

CHAPTER 3.1.7

EPIZOOTIC HAEMORRHAGIC DISEASE (INFECTION WITH EPIZOOTIC HEMORRHAGIC DISEASE VIRUS)

SUMMARY

Epizootic haemorrhagic disease (EHD) is a vector-borne infectious noncontagious viral disease of domestic and wild ruminants, primarily white-tailed deer (*Odocoileus virginianus*) and cattle. Sheep, goats and camelids might also be susceptible, but usually do not develop overt disease.

EHD virus (EHDV) is transmitted between ruminant hosts by species of *Culicoides* biting midges, thus EHD infections are strongly seasonal. White-tailed deer is the most severely affected species, with the peracute form having a high mortality rate. In cattle, clinical signs occur rarely but fever, anorexia, dysphagia, emaciation, ulcerative stomatitis, lameness, respiratory distress and erythema of the udder have been reported.

Detection of the agent: EHDV belongs to the family Reoviridae, genus Orbivirus, and shares many morphological and structural characteristics with the other members of the genus, in particular bluetongue virus (BTV).

EHDV particles are non-enveloped but have a double capsid with an icosahedral symmetry. Within the virus core, 10 double-stranded RNA genomic segments code for seven structural proteins (VP) and at least four nonstructural proteins (NS). The protein VP2 of the outer core is the major determinant of serotype specificity, while the VP7 of the inner core possesses the serogroup-specific antigens. At least seven distinct serotypes have been identified and two new putative serotypes; there is however, some uncertainty regarding the exact number of serotypes and a panel of reference strains of EHDV is not yet officially recognised.

Assays for detection of EHDV in field samples include virus isolation in cell culture and EHD serogroup-specific reverse-transcription polymerase chain reaction (RT-PCR) tests. Suspected isolates can be identified by competitive (antigen-capture) and sandwich enzyme-linked immunosorbent assays (ELISAs). Serotype-specific RT-PCR assays have been developed for serotype identification of cell culture isolates. Isolates may also be identified by high throughput sequencing or virus neutralisation tests.

Serological tests: Antibodies to EHDV may be first detectable from 8 days post-exposure. Neutralising antibodies and the virus can co-exist in the infected animal, likely because of the strong association between the EHDV and the red blood cells.

For the detection of anti-EHDV antibodies in the sera of exposed animals, a specific monoclonal antibody-based competitive ELISA (C-ELISA) is recommended. The C-ELISA is a rapid test, detecting antibodies against the VP7 protein. Virus neutralisation (VN) tests may also be performed. VN testing is usually performed to identify exposure to specific EHDV serotypes. The VN test is more time-consuming (3–5 days) and labour intensive, and cross reactions among serotypes may preclude optimal results. Tests such as agar gel immunodiffusion and the indirect ELISA can be used, but have the major drawback of being unable to distinguish between antibodies to EHDV and BTV.

Requirements for vaccines: In the USA, an autogenous vaccine that can be used only in captive wild deer has been administered. In Japan, a vaccine has been developed for use in cattle. Apart from these two limited settings, there has been little interest from laboratories and vaccine companies elsewhere in developing vaccines to control the disease or EHDV circulation.

A. INTRODUCTION

Epizootic haemorrhagic disease (EHD) is an infectious noncontagious viral disease transmitted by insects of the genus *Culicoides*. Available data suggest that the species of *Culicoides* that transmit EHD virus (EHDV) are likely to be similar though not necessarily the same as those that transmit bluetongue virus (BTV) (Carpenter *et al.*, 2008). The disease affects both wild and domestic ruminants, particularly North American cervids, and, to a lesser degree, cattle (Bréard *et al.*, 2004), although many countries describe only asymptomatic infection (Gard & Melville, 1992; St George *et al.*, 1983). Sheep, goats and camelids may be susceptible to EHDV infection but their role as hosts is uncertain.

In susceptible species, EHDV may cause a disease with clinical manifestations similar to BTV infection. White-tailed deer (*Odocoileus virginianus*) are the species most severely affected with the peracute form characterised by fever, anorexia, respiratory distress, and severe oedema of the head and neck. Swelling of the tongue and conjunctivae can also be present. In the acute (or classical) form, these clinical signs may be accompanied by haemorrhages in many tissues including skin and heart, and animals may develop ulcers or erosions of the tongue, dental pad, palate, rumen and abomasum (Savini *et al.*, 2011).

