial VP2 sequence comparisons of 304-06 with the single EHDV-6 (Indiana) isolate from 2007 (433-07) revealed 98 % (482/490) nucleotide and 99 % (162/163) amino acid identities (not shown). Seven of the eight nucleotide substitutions were synonymous third-base transitions. The single non-synonymous nucleotide change resulted in a



**Fig. 1.** Comparative partial alignment of selected structural proteins between EHDV-1 (New Jersey), EHDV-2 (Alberta) and EHDV-6 (Indiana) (isolate 304-06). VP1 (a) is the RNA-dependent RNA polymerase, VP2 (b) is the major surface antigen determining serotype specificity and VP3 (c) is the capsid protein forming the core particle encasing the viral dsRNA and associated replicative enzymes. Shaded asterisks denote identity; colons and dots represent conserved and semi-conserved amino acids, respectively.

conservative substitution of basic amino acids (K→R) between 304-06 and 433-07, respectively. Additional analysis of other EHDV-6 (Indiana) isolates from 2006–2008 indicated similar nucleotide identities in the VP2 gene (≥98%) irrespective of geographical location. Sequence analysis of multiple genes from all 12 isolates suggested that each had the same reassortment configuration (i.e. L2 and M6 were derived from EHDV-6, whilst the remaining RNA segments were derived from EHDV-2; not shown), although

these results are preliminary and require a more comprehensive analysis.

## DISCUSSION

Sequence data obtained from the US EHDV isolates indicated that they were all derived from the reassortment of at least two serotypes: EHDV-2 and EHDV-6. The L1, L3, M5, S7 and S10 RNA segments of the reassortant,

**Table 1.** Nucleotide and amino acid identities (%) of selected non-structural (NS) and structural (VP) genes of EHDV-6 (Indiana) (isolate 304-06) compared with the cognate region in endemic North American serotypes (EHDV-1 and EHDV-2) and an exotic EHDV-6 isolate from South Africa (M44/96)

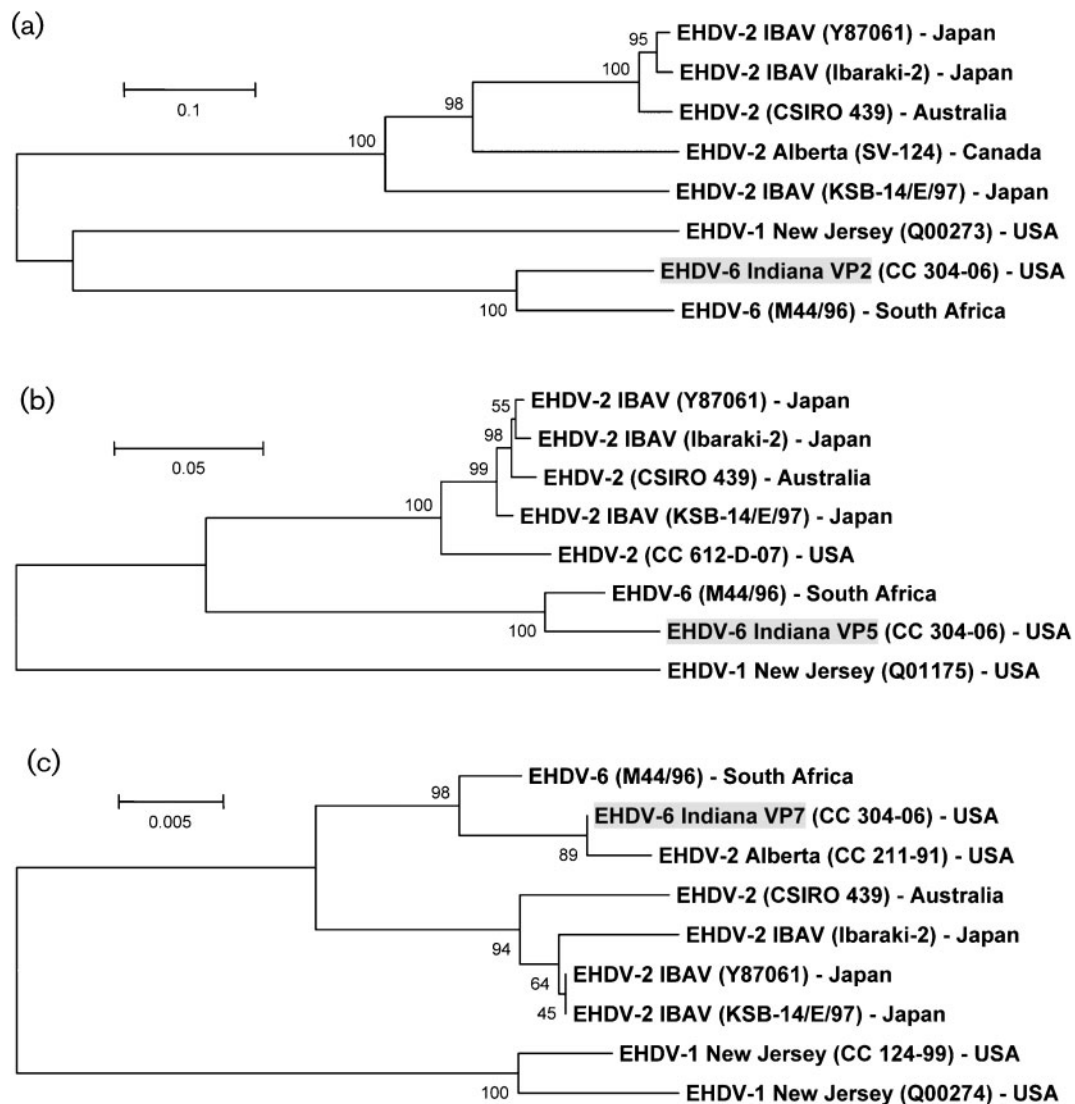
| EHDV-6 (Indiana) gene | EHDV-1 (New Jersey) |     | EHDV-2 (Alberta) |     | EHDV-6 (South Africa) |    |
|-----------------------|---------------------|-----|------------------|-----|-----------------------|----|
|                       | nt                  | aa  | nt               | aa  | nt                    | aa |
| NS1                   | 96                  | 98  | 97               | 97  | 89                    | 97 |
| NS3                   | 97                  | 100 | 99               | 100 | 86                    | 97 |
| VP1                   | 96                  | 98  | 96               | 97  | 88                    | 95 |
| VP2                   | 29                  | 40  | 30               | 38  | 73                    | 79 |
| VP3                   | 96                  | 99  | 98               | 100 | 89                    | 99 |
| VP5                   | 64                  | 65  | 69               | 75  | 80                    | 94 |
| VP7                   | 78                  | 94  | 99               | 100 | 89                    | 99 |

