Epizootic Haemorrhagic Disease

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Part 1. Diagnostic Overview

Summary

Epizootic haemorrhagic disease (EHD) is caused by an infectious, noncontagious, insect-borne virus that infects most ruminants. EHD virus (EHDV) has been isolated in North and South America, Asia, Africa, the Middle East and Australia. Clinical signs of disease have been observed overseas mainly in deer and sometimes in cattle. These include a febrile response, erosive and ulcerative lesions of the oral and oesophageal mucosa, stiffness, lameness, and thickened, oedematous skin. These signs are indistinguishable from those caused by bluetongue disease.

EHDV is a member of the Orbivirus genus of the family Reoviridae. Within the Orbivirus genus there are 22 distinct virus species.[1] *The EHDV serogroup contains 7 recognised serotypes (numbered 1,2,4,5,6,7,8),*[2] *six of which occur in Australia. The previously designated EHDV serotype 3 has been included in EHDV serotype 1. In Australia, the six Australian serotypes of EHDV have not been shown to cause natural clinical disease in any of the species mentioned, although mild disease has been demonstrated experimentally in cattle and sheep. The emergence of EHD in EHDV-free zones and reports of clinical disease in cattle from EHDV-free zones prompted the inclusion of EHD in the OIE list of notifiable diseases in May 2008. The OIE list of notifiable diseases 2014 has classified EHD under multiple species disease.*

EHDV infection can readily be detected by direct isolation of virus or indirectly through the detection of group- or type-specific antibodies. The diagnostic tests currently used in Australia and New Zealand are: virus isolation, virus neutralisation (VNT), agarose gel immunodiffusion (AGID) and enzyme linked immunosorbent assay (ELISA).

Neither EHDV nor EHD antibody has been detected in animals in New Zealand.

Aetiology

Epizootic haemorrhagic disease is caused by a virus belonging to the *Orbivirus* genus of the family *Reoviridae*. Within the genus, EHDV is very closely related to bluetongue virus (BTV) group and shows immunological cross reactivity with bluetongue virus. Typically, viruses belonging to this genus are non-enveloped particles, 50-80 nm in diameter, icosahedral in symmetry and have a fibrillar outer coat.[3] The virion contains 10 strands of double stranded RNA and is resistant to lipid solvents, which is typical of non-enveloped viruses.[4,5,6] However, the virus is readily inactivated by acids and alkalis, sodium hypochlorite and phenolic compounds. The virus is sensitive to pH <6.0 and >8.0. The virus is stable in blood and tissue specimens at 20°C, 4°C and -70°C, but not at -20°C. However, the virus is inactivated at temperatures of 50°C for 3 hours, 60°C for 15 minutes and 121°C for 15 minutes.

Clinical Signs

All clinical signs described herein are those recorded from disease outbreaks or observations overseas. Naturally occurring disease has not been observed in cattle, deer or other susceptible species in Australia or New Zealand. The extent of clinical signs can vary from asymptomatic to severe depending on the serotype/strain involved, breed of ruminant and geographical location.[7] Generally, EHDV infections in domestic ruminants are mild and regress quickly, without complications. However, the Ibaraki strain (isolated in 1959 in Japan), classified as EHDV2, has been associated with sporadic outbreaks of severe disease of cattle in East Asia, with mortality rates of up to 10%.[8,9] Clinical signs develop in white-tailed deer (*Odocoileus virginianus*) about 7 days after exposure to the virus and include fever, erosive and ulcerative lesions of the oral and oesophageal mucosa, loss of appetite, excessive salivation, muscle weakness, stiffness, lameness, oedematous skin particularly around the face, depression and rapid pulse and respiration rates. Within 36 hours of the onset of signs, animals may enter a 'shock-like' state, become prostrate and die. In pregnant cows, EHD can result in foetal resorption or hydranencephaly if infection occurs between days 70 and 120 of gestation.[10]