Histopathological lesions include widespread vasculitis with thrombosis, endothelial swelling, haemorrhages and necrosis in many organs especially the tongue, salivary glands, fore-stomachs, aorta and papillary muscle of the left ventricle of the myocardium. Scattered grey plaques on the surface of the gall bladder mucosa were also described (Noon *et al.*, 2002).

While the majority of infections in cattle are subclinical, the serotypes 1, 2, 6 and 7 have been described as potentially pathogenic (Bréard *et al.*, 2004; Cêtre-Sossah *et al.*, 2014; Temizel *et al.*, 2009). The disease is characterised by fever, anorexia, ulcerative stomatitis, swelling of eyelids, respiratory distress, nasal and ocular discharge, redness and scaling of muzzle and lips, lameness, erythema of the udder and difficulty swallowing (Temizel *et al.*, 2009). Ibaraki disease in cattle is caused by a strain of EHDV-2 (Anthony *et al.*, 2009). Animals become dehydrated and emaciated, and in some cases death occurs due to aspiration pneumonia. The lesions are histologically characterised by hyaline degeneration, necrosis and mineralisation of striated muscle accompanied by an infiltration of neutrophils, lymphocytes and histiocytes (Ohashi *et al.*, 1999; Savini *et al.*, 2011).

Taxonomically, EHDV is classified in the *Orbivirus* genus of the family *Reoviridae* (Maclachlan & Osburn, 2004). It is a double-stranded RNA virus with a genome of 10 segments. At least some of the genome segments are able to undergo reassortment during mixed infections in the field (Allison *et al.*, 2010; Anbalagan *et al.*, 2014). Seven serotypes (and two new putative serotypes) are currently recognised, but there is not yet a widely accepted consensus on the exact number of serotypes (Anthony *et al.*, 2010; Maan *et al.*, 2017; Shirafuji *et al.*, 2017; Wright, 2013). The virus is stable at -70°C and in blood, tissue suspension or washed blood cells held at 4°C . EHDV on laboratory surfaces is susceptible to 95% ethanol and 0.5% sodium hypochlorite solution.

EHDV particles are composed of three protein layers. The outer capsid consists of two proteins, VP2 and VP5. Like BTV, VP2 is the primary determinant of serotype specificity. VP5, the other external protein, might also elicit neutralising antibodies (Savini *et al.*, 2011; Schwartz-Cornill *et al.*, 2008). This outer capsid is dissociated readily from the core particle, and leaves a bi-layered icosahedral core particle composed of two major proteins, VP7 and VP3, surrounding the transcriptase complex (VP1, VP4, and VP6) and the genomic RNA segments. VP7 is the serogroup-specific immuno-dominant protein and the protein used in serogroup specific enzyme-linked immunosorbent assays (ELISAs) (Saif, 2011). The viral RNA also encodes five nonstructural proteins (NS1 to NS5) (Belhouchet *et al.*, 2011).

As a vector-borne viral disease, the distribution of EHD is limited to the distribution of competent *Culicoides* vectors (Mellor *et al.*, 2008). The EHDV has been isolated from wild and domestic ruminants and arthropods in North America, Ecuador (Verdezoto *et al.*, 2018), the Caribbean region (Brown-Joseph *et al.*, 2019), French Guiana (Viarouge *et al.*, 2014), Asia, Africa and Australia, and more recently in countries surrounding the Mediterranean Basin including Algeria, Israel, Jordan, Morocco, Tunisia, and Turkey. No cases of EHDV infection have been reported in Europe. Outbreaks generally coincide with the peak of vector population abundance, so most cases of EHD occur in the late summer and autumn (Mellor *et al.*, 2008; Stallknecht & Howerth, 2004).

As EHDV is a vector-borne infection it may be difficult to control or eradicate once established. Unpredicted and uncontrollable variables such as climatic and geographical factors, as well as abundance of suitable EHDV insect vectors are all important for the outcome and persistence (or reappearance) of EHDV in an area. Furthermore, to date, there are no detailed studies on the effect of control measures applied in the countries where the disease has affected cattle. Sera prepared from viraemic animals may represent some risk if introduced parenterally into naive

animals. The most significant threat from EHDV occurs when virus is inoculated parenterally into susceptible animals. If appropriate *Culicoides* are present, virus can be transmitted to other hosts. Therefore, EHDV-infected animals must be controlled for the period of viraemia and protected against *Culicoides* by physical means.