encoding the VP1, VP3, NS1, VP7 and NS3 genes, respectively, were apparently derived from EHDV-2 (Alberta) (Table 1). As VP1, VP3, NS1 and NS3 were highly conserved between EHDV-1 and EHDV-2 (Fig. 1a, c; Table 1), ascribing EHDV-2 as the parental virus from which their respective segments were obtained is tentative. However, as partial sequencing of VP7 (975 nt) indicated that EHDV-6 (Indiana) shared 78 and 99% nucleotide identity with EHDV-1 and EHDV-2, respectively (Table 1), it is presumed that EHDV-2 was probably the parental virus of other less variable RNA segments that share a very close identity between EHDV-1 and EHDV-2. Currently, other than the M5 (NS1) and S10 (NS3) sequences of the prototype EHDV strains recently reported by Wilson *et al.* (2009), there are no gene sequences for any other RNA segments for EHDV serotypes 3 (1), 4, 5, 6, 7 or 8 available in public databases to allow a comparison.

Based on genetic comparisons with a bovine EHDV-6 isolate from South Africa (M44/96), in conjunction with virus neutralization testing with exotic EHDV antisera, the L2 RNA segment (encoding the VP2 gene predominantly responsible for serotype specificity and mammalian receptor binding/cell entry) of the 2006 isolates was derived from EHDV-6 (Iwata *et al.*, 1992; Hassan & Roy, 1999). Further genetic comparisons of the VP2 gene of all 12 isolates recovered from 2006–2008 indicated that they all share the same L2 RNA segment and are probably derived from the same reassortment event. Although the VP2 amino acid identity shared between 304-06 and M44/96 (covering approximately one-third of the coding region of VP2) was only 79%, it is roughly equivalent to the divergence observed between topotypes of EHDV-2 (e.g. Australian and North American EHDV-2 VP2 amino acid sequences share 73% identity) and is also suggestive that the North American and African strains of EHDV-6 did not recently diverge from one another (i.e. M44/96, or a virus very closely related to it, is not the immediate parental virus of 304-06). Additionally, the M6 RNA segment, encoding the VP5 gene, is also apparently derived from EHDV-6. The amino acid identity of the complete coding region of VP5 (527 aa) between 304-06 and M44/96

was relatively higher (94%) in comparison with that of VP2, indicative of the reduced selection pressure that VP5 is under from the host immune response due to its presumably buried location in the outer capsid (Iwata *et al.*, 1991). The fact that VP2 and VP5 must interact directly with one other (albeit not extensively) to form the outer capsid, as demonstrated in the closely related BTV (Nason *et al.*, 2004), and that VP2 (Fig. 1b) and to a lesser extent VP5 are more variable in amino acid sequence than other EHDV proteins (Iwata *et al.*, 1991, 1992), may impart some degree of functional and/or conformational constraints that inhibit the survival of reassortants bearing outer surface proteins derived from two different EHDV serotypes. Additionally, reassortants containing chimeric outer capsid proteins have been shown to be difficult to produce and isolate *in vitro*, suggesting that reassortants containing VP2 and VP5 derived from contrasting serotypes may be exceedingly rare in nature (Mertens, 1999).