Mule deer (black-tailed deer) (*Odocoileus hemionus*) display similar signs to those seen in white-tailed deer but are less severely affected.[11] Clinical signs in pronghorn antelope (*Antilocapra americana*) include disorientation, lethargy and bleeding from the oronasal cavity, urogenital tract and the rectum. Haemorrhaging animals usually die within 24 hours.[12] Affected bighorn sheep (*Ovis canadensis cremnobates* and *Ovis canadensis canadensis*) display a swollen head, face and lips, appear lethargic and, in severe cases, show a gradual deterioration leading to death.[13] In recent years, EHDV-associated sporadic outbreaks in cattle in many parts of the world have increased dramatically, suggesting an increased geographic distribution of EHDV.[14]

Experimental infection of cattle with Australian EHDV serotypes 2, 5, 6, 7 and 8 caused no clinical signs. Experimental infection of sheep with EHDV serotypes 2, 5, 6 and 8 caused a rise in temperature, with those sheep infected with EHDV serotypes 5, 6 and 8 displaying mild buccal hyperaemia and/or ulceration. No clinical signs were observed in the animals infected with EHDV serotype 7.[15]

Epidemiology

EHDV is spread between susceptible animals via a haematophagous insect host (*Culicoides* spp, midges). In the Northern Territory (NT), cattle can become infected between August and May each year during the summer, or wet season, when *Culicoides* numbers are at their peak. Distribution of EHDV closely shadows that of BTV, being present in the Northern Territory (NT), eastern Queensland (QLD) and north-eastern New South Wales (NSW).[16,17]

In surveys carried out by the virology laboratory of the Berrimah Veterinary Laboratories, NT, between 1979 and 2007, the duration of viraemia detected by virus isolation in cattle for each Australian EHDV serotype was:

EHDV-1	Maximum of 6 weeks. Mean 2-3 weeks
EHDV-2	Maximum of 8 weeks. Mean 3-4 weeks
EHDV-5	Maximum of 8 weeks. Mean 2-3 weeks
EHDV-6	Maximum of 2 weeks. Mean 1-2 weeks
EHDV-7	Maximum of 4 weeks. Mean 2-3 weeks
EHDV-8	Maximum of 3 weeks. Mean 1-2 weeks

Occurrence and Distribution

EHDV infection occurs in Australia, Africa, North and South America, Asia and the Middle East, in tropical and subtropical areas. There are seven recognised serotypes of EHDV worldwide (EHDV1,2,4,5,6,7,8) and six of these have been isolated in Australia (EHDV1, 2, 5, 6, 7, 8).[16,18,19,20] The geographic distribution of EHDV is similar to that of BTV. It is transmitted primarily by *Culicoides* spp (midges or gnats).[7,21] In Australia, EHD virus or antibody has been detected in QLD, NT and NSW.[16,20] EHDV is considered to be enzootic in the NT, eastern QLD and northern coastal NSW.

There have been no reports of naturally occurring clinical disease in Australia although the virus is isolated regularly in the NT and QLD under a sentinel cattle program (the National Arbovirus Monitoring Program). EHDV serotypes 1, 2, 5, 6, 7 and 8 occur in the NT while 2, 5, 6 and 7 occur in QLD.

Neither EHDV nor EHD antibody has been reported in New Zealand.

Gross Pathology

A variety of lesions have been described in outbreaks of EHD affecting white-tailed deer (*Odocoileus virginianus*) overseas. Peracute disease is characterised by degenerative changes (focal haemorrhage or grey-white lesions, or both) in striated musculature that are often prominent in the oesophagus, larynx, tongue, and skeletal muscles. Areas of discolouration, suggestive of mucosal erosion, can be present on the tongue and buccal mucosa. The acute disease also includes extensive haemorrhages on the serosal surfaces of the intestine and rumen. The lungs may be oedematous and cyanotic. Haemorrhages can occur at the base of the pulmonary artery and on the epicardial surface. There may also be oedema, hyperaemia, and petechial and/or ecchymotic haemorrhages of the brain, spinal cord, trachea, liver, gall bladder, kidney and lymph nodes.[11,12,13] The widespread haemorrhages in mucous membranes, skin, and viscera are the result of disseminated intravascular clotting. In the chronic disease there can be ulcers, scars, or erosions in the rumen. Extensive damage to the lining of the rumen can also cause emaciation.[22]

In susceptible deer, the disease is similar to bluetongue disease in sheep. The Ibaraki strain of EHDV2 can cause widespread vascular lesions in cattle similar to those described for bluetongue. Experimental infections have shown congenital lesions, such as hydranencephaly, similar to bluetongue. However, there are no reports of the shedding of EHDV in semen that occurs in bluetongue viraemic bulls.[23]

Diagnostic Tests (General)

EHDV infection can readily be detected by direct isolation of virus or indirectly through the detection of group or type-specific antibodies.