There is no known risk of human infection with EHDV. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of EHD and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent^(a)						
Real-time RT-PCR	–	+++	+	++	++	–
RT-PCR	–	++	+	++	++	–
Isolation in cell culture	–	++	–	++	–	–
Detection of immune response						
C-ELISA (serogroup specific)	+++	+++	+++	–	+++	++
VN (serotype specific)	++	++	++	–	++	+++
AGID	+	–	+	–	+	+
CFT	+	–	+	–	+	+

Key: +++ = recommended for this purpose; ++ recommended but has limitations; + = suitable in very limited circumstances; – = not appropriate for this purpose.

RT-PCR = reverse-transcription polymerase chain reaction; C-ELISA = competitive enzyme-linked immunosorbent assay;

VN = virus neutralisation; AGID = agar gel immunodiffusion test; CFT = complement fixation test.

^(a)A combination of agent identification methods applied on the same clinical sample is recommended.

Clinical signs of EHD in wild ruminants and cattle are similar to those of BT in sheep and cattle, and they may be similar to signs found in other cattle diseases like bovine viral diarrhoea/mucosal disease, infectious bovine rhinotracheitis, vesicular stomatitis, malignant catarrhal fever and bovine ephemeral fever. Definitive diagnosis of EHDV infection therefore requires the use of specific laboratory tests.

1. Detection of the EHDV agent

1.1. *In-vitro* culture

1.1.1. Isolation in cell culture

The same diagnostic procedures are used for domestic and wild ruminants. Virus isolation can be attempted from the blood of viraemic animals, tissue samples including spleen, lung and lymph

nodes of infected carcasses, and from *Culicoides* spp. EHDV can be isolated by inoculation of cell cultures such as those of cattle pulmonary artery endothelial, baby hamster kidney (BHK-21), and African green monkey kidney (Vero) (Aradaib *et al.*, 1994), the latter two being the most commonly used for growing the virus. *Aedes albopictus* (e.g. C6/36) and *Culicoides variipennis* (Kc) cell lines may also be used for virus isolation (Batten *et al.*, 2011; Eschbaumer *et al.*, 2012; Gard *et al.*, 1989); as can embryonated chicken eggs, but with less sensitivity (Eschembauer *et al.*, 2012). Cytopathic effect (CPE), which occurs only in mammalian cell lines, usually appears between 2 and 7 days post-inoculation, however a blind passage may be required.

Below is a general virus isolation procedure in cell culture that can be modified according to individual laboratory needs. Incubation of cell cultures for EHDV isolation is usually performed in a humid 5% CO₂ atmosphere.

- i) For tissues from clinical cases, prepare a 10–30% tissue suspension in cell culture or other appropriate medium containing antibiotics. Centrifuge and save the supernatant for virus isolation.
- ii) For uncoagulated whole blood, centrifuge the blood (0.2 ml) to separate the red blood cells (RBC) and plasma. Discard the plasma and replace with phosphate-buffered saline (PBS, pH 7.4). Centrifuge the blood again to separate the RBC. Perform three total washes with PBS. Add 0.4 ml of sterile distilled water to lyse the RBC. Cells may alternatively be lysed by sonication. Add 1.6 ml of PBS to the lysed RBC and save for virus isolation.
- iii) Discard medium from the vessel containing fresh monolayer cells (1–3 days old).
- iv) Inoculate the cells with a fraction of the clarified tissue or lysed RBC suspension, or previous passage cell culture.
- v) Incubate at 34–37°C for 1 hour. Cell culture flask caps should be loosened or vented caps should be used to allow for gas transfer.
- vi) Discard the inoculum and wash the monolayer with medium containing antibiotics once or twice. Add maintenance medium and return to the incubator.
- vii) Observe the cells for CPE regularly. CPE is only observed in mammalian cell lines and usually appears between 2 and 7 days post-inoculation.
- viii) If no CPE appears, a second and third passage should be attempted. Scrape the cells by using a scraper or freeze–thaw the cells once and inoculate fresh cultures.
- ix) If CPE is present suggesting the presence of virus, the identity of the isolate may be confirmed by reverse-transcription polymerase chain reaction (RT-PCR), antigen capture ELISA, immunofluorescence, or virus neutralisation.