Interestingly, Nason *et al.* (2004) demonstrated that, with BTV, the most extensive protein interactions were not between VP2 and VP5, but rather between VP7 and the outer capsid proteins. Comparative analysis of the nucleotide and amino acid sequences of VP7 between M44/96 and 304-06 (with VP7 being derived from EHDV-2 Alberta) demonstrated 89 and 99% identity, respectively (Table 1). In comparison, the VP7 sequence of EHDV-1 is only 78 and 94% identical at the nucleotide and amino acid levels with that of EHDV-2. Additionally, phylogenetic analysis of the VP7 amino acid sequence indicated that EHDV-2 Alberta, along with 304-06, formed a monophyletic group with the EHDV-6 isolate from South Africa, which was distinct from other EHDV-2 topotypes (Fig. 2c). This grouping of EHDV-6 and EHDV-2 Alberta VP7 sequences was supported by high bootstrap values and was also present when the sequences were compared using alternative (maximum-parsimony) methods. That fact that the VP7 sequences of EHDV-2 Alberta and an African EHDV-6 isolate are more closely related to one another than EHDV-2 Alberta is to Australian and Asian EHDV-2 topotypes is intriguing and may also suggest that it was this direct compatibility of the protein–protein interactions



**Fig. 2.** Neighbour-joining phylogenies of the VP2 (a), VP5 (b) and VP7 (c) amino acid sequences of currently available EHDV serotypes. The lengths of the VP2, VP5 and VP7 sequences used in phylogenetic comparisons were 388, 527 and 325 aa, respectively. The particular viral protein used to construct each tree is highlighted for each of the EHDV-6 (Indiana) (isolate 304-06) sequences. Bootstrap confidence values were determined using 500 replicates. Amino acid distances were estimated using the Poisson correction method and are represented as the number of amino acid substitutions per site. Phylogenetic analyses using maximum-parsimony methods yielded similar results. The geographical location of each EHDV isolate/strain is indicated. Isolate/strain designations are in parentheses, with GenBank accession numbers listed if the isolate/strain designation is not known. The VP2, VP5 and VP7 NCBI GenBank sequences used for comparisons were EHDV-2/IBAV Y87061 (BAC20272, BAC20274, BAC20275); EHDV-2/IBAV Ibaraki-2 (BAC20268, BAC20270, BAC20271); EHDV-2/IBAV KSB-14/E/97 (BAC20276, BAC20278, BAC20279); EHDV-2 CSIRO 439 (BAC20280, BAC20281, AAA86263); EHDV-2 SV-124 (L33818); EHDV-2 CC 211-91 (AAP86688); EHDV-2 CC 612-D-07 (GQ387640); EHDV-1 CC 124-99 (AAP86721); EHDV-1 New Jersey (Q00273, Q01175, Q00274); EHDV-6 M44/96 (GQ385940, GQ387639, GQ387642); and EHDV-6 Indiana CC 304-06 (GQ385939, GQ387638, GQ387641).

between VP7 of EHDV-2 and VP2 and VP5 of the parental EHDV-6 that facilitated the viability and survival of the new reassortant in nature.

Although the extremely high nucleotide identity (99.8%) of the partial NS3 sequence from 304-06 indicated that

EHDV-2 is the parental virus of the S10 segment, M44/96 and 304-06 shared 97% amino acid identity in NS3, whilst other exotic serotypes, including the Australian EHDV-6 prototype (CSIRO 753), shared relatively lower identities with 304-06, ranging from 87 to 88% (Table 2). For BTV,

**Table 2.** Nucleotide and amino acid identities (%) of the partial NS1 and NS3 sequences of EHDV-6 (Indiana) (isolate 304-06) compared with the cognate region in the prototype strains of all EHDV serotypes

| Serotype | Strain/isolate | Location       | Year* | NS1 |    | NS3 |    |
|----------|----------------|----------------|-------|-----|----|-----|----|
|          |                |                |       | nt  | aa | nt  | aa |
| EHDV-1   | New Jersey     | USA            | 1955  | 96  | 98 | 95  | 98 |
| EHDV-2   | SV-124         | Canada         | 1962  | 97  | 97 | 97  | 98 |
| EHDV-2   | Ibaraki        | Japan          | 1959  | –†  | –† | 75  | 87 |
| EHDV-3‡  | Ib Ar 22619    | Nigeria        | 1967  | 90  | 95 | 74  | 88 |
| EHDV-4   | Ib Ar 33853    | Nigeria        | 1968  | 91  | 97 | 74  | 88 |
| EHDV-5   | CSIRO 157      | Australia      | 1977  | 76  | 89 | 76  | 88 |
| EHDV-6   | CSIRO 753      | Australia      | 1981  | 77  | 89 | 77  | 87 |
| EHDV-6   | M44/96         | South Africa   | 1996  | 89  | 97 | 86  | 97 |
| EHDV§    | AY351654       | Réunion Island | 2003  | –†  | –† | 86  | 97 |
| EHDV-7   | CSIRO 775      | Australia      | 1981  | 77  | 89 | 74  | 87 |
| EHDV-8   | DPP 59         | Australia      | 1982  | 79  | 92 | 75  | 88 |

\*Year of isolation of compared sequence is given; however, no isolation date for the Ibaraki NS3 sequence was available in GenBank, so the date of the first description of IBAV is listed.