Virus isolation can be carried out on blood clots or unclotted whole blood. The duration of viraemia may depend on serotype and host. The most sensitive method for the detection of virus in the blood of the host is by the intravascular inoculation of lysed blood into embryonated chicken eggs (ECE), followed by passage to C6/36 (*Aedes albopictus*) cells and mammalian cell cultures. Several different mammalian cultures can be used, but BSR (cloned from baby hamster kidney clone 21 (BHK-21)) cells produce approximately 2 $\log_{10}$ higher virus yields than BHK-21 or Vero cells.

Methods currently used for the detection of EHDV antibodies in infected animals are the agarose gel immunodiffusion (AGID) test and the virus neutralisation test (VNT). Cross-reactions between EHDV and the related BTV and Palyam serogroups occur when using the group-reactive AGID test, and care must be taken in interpretation of the test results.

Molecular detection methods are also valid; however, they will not distinguish between viraemic and non-viraemic animals as RNA fragments can be detected for months after a viraemia has ceased. Virus isolation and real-time polymerase chain reaction (RT-PCR) should be used concurrently to validate each test.

A conventional RT-PCR has been developed by the Pirbright laboratory, United Kingdom, that is directed toward the VP2 region of the EHD genome[24] and the Elizabeth Macarthur Agricultural Institute, NSW, has developed a quantitative real-time PCR (qPCR) that can detect all EHD serotypes; however, neither the RT-PCR nor the qPCR is used as a routine diagnostic tool.

Part 2. Diagnostic Test Methods

Isolation of EHD virus

Principle of the Test

Clotted or unclotted (in EDTA or lithium heparin) blood samples are suitable for virus isolation. The samples should be transported, chilled, to the laboratory without delay. The most suitable sample for arbovirus isolation is washed red blood cells. EHDV, like bluetongue virus, rarely replicates in mammalian cells on the first passage. Isolation rates are significantly enhanced by passage of the diluted, lysed blood samples in ECE by intravascular inoculation. The ECE method is best employed to isolate bluetongue virus, hence further identification of the viral isolate is required.

Reagents and Materials

Post-mortem samples (lungs, liver, spleen, heart and kidneys) are homogenised in sterile brain heart infusion broth containing penicillin (5 mg/mL), streptomycin (3 mg/mL) and amphotericin B (12 µg/mL) prior to centrifugation.

Test Procedure

The sample (relevant tissue, clotted or whole blood) is clarified by centrifugation at 2000 ***g*** and 4°C for 10 minutes. The sample is then diluted 1:10 and inoculated intravenously into three ECE that are 9-11 days old.

Washed red blood cells are lysed 1:10 in sterile distilled water and inoculated intravascularly into at least three ECE that are 9-11 days old.

Clots are removed from the collection tube and a small amount (1 g) is lysed 1:10 in sterile distilled water and inoculated intravascularly into at least three ECE that are 9-11 days old. A

set of control eggs is inoculated with sterile distilled water and maintained similarly for further procedures. Use of a positive control is not recommended, to avoid cross contamination. If ECE are unavailable, then samples may be inoculated directly onto mammalian or insect cultures.

The ECE are incubated at 33-37°C for 5 days and candled each day. Embryos dying within 24 hours of inoculation are discarded (assumed to be non-specific deaths). Embryos dying 48-120 hours after inoculation are held at 4°C. At 120 hours, all remaining embryos are euthanased (by holding at -20°C for 40 minutes; do not freeze). All embryos are decapitated and harvested, pooling embryos that died at similar times and those that remained alive as separate pools. Embryos are homogenised in 5 mL of sterile brain heart infusion broth containing antibiotics, or alternate culture media, for use in the following cell culture inoculation.