1.1.2. Serogroup identification of isolates

i) *Molecular methods*

See Section 1.2.1 for PCR methods.

ii) *Immunological methods*

Orbivirus isolates are typically serogrouped on the basis of their reactivity with specific standard antisera that detect proteins, such as VP7, that are conserved within each serogroup. The cross-reactivity between members of the EHD and BT serogroups raises the possibility that an isolate of BTV could be mistaken for EHDV on the basis of a weak immunofluorescence reaction with a polyclonal anti-EHDV antiserum. For this reason, an EHDV serogroup-specific monoclonal antibody (MAb) can be used. A number of laboratories have generated such serogroup-specific reagents (Luo & Sabara, 2005; Mecham & Jochim, 2000; Mecham & Wilson, 2004; White *et al.*, 1991). Commonly used methods for the identification of viruses to serogroup level are as follows.

a) Immunofluorescence

Monolayers of BHK-21 or Vero cells on chamber slides (glass cover-slips) are infected with either tissue culture-adapted virus or virus in insect cell lysates. After 24–48 hours at 37°C,

or after the appearance of a mild CPE, infected cells are fixed with agents such as paraformaldehyde, acetone or methanol, dried and viral antigen detected using anti-EHDV antiserum or EHDV-specific MAbs and standard immunofluorescent procedures.

b) Serogroup-specific sandwich ELISA

The serogroup-specific sandwich ELISA is able to detect EHDV in infected insects and tissue culture preparations (Thevasagayam *et al.*, 1996). The assay is EHDV specific, with no cross-reactions with other orbiviruses such as BTV and African horse sickness virus (AHSV).

1.1.3. Serotype identification of EHDV isolates

i) *Molecular methods*

a) Polymerase chain reaction

Recent genome identification of EHDV isolates has enabled molecular identification of serotype and/or topotype by RT-PCR using serotype-specific primers followed by sequencing (Maan *et al.*, 2010; Sailleau *et al.*, 2012).

b) High throughput sequencing

High throughput sequencing may be performed on isolates with or without serotype-specific primers. Sequences may be compared with the GenBank library for serotype identity.

ii) *Immunological techniques*

a) Serotyping by virus neutralisation

There is a variety of tissue culture-based methods available to detect the presence of neutralising anti-EHDV antibodies. Cell lines commonly used are BHK-21 and Vero. Two methods to serotype EHDV are outlined briefly below. EHDV serotype-specific antisera generated in guinea-pigs or rabbits have been reported to have less serotype cross-reactivity than those made in cattle or sheep. It is important that antiserum controls be included. The WOAHA Reference Laboratory can supply control positive sera.

- Plaque reduction

The virus to be serotyped is serially diluted and incubated with either no antiserum or with a constant dilution of individual standard antisera to a panel of EHDV serotypes. Virus/antiserum mixtures are added to monolayers of cells. After 1 hour adsorption at 37°C and 5% CO₂, and removal of inoculum, monolayers are overlaid with cell culture medium containing 0.8–0.9% agarose. Plates/flasks are incubated at 37°C and 5% CO₂. After 4 days' incubation, a second overlay containing 0.01% (1 part per 10,000) neutral red and 0.8–0.9% agarose in cell culture medium is applied and the plates/flasks are incubated at 37°C and 5% CO₂. Flasks are examined daily for visible plaques for up to 3 more days. The neutralising antibody titres are determined as the reciprocal of the serum dilution that causes a fixed reduction (e.g. 90%) in the number of plaque-forming units. The unidentified virus is considered serologically identical to a standard serotype if the latter is run in parallel with the untyped virus in the test, and is similarly neutralised.

- Microtitre neutralisation

Approximately 100 TCID₅₀ (50% cell culture infective dose) of the standard or serial dilution of the untyped virus is added in 50 µl volumes to test wells of a flat-bottomed microtitre plate and mixed with an equal volume of a constant dilution of standard antiserum in tissue culture medium. After 1 hour incubation at 37°C and 5% CO₂ approximately 10⁴ cells are added per well in a volume of 100 µl, and the plates incubated for 3–5 days at 37°C and 5% CO₂. The test is read using an inverted microscope. Wells are scored for the degree of CPE observed. Those wells that contain cells only or cells and antiserum, should show no CPE. In contrast, wells containing cells and virus should show 75–100% CPE. The unidentified virus is considered to be serologically identical to a standard EHDV serotype if both are

neutralised in the test to a similar extent, i.e. 75% and preferably 100% protection of the monolayer is observed.