†Sequence not available.

‡For clarity, the serotype designation for EHDV-3 is based on the current ICTV classification (Mertens *et al.*, 2005).

§Untyped EHDV. Although VP2 sequence was obtained for an EHDV isolate from the Réunion Island outbreak (Bréard *et al.*, 2004), the sequence is currently not available in GenBank; hence, no serotype designation can be given. However, VP7 and NS3 amino acid sequences of the Réunion Island isolate share 100% identity to EHDV-6 M44/96 from South Africa.

NS3 has been demonstrated to play a functional role in viral egress from *Culicoides* cells by binding not only to cellular proteins involved in exocytosis but also to VP2, thereby potentially acting as a bridging molecule between cellular export proteins and assembled virions (Beaton *et al.*, 2002; Celma & Roy, 2009). Although the interaction between NS3 and VP2 may potentially be limited to short conserved motifs in both proteins (Celma & Roy, 2009), the high amino acid identity of 304-06 NS3 compared with M44/96 NS3 (97%) in comparison with other serotypes (87–88%) may suggest that this could have facilitated the functional interaction between NS3 of EHDV-2 and VP2 of the parental EHDV-6 during viral egress in North American vectors (i.e. *Culicoides sonorensis*). In addition, as VP7, which exhibits a very high amino acid identity (99%) between M44/96 and EHDV-2, has been demonstrated to be the viral protein involved in receptor attachment and subsequent infection of *Culicoides* cells by BTV (Mertens *et al.*, 1996; Xu *et al.*, 1997), this not only potentially suggests that the structural viability of the reassortant (i.e. VP2/VP5 interaction with VP7) was facilitated by the overall compatibility of the proteins between EHDV-2 and EHDV-6, but also that its subsequent transmission (i.e. VP7 interaction with North American *Culicoides* receptors; NS3 interaction with VP2) may have been dependent, in part, upon the compatibility of these protein interactions.

The reassortment event between EHDV-6 and EHDV-2 presumably took place in the USA (assuming the Alberta

strain of EHDV-2 is not circulating outside North America), giving rise to EHDV-6 (Indiana). Whether the parental EHDV-6 is also circulating in the USA is unknown. Alternatively, reassortment may have been a necessary prerequisite for the efficient transmission and consequential survival of parental EHDV-6 in North America. Whether other reassortants containing different gene segment configurations of the two parental viruses are also circulating is speculative and may be difficult to detect if they contain only highly conserved (non-serotype-specific) RNA segments from EHDV-6. The fact that an EHDV-6 isolate from South Africa (M44/96) shares  $\geq 86\%$  nucleotide identity in partial sequences of NS1, NS3, VP1, VP3 and VP7 with EHDV-2 (Alberta) reiterates this assumption. However, as the geographical distributions of most EHDV serotypes are not well delineated and there is some serological evidence of EHDV-2 (Alberta) infection in cattle from Guyana and Columbia (Gumm *et al.*, 1984; Homan *et al.*, 1985), it is also plausible that the reassortment event may have occurred outside the USA (e.g. Central or South America).

Although the natural reassortment of different serotypes within an orbivirus serogroup (species) has been described previously (e.g. Collisson & Roy, 1983), to our knowledge, this is the first report of the field isolation of a reassortant EHDV containing RNA segments from both indigenous and exotic serotypes. Reassortment is likely to have played an important role in orbivirus evolution, contributing to the genotypic and phenotypic diversity observed within each serogroup of the genus (Sugiyama *et al.*, 1982; Samal *et al.*, 1987a, b; Stott *et al.*, 1987; Brown *et al.*, 1988; Maia &

Osburn, 1992). However, the comparative effects that reassortment (i.e. compared with parental viruses) has on virus–host interactions (e.g. virus fitness, host virulence, vector competence and transmissibility) has not been studied extensively and requires further research.