Each sample is inoculated (15 µL/well) into two wells of a 96-well flat-bottomed cell culture microtitre plate containing a confluent monolayer of C6/36 (*Aedes albopictus*) cells. The medium is not changed prior to or after inoculation. Other suitable culture vessels can be used with appropriate volume of inoculum (e.g. tubes, 24-well plates or flasks). A pair of control wells without inoculum is kept as cell controls with every set of samples being tested. The samples are incubated at 26-28°C for 7 days. Each pair of wells is scraped using plastic or filter tips attached to an eight-channel pipette, and 15 µL of supernatant fluid is transferred to fresh plates containing confluent monolayers of C6/36, BSR[25] and porcine stable equine kidney (PSEK) cultures. In the author's experience, other mammalian cell lines, (BHK-21, Vero and hamster lung cell line (HmLu-1) are less sensitive than PSEK and BSR for the culture of the majority of Australian arboviruses.

The BSR and PSEK cell culture plates are incubated at 37°C, in 5% CO_2, in a humidified container for 7 days. Each well is inspected for the presence of cytopathic effect (CPE) from day 3 to 7 after inoculation. If CPE is not detected, a further passage from the second passage in C6/36 cells to fresh BSR and PSEK cell cultures is undertaken. Plates containing mammalian cultures and not showing CPE are discarded.

If no CPE is detected in the wells of the second mammalian cell culture pass (passage 3), the samples are presumed to be negative. If CPE is detected, 100 µL of the CPE-positive supernatant is transferred to a 25 cm^2 cell culture flask containing BSR cells. The supernatant and cells should be harvested at approximately 90% CPE to produce a virus stock that will later be used for virus identification. Controls should include a cell control (uninoculated monolayer of the same age and type of cells).

Interpretation of Results

A valid test requires the absence of CPE in the negative cell controls. CPE as a result of a positive test sample can usually be detected from day 3 after inoculation of the first mammalian cell culture passage.

Identification of EHD virus

All unidentified viral isolates are subjected to a serogrouping ELISA, indirect fluorescent antibody (IFA) serogrouping test, or VNT. The IFA test incorporates group monoclonal antibodies or polyclonal antibodies specific for each of the group members. Virus neutralisation serotyping tests are performed using antibodies to each member of the EHDV serogroup.

Virus identification by serogrouping ELISA[26]

Principle of the Test

The antigen from cell homogenate is coated onto microtitre plates. The group specific antibodies are raised in mice or rabbits as suitable animal models. Upon incubation with the antigen, an antigen-antibody complex will form. The presence of this complex is detectable using anti-species antibody labelled with horse-radish peroxidase (HRP). The unbound conjugate is washed away and a substrate/chromogen solution is added. In the presence of enzyme, substrate is converted into a product that reacts with the chromogen to generate a colour indicating presence of antigen in cell homogenate.

Reagents and Materials

Tetramethylbenzidine Substrate: Concentrate (wear gloves when working with this reagent)

Tetramethylbenzidine (TMB) powder	0.1 g
Dimethylsulphoxide (DMSO)	10 mL

Dissolve TMB in DMSO.

Dispense in 1 mL amounts into 5 mL vials wrapped in aluminium foil because it is sensitive to light.

Store at 4°C

TMB Substrate: Working

	1 plate
Double-distilled H_20 (DDH_2O)	4.5 mL
Citric Acid/Acetate Buffer	0.5 mL
TMB Substrate: Concentrate	50 µL
6.25 µL of 30% H_2O_2 to 56.25 µL DDH_2O	6.25 µL

Test Procedure

Wash the cells by resuspending the cell pellet in 1 mL of Dulbecco's phosphate-buffered saline (PBS), transfer to a 1.5 mL centrifuge tube and pellet by centrifugation at 6500 rpm for 30 seconds. Decant the PBS, invert the tube and allow to drain. Add 100 µL of 1M Tris buffer (pH 8.0), containing 34 mM (1.0% w/v) sodium deoxycholate, to the cell pellet, and homogenise the pellet

Coat the ELISA plates by adding 10 µL of the unknown antigen (cell homogenate from an infected cell culture) to 990 µL of ELISA coating buffer (150 mM Tris, 500 mM Tris HCl, pH 9). Add 50 µL per well and incubate and shake at 37°C for 60 minutes. Note: If pellets have been stored at 4°C in 50% glycerol, dilute 1:50 using coating buffer.