1.2. Molecular methods – detection of nucleic acid

1.2.1. Conventional reverse-transcription polymerase chain reaction

In recent years, several efforts were directed towards the development of innovative molecular techniques such as the RT-PCR for the rapid detection of EHDV nucleic acid (Aradaib *et al.* 2003; Clavijo *et al.*, 2010; Wilson *et al.*, 2009; Viarouge *et al.*, 2014). RT-PCR allows the detection of EHDV RNA in blood samples and other tissues. Moreover, serotype-specific RT-PCRs targeting segment 2 of the viral RNA have been developed (Brodie *et al.*, 1998; Maan *et al.*, 2010; Sailleau *et al.*, 2012), as well as multiplex real-time RT-PCRs for the discrimination between EHDV and BTM (Wilson *et al.*, 2009; Yin *et al.*, 2010). Although RT-PCR has high sensitivity and specificity, RT-PCR-based diagnosis should be interpreted with caution: the RT-PCR technique detects viral RNA with a very high level of sensitivity, but this does not necessarily indicate the presence of infectious virus. The duration of EHDV positivity by RT-PCR in blood is not known, but there is evidence that it lasts longer than the period over which infectious virus can be isolated, as demonstrated for BTM.

The capacity of RT-PCR assays to detect very small numbers of nucleic acid molecules means that such tests are exquisitely sensitive to contamination by extraneous nucleic acids. The latter may include any primers in use in the laboratory or previously amplified polynucleotides. It is critical therefore to have a 'clean' area containing all equipment necessary for reagent and test preparation and a separate area with its own equipment for amplification. Impervious gloves should be worn and changed frequently at all stages of the procedure, particularly after working with sample RNA or amplified DNA. This will help protect reagents and samples from contamination by ubiquitous RNases and other agents and from cross-contamination by DNA.

i) RNA extraction from blood, insects and tissue samples

A large variety of commercial kits are available; the RNA extraction step should be appropriate to the sample to be tested (e.g. blood vs tissue) and can be performed following directions suggested by the manufacturers.

ii) Reverse-transcription polymerase chain reaction

Several kits for one-step RT-PCR are currently available on the market. Alternatively, a double step procedure consisting of an initial RT step followed by cDNA amplification could be performed. Most of these methods still require a proper validation protocol. General recommendations that might be modified depending upon local/case-specific needs are indicated below. The method described by Aradaib *et al.* (2003) directed to S5 of EHDV has been taken as an example of conventional RT-PCR.

Primer sequences

For: 5'-TCG-AAG-AGG-TGA-TGA-ATC-GC-3';

Rev: 5'-TCA-TCT-ACT-GCA-TCT-GGC-TG-3'

- a) Primer stock solutions are diluted to a final concentration of 10 pmol/μl.
- b) One-step RT-PCR reaction tubes are labelled and 4.0 μl of primer mix is added to each tube. The tubes are held on ice.
- c) Next, 4 μl of test, positive and negative control RNA samples are added to 4 μl of the primer mix in RT-PCR tubes.
- d) Heat denaturation: 95°C for 5 minutes, then ice for further 3 minutes.
- e) One-step RT-PCR mix is prepared, based on direction of the manufacturer, containing reagents in sufficient volume for the number of samples being tested.
- f) One-step mix is added to the denatured mix to a final volume of 50 μl.
- g) The tubes are placed in a thermal cycler programmed for reverse transcription and cDNA amplification as suggested by the manufacturer.

iii) *Electrophoretic analysis of RT-PCR product*

The presence of EHDV in the RT-PCR products could then be visualised by using standard electrophoretic analysis procedures.

1.2.2. Real-time reverse-transcription polymerase chain reaction

Assays based on RT-PCR have been used to detect EHDV RNA in clinical specimens. Serogroup-specific RT-PCRs are directed toward the highly conserved genes of EHDV such as S9 or S10 or less highly conserved genes including S3. However, none of these assays is able to discriminate simultaneously all EHDV serotypes. There is limited (or no) information regarding the detectability of published assays for all serotypes, a real-time RT-PCR (Clavijo *et al.*, 2010) is reported to recognise and quantify all EHDV serotypes. The targeted gene of this method is represented by the NS1 gene. More recently, new real-time RT-PCRs have been described for the detection and typing of seven serotypes of EHDV (Maan *et al.*, 2017; Viarouge *et al.*, 2015).

The real-time RT-PCR described below (Viarouge *et al.*, 2015) is a duplex pan-EHDV real-time RT-PCR assay that amplifies a portion of segment 9 of all EHDV strains coupled with a real-time RT-PCR that amplifies the beta-actin RNA of ruminant cells (housekeeping gene). Moreover, a set of seven duplex serotyping real-time RT-PCR assays that specifically amplify both the EHD segment 2 portion of each serotype and the beta-actin gene is described. The Ct (cycle threshold) value obtained with the real-time RT-PCR that amplifies the housekeeping gene is used to verify the integrity of the total RNA extracted and the absence of inhibitors that can interact during the RT-PCR steps for each biological sample tested. These assays were developed and validated to detect and serotype EHDV for application to biological samples from the field.