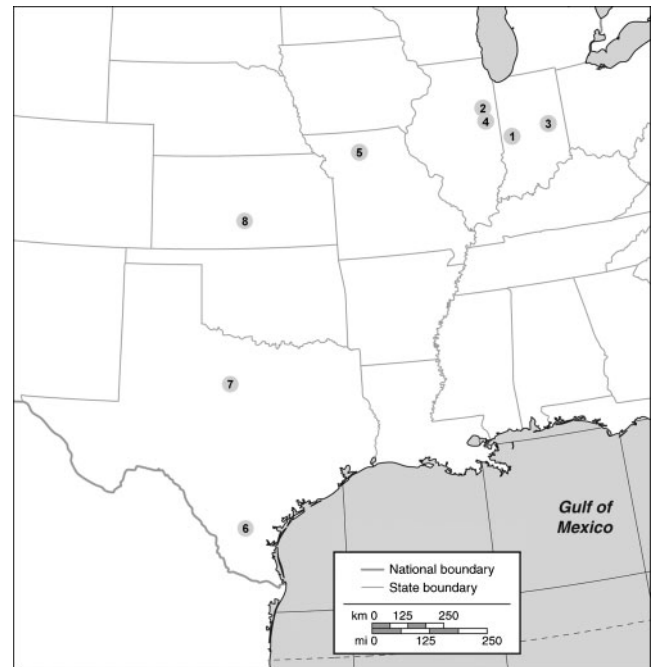
The recognition of EHDV-6 (Indiana) in the USA was predominantly afforded through the presence of a sentinel species (white-tailed deer) that is highly susceptible to EHDV-induced clinical disease. The lack of reported EHDV-induced disease in deer populations outside North America may consequently be partially due to species composition, reporting bias and/or the relative population size. Therefore, in areas where large populations of ruminant species that are highly susceptible to clinical disease are absent, EHDV serotypes may circulate undetected unless a proactive surveillance system (e.g. sentinel cattle) is in place for their detection. Additionally, the recognition of EHDV-6 (Indiana) in the USA would have been missed by serogroup-based diagnostic protocols, underscoring the importance of serotyping EHDV isolates by virus neutralization and/or serotype-specific RT-PCR to ensure proper identification. Currently, the geographical distribution of EHDV-6 (Indiana) in the USA is unknown, but presumably stretches from at least east/central Indiana west to Kansas and south to Texas (Fig. 3). Continued haemorrhagic disease surveillance and virus isolation from clinical submissions, in addition to current and retroactive serological testing of white-tailed deer or domestic ruminant populations from different geographical regions, is likely to help define the distribution of EHDV-6 (Indiana) in the USA.

## METHODS

**Case histories.** EHDV-6 (Indiana) was isolated from 12 individual white-tailed deer on eight occasions during 2006–2008. Four cases (cases 1–4), involving six deer, occurred in Indiana and Illinois in 2006; one case (case 5), involving a single deer, occurred in Missouri in 2007; and three cases (cases 6–8), involving five deer, occurred in Texas and Kansas in 2008 (Fig. 3). Clinical case isolate 304-06 (Henry Co., Indiana, 2006), shown as case 3 in Fig. 3, represented the only virus recovered from a free-ranging white-tailed deer; the remaining 11 isolates were obtained from captive deer herds.

**Virus isolation and L2 RNA segment purification.** Virus isolation was performed on tissue and blood samples using cattle pulmonary artery endothelium (CPAE) cells (ATCC). CPAE cells were propagated in maintenance medium [ $1 \times$  minimal essential media (MEM), 2.2 g  $\text{NaHCO}_3 \text{ l}^{-1}$ , 10% fetal bovine serum (FBS), 100 U penicillin  $\text{ml}^{-1}$ , 100  $\mu\text{g}$  streptomycin  $\text{ml}^{-1}$ , 0.25  $\mu\text{g}$  amphotericin B (Sigma)  $\text{ml}^{-1}$ ].

For virus isolation from tissue, samples (approx.  $0.5 \text{ cm}^3$ ) were mechanically homogenized in 650  $\mu\text{l}$  virus isolation (VI) medium ( $1 \times$  MEM, 2.2 g  $\text{NaHCO}_3 \text{ l}^{-1}$ , 20% FBS, 400 U penicillin  $\text{ml}^{-1}$ , 400  $\mu\text{g}$  streptomycin  $\text{ml}^{-1}$ , 1  $\mu\text{g}$  amphotericin B  $\text{ml}^{-1}$ ). Homogenized tissues were centrifuged (6700 g for 10 min) to pellet debris and an aliquot (100  $\mu\text{l}$ ) of clarified supernatant was used to inoculate a 3-day-old CPAE cell culture in a 12.5  $\text{cm}^2$  flask or a 12-well plate format.



**Fig. 3.** Geographical locations of EHDV-6 (Indiana) isolates from free-ranging and captive white-tailed deer recovered by the Southeastern Cooperative Wildlife Disease Study in the USA during 2006–2008. EHDV-6 (Indiana) isolates obtained during 2006, 2007 and 2008 are represented as cases 1–4 (Indiana and Illinois), case 5 (Missouri) and cases 6–8 (Texas and Kansas), respectively. Numerical designations refer to the chronological order of isolation. © Houghton Mifflin Company. Reprinted by permission of Houghton Mifflin Harcourt Publishing Company. All rights reserved. Any further duplication is strictly prohibited unless written permission is obtained from Houghton Mifflin Harcourt Publishing Company ([http://www.eduplace.com/ss/maps/pdf/midwestus\\_nl.pdf](http://www.eduplace.com/ss/maps/pdf/midwestus_nl.pdf)).