Wash plates three times between each step using PBS containing 0.1% Tween 20 (PBST). Add 50 µL/well of each titrated serogroup antibody to the appropriate wells. Add the appropriate negative control to each plate. A representative positive control panel is required per test. Incubate at 37°C, shaking, for 60 minutes. At the end of the antibody step, wash the plates three times with PSBT and tap dry. Do not allow plates to dry completely.

Dilute the conjugate in PBST with 5% skim milk powder (use protein G-horse HRP for polyclonal antibodies, and anti-mouse-HRP for mouse immune serum and monoclonal antibodies) and add 50 µL to each well. Incubate at 37°C, shaking, for 30 minutes.

Prepare substrate. Do not add H_2O_2.

At the end of the conjugate step, wash the plates three times with PSBT and tap dry. Do not allow plates to dry completely. Add calculated amount of H_2O_2 to TMB. Add 50 µL of substrate (TMB working solution) to each well. Incubate at room temperature for 10 minutes.

Stop the reaction by the addition of 50 μL of 1 M sulphuric acid to each well. Read the plate on a plate reader at 450 nm.

Interpretation of Results

Nett optical density (OD) = virus pellet OD – negative cell control OD
A Nett OD over 0.2 (when compared with the negative control) is considered significant. Cross-reactions do occur between BTV and EHD. The serogroup with the highest Nett OD is considered the specific serogroup for the specific virus under investigation.

Virus identification by IFA serogrouping test

Principle of the Test

The IFA test incorporates group monoclonal antibodies, or polyclonal antibodies specific for each of the group members. These antibodies will bind to the virus particle to form an antigen-antibody complex. Using anti-species antibody conjugated with fluorescein isothiocyanate (FITC) the antigen-antibody complex is visualised using a fluorescence microscope.

Reagents and Materials

Pretitrated group specific antibodies raised in mouse or rabbit models.

Pretitrated Anti-mouse/-rabbit FITC antibody with 0.4% Trypan blue.

Phosphate buffered saline.

Test Procedure

Infect a separate 25 cm^2 tissue culture flask containing BSR cells with 100 tissue culture infective dose ($TCID_{50}$) of each unidentified virus and incubate at 37°C. Allow the CPE to progress to 50%, then remove the supernatant and scrape the remaining cells from the flask and combine with the decanted supernatant. Centrifuge the suspension at 2000 ***g*** at 4°C for 15 minutes and resuspend the pellet in 4 mL of PBS containing 5% foetal bovine serum.

Prepare spot slides as described.[27] Briefly, add 30 μL of cell suspension to each spot of ten, twelve-spot slides. Air-dry the slides then immerse and fix in cold acetone for 15 minutes. Remove the slides from acetone and air-dry before rinsing in PBS (to remove crystalline deposits) and then acetone (to aid drying), and store at -20°C.

Dilute group specific (mouse or rabbit) antibodies 1:25 and add to a pre-recorded spot on each slide for each unidentified virus. Incubate all slides in a humidified container (150 mm disposable Petri dishes containing damp paper towel) at 37°C for 1 hour. After incubation, rinse the slides in PBS and then wash in PBS for 5 minutes. Air-dry the slides and add 30 μL of reconstituted anti-species fluorescein isothiocyanate conjugate containing 0.4% Trypan blue to each spot. Return the slides to the humidified container and place at 37°C for 1 hour. After incubation, wash the slides as previously described, but do not allow to dry. Lay a commercially available mountant (~100 μL) in a strip down the centre of each wet slide and place a large coverslip (60 x 24 mm) over the slide and remove air bubbles.[28]

Interpretation of Results

Fluorescence is visualised using a fluorescent microscope and subjectively estimated from 0 (negative) to 4+ (strong positive) and recorded for each spot. Spots recorded as 2+ or greater are considered positive.