Assay type	Primers/Probe Name	Sequence (5'-3')
Pan EHDV	EHDV_Seg9_F_7-25	AAT-TGC-GCA-TGT-CAG-CTG-C
	EHDV_Seg9_R_76-55	TTT-AAT-TCC-TCG-GTC-GAA-CGT-T
	EHDV_Seg9_P_29-44	FAM-TTT-GCT-CGC-ACC-CGG-T-MGB
Type EHDV1	EHDV1_Seg 2_F_8-31	TGT-GTC-AGG-ATG-GAG-GAC-ATT-AAC
	EHDV1_Seg 2_R_231-255	CGA-ATT-ATT-CCR-GTR-CTT-AAC-GCT-T
	EHDV1_Seg2_P_128-143	FAM-CCG-CAT-CAA-ATG-TAT-G-MGB
	EHDV1_Seg2_P'_128-141	FAM-CCG-CAC-CAG-ATG-TAT-MGB
Type EHDV2	EHDV2_Seg2_F_1642-1665 (W)	CCT-TTA-AGA-TAA-GAC-GGG-TCG-AGA
	EHDV2_Seg2_F'_1640-1666 (E)	GTC-CTT-TAA-GGT-AAG-ACG-GGT-AGA-GAT
	EHDV2_Seg2_R_1798-1770	CTC-AAG-ATA-TTA-CCG-GTT-AAG-CAT-AGA-GT
	EHDV2_Seg2_R'_1788-1170	TGC-CGG-TCA-TAC-AGA-ACG-C
	EHDV2_Seg2_P_1728-1743	FAM-AAC-GAG-ATG-TGG-CTT-C-MGB
Type EHDV4	EHDV4_Seg2_F_2573-2591	TAT-CAA-GCG-ACC-CAG-TCG-C
	EHDV4_Seg2_R_2771-2743	CGT-ATG-ACA-TTC-TGC-AAG-TCA-GC
	EHDV4_Seg2_P_2635-2653	FAM-CAC-ATC-TAC-GGA-TAC-TGT-G-MGB
Type EHDV5	EHDV5_Seg2_F_403-424	ACG-AAT-CGG-AGG-ATA-CGG-ATC
	EHDV5_Seg2_R_522-501	TCG-CGT-ATG-ATC-ACA-CTG-GTC-T
	EHDV5_Seg2_P_471-489	FAM-ACT-ATC-GGT-AGT-GGT-GTT-C-MGB
Type EHDV6	EHDV6_Seg2_F_560-582	GGA-TCT-GGA-ACG-TGC-TAT-GAT-CT
	EHDV6_Seg2_F'_561-582	GTT-CCG-GGA-CAT-GCT-ATG-ATC-T
	EHDV6_Seg2_R_734-714	CAG-CCT-GAA-TCT-TCG-TTT-GCT
	EHDV6_Seg2_R'_734-715	CCG-CCT-GAA-TTT-TTG-TTT-GC
	EHDV6_Seg2_P_686-702	FAM-ATA-ACG-AAC-AGG-GAG-CC-MGB

Assay type	Primers/Probe Name	Sequence (5'–3')
Type EHDV7	EHDV7_Seg2_F_2490-2508	CGA-GAG-GAA-CCG-ACC-GAA-G
	EHDV7_Seg2_F'_2490-2512	CGA-GAA-GAG-CCG-ATT-GAA-GAA-G
	EHDV7_Seg2_R_2627-2605	GCT-TAA-ATG-CGT-ATT-CAT-GGG-AT
	EHDV7_Seg2_R'_2629-2605	GTG-CTT-AAA-TGC-GTA-TTC-ATA-GGG-T
	EHDV7_Seg2_P_2520-2537	FAM-ACT-GTA-TGG-CCG-TAT-CTA-MGB
Type EHDV8	EHDV8_Seg2_F_822-846	ACG-ATC-CTA-TAA-TAT-CAC-GCT-TGG-A
	EHDV8_Seg2_R_897-878	TCT-TCG-ATC-CGC-TCA-CTG-C
	EHDV8_Seg2_P_853-869	FAM-AGC-TGA-TGA-ATG-GAT-GC-MGB

Primer and probe sequences for endogenous gene (Toussaint *et al.*, 2007)

Primers:

ACT_F_1005–1029 (5'-CAG-CAC-AAT-GAA-GAT-CAA-GAT-CAT-C-3'), ACT_R_1135–1114 (5'-CGG-ACT-CAT-CGT-ACT-CCT-GCT-T-3').