For virus isolation from blood, heparinized whole blood (1 ml) was washed in Dulbecco's PBS and cells were pelleted by light centrifugation (720 g for 4 min). The wash step was repeated three times and an aliquot (100  $\mu\text{l}$ ) of the final cell pellet was diluted 1 : 10 in VI medium and sonicated for approximately 20 s at 5 W using a VirSonic 100 Ultrasonic Cell Disrupter (VirTis). An aliquot of the sonicated solution (100  $\mu\text{l}$ ) was then used to inoculate CPAE cell culture as for the tissue samples.

For wells exhibiting cytopathology, cell culture supernatant was harvested for: (i) virus neutralization testing; (ii) RNA extraction using a QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions; and (iii) stock virus. In order to gain sequence information for the VP2 gene, stock virus was inoculated into three 75  $\text{cm}^2$  flasks of Madin–Darby bovine kidney cells (ATCC) and virus was precipitated on day 6 post-inoculation using polyethylene glycol as described by Killington *et al.* (1996). Extracted RNA was electrophoresed on a 1% agarose ethidium bromide-stained TAE gel and the L2 RNA segment was excised and purified from the agarose using a QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's instructions.

**Virus serotyping.** Virus neutralization tests against EHDV and BTV serotypes currently considered indigenous to the USA (EHDV



serotypes 1 and 2; BTV serotypes 2, 10, 11, 13 and 17) were performed as described previously (Abdy *et al.*, 1999). For virus neutralization tests using exotic EHDV antiserum, the 2006 isolates were sent to the NVSL, Ames, IA, USA. The isolates were then sent to the IAH, Pirbright Laboratory, Woking, UK, for confirmatory testing using VP2-specific primers directed against all EHDV serotypes.

**RT-PCR and phylogenetic analysis.** All EHDV isolates were analysed by RT-PCR using serogroup-specific (i.e. amplifying both EHDV-1 and EHDV-2) and serotype-specific [i.e. amplifying EHDV-1, EHDV-2 or EHDV-6 (Indiana)] primers (Supplementary Table S1). Serogroup-specific primers to NS1, NS3, VP1, VP3, VP5 and VP7 listed in Supplementary Table S1 have not been tested on EHDV serotypes other than 1 and 2. Serotype-specific primers against VP2 (primary and nested) were developed for EHDV-1, EHDV-2 and EHDV-6 (Indiana). Serotype-specific primers against VP5 (primary only) were also produced for EHDV-6 (Indiana). Decamer oligonucleotides (not shown) were originally used in order to obtain the VP2 sequence for EHDV-6 (Indiana). Primers based on the sequence of the South African EHDV-6 M44/96 isolate (not shown) were originally used in order to obtain the VP5 sequence for EHDV-6 (Indiana).

Single-tube RT-PCRs (50 µl) were set up using 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl<sub>2</sub>, 250 µM dNTPs, 0.5 µM each primer, 2 U avian myeloblastosis virus reverse transcriptase (Promega), 1 U *Taq* DNA polymerase (Promega) and 5 µl extracted RNA. Cycling parameters were reverse transcription at 42 °C for 30 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min, with a final extension step at 72 °C for 10 min. Amplicons were electrophoresed on a 2% agarose ethidium bromide-stained TAE gel, excised and purified from the agarose, and cloned using a PCR Cloning<sup>plus</sup> kit (Qiagen), and recombinant plasmid was subsequently purified using a QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. Sequencing was performed using a 3100 Genetic Analyzer (Applied Biosystems). Phylogenetic analysis of VP2, VP5 and VP7 amino acid sequences by neighbour-joining and maximum-parsimony methods was conducted using the MEGA4 program (Tamura *et al.*, 2007).

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## REFERENCES

Abdy, M. J., Howerth, E. W. & Stallknecht, D. E. (1999). Experimental infection of calves with epizootic hemorrhagic disease virus. *Am J Vet Res* **60**, 621–626.

Anthony, S. J., Maan, S., Maan, N., Batten, C., Darpel, K. E., Sutton, G., Attoui, H. & Mertens, P. P. C. (2009). Genetic and phylogenetic analysis of the outer-coat proteins VP2 and VP5 of epizootic hemorrhagic disease virus (EHDV): comparison of genetic and serological data to characterise the EHDV serogroup. *Virus Res* **145**, 200–210.

Bak, U. B., Cheong, C. K., Choi, H. I., Lee, C. W. & Oh, H. S. (1983). An outbreak of Ibaraki disease in Korea. *Korean J Vet Res* **23**, 81–89.

Barber, T. L. & Jochim, M. M. (1975). Serotyping bluetongue and epizootic hemorrhagic disease virus strains. *Proc Am Assoc Vet Lab Diagn* **18**, 149–157.

Beaton, A. R., Rodriguez, J., Reddy, Y. K. & Roy, P. (2002). The membrane trafficking protein calpactin forms a complex with bluetongue virus protein NS3 and mediates virus release. *Proc Natl Acad Sci U S A* **99**, 13154–13159.