Probe sequences

Probe: ACT_P_1081–1105 (VIC-TCG-CTG-TCC-ACC-TTC-CAG-CAG-ATG-T-TAMRA).

The PCR amplification is performed using a real-time one-step RT-PCR kit. The mixtures contain 1× buffer, 0.5 µM of each EHDV-primer, 0.25 µM EHDV-probe, 0.375 µM of each beta-actin-primers, 0.25µM beta-actin-probe, 1 µl of enzyme mix, and 5 µl of denaturated template in a total of 20 µl of reaction volume. After RNA denaturation by heating at 95°C for 3 minutes, 5 µl of denaturated RNA was added to the mix and the amplifications were carried out using the same cycling parameters for all of the assays: 45°C for 10 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Fluorescence was measured during the 60°C annealing/extension step. Ct values were measured at the point at which the sample fluorescence signal crossed a threshold value (the background level). Negative results (for assays that did not exceed this signal level) are reported as 'No Ct'.

The Viarouge *et al.* (2015) method was validated according to the WOAH Validation Standard (except for the proficiency test, which was not undertaken). The real-time RT-PCR does not detect any of the 27 serotypes of BTV.

2. Serological methods

Following infection with EHDV, antibodies may be detectable from 8 days post-infection (Breard *et al.*, 2013; Eschbaumer *et al.*, 2012; Quist *et al.*, 1997). Like BTV, EHDV-infected animals may have neutralising antibodies and EHD viraemia at the same time; this is likely to be because of the strong association between the viruses and the RBCs. Duration of acquired immunity is still unknown but evidence from natural infections suggests it may last for life. A variety of serological methods, varying in sensitivity and specificity, are available to detect EHDV serogroup- and serotype-specific antibodies.

2.1. Competitive enzyme-linked immunosorbent assay (C-ELISA)

The EHD competitive ELISA (C-ELISA) was developed to measure EHDV-specific antibody without detecting cross-reacting antibody to other orbiviruses. These techniques making use of MAbs against EHDV VP7 are able to detect EHDV serogroup-specific antibodies (Luo & Sabara, 2005; Mecham & Jochim, 2000; Mecham & Wilson, 2004; White *et al.*, 1991), and currently are the preferred technique.

2.1.1. Test procedure

There are several test procedures described, and commercial kits are available. The following procedure gives one example of an EHD C-ELISA. The technique is similar to others except that in this technique the recombinant *Baculovirus*-expressed VP7 is captured by rabbit polyclonal anti-VP7-antibodies previously adsorbed to the wells (Mecham & Wilson, 2004).

- i) 96-well microtitre plates are coated with 100 µl of the recombinant VP7 expressed in *Baculovirus* overnight at 4°C (Luo & Sabara, 2005), and diluted in 0.05 M carbonate buffer, pH 9.6.
- ii) The plates are washed three times with PBST (0.01 M PBS containing 0.05% or 0.1% Tween 20, pH 7.4) and then blocked for 1 hour with 5% dry milk at room temperature.
- iii) After being washed with PBST, 100 µl of test sera is added in duplicate at a single dilution, either 1/5, 1/10 or 1/20 in PBST containing 2.5% dry milk.
- iv) Immediately, 100 µl of a predetermined dilution of MAb diluted in PBST is added to each well. MAb control wells contain diluent buffer in place of test serum.
- v) Plates are incubated for 1 hour at 37°C and then further washed with PBST as described above
- vi) After washing, wells are filled with 100 µl of an appropriate dilution of horseradish peroxidase-secondary antibody in PBST.
- vii) Following incubation for 1 hour at 37°C, the conjugate solution is discarded and plates are washed three times using PBST. Wells are filled with 100 µl substrate solution and the plates are shaken at room temperature for 30 minutes.
- viii) The reaction is stopped by addition of appropriate stopping reagent, and after blanking the ELISA reader on wells containing substrate and stopping reagent, the absorbance values are measured by using appropriate filters.
- ix) Results are expressed as per cent inhibition and are derived from the mean absorbance values for each sample by the following formula:
$$\% \text{ inhibition} = 100 - [(\text{mean absorbance test sample}) / (\text{mean absorbance MAb control}) \times 100].$$

NB: Some laboratories prefer to use a negative control serum that has previously been shown to have a percentage inhibition of zero as an alternative to the MAb control.
- x) Percentage inhibition values >50% are considered to be positive. Inhibition between 40% and 50% is considered to be suspicious. The results of the test sera duplicates can vary as long as the results do not lie either side of the positive cut-off.
- xi) Strong and weak positive sera and a negative serum should be included on each plate. The weak positive should give 60–80% inhibition and the negative should give less than 40% inhibition.