Bréard, E., Sailleau, C., Hamblin, C., Graham, S. D., Gourreau, J. M. & Zientara, S. (2004). Outbreak of epizootic hemorrhagic disease on the island of Réunion. *Vet Rec* **155**, 422–423.

Brown, S. E., Gonzalez, H. A., Bodkin, D. K., Tesh, R. B. & Knudson, D. L. (1988). Intra- and inter-serogroup genetic relatedness of orbiviruses. II. Blot hybridization and reassortment *in vitro* of epizootic hemorrhagic disease serogroup, bluetongue type 10 and Pata viruses. *J Gen Virol* **69**, 135–147.

Celma, C. C. P. & Roy, P. (2009). A viral nonstructural protein regulates bluetongue virus trafficking and release. *J Virol* **83**, 6806–6816.

Chalmers, G. A., Vance, H. N. & Mitchell, G. J. (1964). An outbreak of hemorrhagic epizootic disease in wild ungulates in Alberta. *J Wildl Dis* **15**, 1–6.

Collisson, E. & Roy, P. (1983). Analysis of the genomes of bluetongue virus serotype 10 vaccines and a recent BTV-10 isolate from Washington. *Am J Vet Res* **44**, 235–237.

Gould, A. R. & Pritchard, L. I. (1991). Phylogenetic analyses of the complete nucleotide sequence of the capsid protein (VP3) of Australian epizootic hemorrhagic disease of deer virus (serotype 2) and cognate genes from other orbiviruses. *Virus Res* **21**, 1–18.

Gumm, I. D., Taylor, W. P., Roach, C. J., Alexander, F. C., Greiner, E. C. & Gibbs, E. P. (1984). Serological survey of ruminants in some Caribbean and South American countries for type-specific antibody to bluetongue and epizootic hemorrhagic disease viruses. *Vet Rec* **114**, 635–638.

Hassan, S. S. & Roy, P. (1999). Expression and functional characterization of bluetongue virus VP2 protein: role in cell entry. *J Virol* **73**, 9832–9842.

Homan, E. J., Taylor, W. P., De Ruiz, H. L. & Yuill, T. M. (1985). Bluetongue virus and epizootic hemorrhagic disease of deer virus serotypes in northern Colombian cattle. *J Hyg (Lond)* **95**, 165–172.

Iwata, H., Hirasawa, T. & Roy, P. (1991). Complete nucleotide sequence of segment 5 of epizootic hemorrhagic disease virus; the outer capsid protein VP5 is homologous to the VP5 protein of bluetongue virus. *Virus Res* **20**, 273–281.

Iwata, H., Chuma, T. & Roy, P. (1992). Characterization of the genes encoding two of the major capsid proteins of epizootic hemorrhagic disease virus indicates a close genetic relationship to bluetongue virus. *J Gen Virol* **73**, 915–924.

Killington, R. A., Stokes, A. & Hierholzer, J. C. (1996). Virus purification. In *Virology Methods Manual*, pp. 73–74. Edited by B. W. J. Mahy & H. O. Kangro. New York: Academic Press.

Lee, V. H., Causey, O. R. & Moore, D. L. (1974). Bluetongue and related viruses in Ibadan, Nigeria: isolation and preliminary identification of viruses. *Am J Vet Res* **35**, 1105–1108.

Liao, Y. K., Lu, Y. S., Huang, S. T. & Lee, S. H. (1996). The etiological and epidemiological studies on the Ibaraki disease in Taiwan. *Zhonghua Min Guo Shou Yi Xue Hui Za Zhi* **32**, 49–57 (in Chinese).

MacLachlan, N. J. & Osburn, B. I. (2004). Epizootic hemorrhagic disease of deer. In *Infectious Diseases of Livestock*, vol. 2, 2nd edn, pp.

1227–1230. Edited by J. A. W. Coetzer & R. C. Tustin. Cape Town: Oxford University Press Southern Africa.