Commercially produced C-ELISAs based on recombinant VP7 and anti-VP7 MAb are now available. These commercial assays are routinely used in many laboratories across the world.

2.2. Virus neutralisation

The reference method for the identification and quantification of antibodies against EHDV serotypes present in test samples is the VN test. This technique detects and quantifies serotype-specific antibodies. The main disadvantage of the technique is that all suspected virus serotypes must be included in the assay, consequently, it can be a very time consuming and labour intensive test to perform. The VN test requires 3–5 days to be completed (Pearson *et al.*, 1992).

2.2.1. Test procedure

Cell lines commonly used are BHK-21 and Vero cells. A titre greater than or equal to 1/10 is usually considered specific for EHDV.

EHDV serotype-specific antisera generated in guinea-pigs or rabbits have been reported to have less serotype cross-reactivity than those made in cattle or sheep. It is important that antiserum controls be included.

- i) 50µl of serial sera dilutions, from 1/10 to 1/1280, are added to each test well of flat-bottomed microtitre plates and each mixed with an equal volume of known EHDV serotypes (100 CID₅₀). The plates are incubated at 37°C in 5% CO₂.

- ii) After 1 hour of incubation, approximately 10^4 Vero cells are added per well in a volume of 100 μ l, of minimal essential medium (MEM) containing antibiotics and, after incubation for 4–6 days, the test is read using an inverted microscope.
- iii) Wells are scored for the degree of CPE observed. A sample is considered positive when it shows 75% to preferably 100% CPE inhibition at the lowest dilution (1/10). The serum titre represents the highest serum dilution capable of reducing more than 75% CPE in cell culture.

2.3. Complement fixation

The complement fixation test (CFT) is sensitive and specific for EHDV diagnosis and was used until 1980 for diagnosis and certification of animals for export. The test is serogroup-specific and inexpensive with a sensitivity similar to the virus neutralisation (VN) and agar gel immunodiffusion (AGID) tests. CFT allows detection and quantification of antibodies for 4–12 months after infection but is less reliable after this period (Pearson *et al.*, 1992). CFT is particularly useful for detecting recent EHDV infections but not for detecting older infections.

2.4. Agar gel immunodiffusion

The AGID was widely used to detect EHDV antibodies from sera of infected animals (Dubay *et al.*, 2004). In the past this test was used for animal trade. It is simple, economical and the antigen used in the assay is relatively easy to generate. However, the disadvantage of the AGID is its lack of specificity in that it cannot discriminate between BTV and EHDV. Thus AGID positive sera should be retested using a serogroup-specific test, at least in those areas where BTV and EHDV may be co-circulating. Furthermore, although semi-quantitative the result of AGID is generally reported out as positive or negative. The AGID can detect antibodies from 5–15 days after infection to 2 years or more (Pearson *et al.*, 1992).

C. REQUIREMENTS FOR VACCINES

As an effort to control the disease, vaccines were developed in USA for captive wildlife deer farmers and in Japan to be used in cattle. Apart from these two limited settings, there has only been minor interest from laboratories and biologicals companies elsewhere to develop vaccines to control the disease or the virus circulation. Autogenous inactivated vaccines have been prepared in North America from EHDV isolates originating from ill or dead animals in affected premises. Their use must be approved by government authorities. Prior to release, these vaccines are tested for purity and safety. To make the products available expediently, autogenous vaccines are not tested for efficacy. Most of the applications are by deer farmers. As the vaccines use inactivated virus, two doses given 2–4 weeks apart are generally necessary to initially immunise the animals and a yearly booster is recommended.

In Japan, both live modified and inactivated vaccines have been developed to control Ibaraki disease. The live attenuated vaccine derived from the Ibaraki-2 strain was used following the outbreaks in 1980s and has been demonstrated to be safe and effective. The vaccine has to be administered once subcutaneously during the low vector season. The inactivated vaccine includes bovine ephemeral fever and Ibaraki viruses grown in cell cultures and inactivated by formalin, as an aluminium-gel adjuvanted vaccine. In Japan both vaccines are used on a voluntary basis according to the epidemiological situation.

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NB: There is a WOAHP Reference Laboratory for epizootic haemorrhagic disease
(please consult the WOAHP Web site:

<https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

Please contact the WOAHP Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for epizootic haemorrhagic disease

NB: FIRST ADOPTED IN 2014; MOST RECENT UPDATES ADOPTED IN 2021.