- Maia, M. S. & Osburn, B. I. (1992).** Differential serological responses to reassortant bluetongue viruses recovered from a bull. In *Bluetongue, African Horse Sickness, and related Orbiviruses: Proceedings of the Second International Symposium*, pp. 776–780. Edited by T. E. Walton & B. I. Osburn. Boca Raton, FL: CRC Press.
- Mertens, P. P. C. (1999).** Orbiviruses and coltivirus (Reoviridae): general features. In *Encyclopedia of Virology*, 2nd edn, pp. 1043–1061. Edited by A. Granoff & R. G. Webster. San Diego, CA: Elsevier Academic Press.
- Mertens, P. P., Burroughs, J. N., Walton, A., Wellby, M. P., Fu, H., O'Hara, R. S., Brookes, S. M. & Mellor, P. S. (1996).** Enhanced infectivity of modified bluetongue virus particles for two insect cell lines and for two *Culicoides* vector species. *Virology* **217**, 582–593.
- Mertens, P. P. C., Maan, S., Samuel, A. & Attoui, H. (2005).** Genus *Orbivirus*, family *Reoviridae*. In *Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses*, pp. 466–483. Edited by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & L. A. Ball. San Diego, CA: Elsevier Academic Press.
- Mohammed, M. E. & Mellor, P. S. (1990).** Further studies on bluetongue and bluetongue-related orbiviruses in the Sudan. *Epidemiol Infect* **105**, 619–632.
- Murphy, M. D., Howerth, E. W., MacLachlan, N. J. & Stallknecht, D. E. (2005).** Genetic variation among epizootic hemorrhagic disease viruses in the southeastern United States: 1978–2001. *Infect Genet Evol* **5**, 157–165.
- Nason, E. L., Rothagel, R., Mukherjee, S. K., Kar, A. K., Forzan, M., Prasad, B. V. & Roy, P. (2004).** Interactions between the inner and outer capsids of bluetongue virus. *J Virol* **78**, 8059–8067.
- Omori, T., Inaba, Y., Morimoto, T., Tanaka, Y., Ishitani, R., Kurogi, H., Munakata, K., Matsuda, K. & Matumoto, M. (1969).** Ibaraki virus, an agent of epizootic disease of cattle resembling bluetongue. I. Epidemiologic, clinical and pathologic observations and experimental transmission to calves. *Jpn J Microbiol* **13**, 139–157.
- Rabenau, H., Ohlinger, V., Anderson, J., Selb, B., Cinatl, J., Wolf, W., Frost, J., Mellor, P. & Doerr, H. W. (1993).** Contamination of genetically engineered CHO-cells by epizootic haemorrhagic disease virus (EHDV). *Biologicals* **21**, 207–214.
- Samal, S. K., Livingston, C. W., Jr, McConnell, S. & Ramig, R. F. (1987a).** Analysis of mixed infection of sheep with bluetongue virus serotypes 10 and 17: evidence for genetic reassortment in the vertebrate host. *J Virol* **61**, 1086–1091.
- Samal, S. K., el-Hussein, A., Holbrook, F. R., Beaty, B. J. & Ramig, R. F. (1987b).** Mixed infection of *Culicoides variipennis* with bluetongue virus serotypes 10 and 17: evidence for high frequency reassortment in the vector. *J Gen Virol* **68**, 2319–2329.
- Shope, R. E., MacNamara, L. G. & Mangold, R. (1955).** Report on the deer mortality, epizootic hemorrhagic disease of deer. *N J Outdoors* **6**, 17–21.
- St. George, T. D., Cybinski, D. H., Standfast, H. A., Gard, G. P. & Della-Porta, A. J. (1983).** The isolation of five different viruses of the epizootic haemorrhagic disease of deer serogroup. *Aust Vet J* **60**, 216–217.
- Stott, J. L., Oberst, R. D., Channell, M. B. & Osburn, B. I. (1987).** Genome segment reassortment between two serotypes of bluetongue virus in a natural host. *J Virol* **61**, 2670–2674.
- Sugiyama, K., Bishop, D. H. L. & Roy, P. (1982).** Analyses of the genomes of bluetongue viruses recovered from different states of the United States and at different times. *Am J Epidemiol* **115**, 332–347.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007).** MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Temizel, E. M., Yesilbag, K., Batten, C., Senturk, S., Maan, N. S., Clement-Mertens, P. P. & Batmaz, H. (2009).** Epizootic hemorrhagic disease in cattle, Western Turkey. *Emerg Infect Dis* **15**, 317–319.
- Uchinuno, Y., Ito, T., Goto, Y., Miura, Y., Ishibashi, K., Itou, T. & Sakai, T. (2003).** Differences in Ibaraki virus RNA segment 3 sequences from three epidemics. *J Vet Med Sci* **65**, 1257–1263.
- Weir, R. P., Harmsen, M. B., Hunt, N. T., Blacksell, S. D., Lunt, R. A., Pritchard, L. I., Newberry, K. M., Hyatt, A. D., Gould, A. R. & Melville, L. F. (1997).** EHDV-1, a new Australian serotype of epizootic haemorrhagic disease virus isolated from sentinel cattle in the Northern Territory. *Vet Microbiol* **58**, 135–143.
- Wilson, W. C., O'Hearn, E. S., Tellgren-Roth, C., Stallknecht, D. E., Mead, D. G. & Mecham, J. O. (2009).** Detection of all eight serotypes of epizootic hemorrhagic disease virus by real-time reverse transcription polymerase chain reaction. *J Vet Diagn Invest* **21**, 220–225.
- Xu, G., Wilson, W., Mecham, J., Murphy, K., Zhou, E. M. & Tabachnick, W. (1997).** VP7: an attachment protein of bluetongue virus for cellular receptors in *Culicoides variipennis*. *J Gen Virol* **78**, 1617–1623.
- Yadin, H., Brenner, J., Bumbrov, V., Oved, Z., Stram, Y., Klement, E., Perl, S., Anthony, S., Maan, S. & other authors (2008).** Epizootic haemorrhagic disease virus type 7 infection in cattle in Israel. *Vet Rec* **162**, 53–56.