Virus identification by VNT

Principle of the Test

The agent causing CPE in cell cultures can be serotyped using the Varying Virus – Constant Serum VNT. The unknown virus is diluted 10^{-1} to 10^{-6} and each dilution is incubated with a constant dilution of reference serum (to specific arbovirus/orbivirus isolates) and a known negative serum (usually foetal calf serum can be used). The test is read at 5-10 days and significant reduction is deemed to have occurred if the virus is neutralised 100-fold or greater. The virus is considered serologically identical to a standard reference strain of virus if the known virus is run in parallel with the unknown virus and is similarly neutralised.

Test Procedure

Use pre-titrated EHDV type-specific antibody to all Australian EHDV serotypes that will neutralise 2-$\log_{10}$ virus after 5 days of incubation. Using a 96-well microtitre plate add 50 µL of each serotype-specific antibody to each column down the plate. For example; EHDV1 to column 1, EHDV2 to column 2, EHDV5 to column 3 etc, until column 7, which receives 50 µL of diluent (cell line maintenance medium) and 50µL of virus (positive control), and column 8, which receives 100 µL of diluent (cell control).

Dilute each isolate, identified as an EHD virus, 1:30. Add 50 µL diluted virus across the plate to rows A1-7 to H1-7 (one unidentified virus per row). The last 6 viruses tested will be the EHDV prototype viruses as an antibody control, and the last row of the test will contain type-specific antibody and diluent as an antibody toxicity control. Incubate at 37°C for a minimum of 60 minutes. Add 100 µL of 2 x 10^5/mL BSR (or Vero, or BHK-21) cells to each well and return plates to the incubator in a sealed container. Read the plate on the third to fifth day after inoculation. The test should be read when virus titration shows 2 $\log_{10}$.

Interpretation of Results

Positive Test: One well should not show any CPE. The positive control well (Column 7) must show CPE, or in the event of cross-reactive or closely related viruses (e.g. EHDV 2 & 7), two wells will show no CPE.

Negative test: All wells, including the positive control must show no CPE.

Toxicity: One or more wells in the toxicity control will display CPE-like cell morphology or cell death.

Antibody detection by AGID

Principle of the Test

The AGID test to detect anti-EHDV antibodies is simple to perform and the antigen used in the assay is relatively easy to prepare. However, a disadvantage of this test is its lack of specificity because it detects antibodies to other orbiviruses, particularly those in the BTV serogroup. Thus sera that are positive by this test may have to be retested using an EHD serogroup-specific assay.

Wells of 4.0 mm in diameter (distance between wells is 2.4 mm) or 6.5 mm diameter (distance between wells is 3 mm) are cut in agarose plates in patterns of 7 wells consisting of a circular centre well and six surrounding circular wells forming the points and centre of a regular hexagon. Four patterns may be cut around the periphery of the agarose plate using a template for spacing. Antigen is placed in the centre well and test and reference sera are placed in the 6 surrounding wells. Antigen and serum antibody diffuse toward each other through the agarose. Where specific antibody and antigen meet at approximately equal concentration, a visible precipitin line forms.

Reagents and Materials

Dispense 15 mL of borate buffered agarose (2 g NaOH, 9.18 g boric acid, 0.1 g sodium azide and 10 g agarose in 1 L distilled water) into plastic 90 mm petri dishes to produce a 2.8 mm layer of agarose and allow to set. Cut circular wells, 4.0 mm in diameter and 2.4 mm apart, or 6.5 mm diameter and 3 mm apart in the agarose with six wells arranged around a central well. Prepare viral antigen as a soluble preparation from BSR, BHK or Vero cells infected with a single EHD serotype 24–48 hours previously. Antigen can be concentrated by precipitation, ultrafiltration or dialysis. Place three positive and three test sera in alternate wells surrounding antigen in the central well and incubate the plates at 20–25°C in a humid environment for 72-96 hours. A series of precipitin lines form between the wells containing antigen and known positive sera. Lines generated by strong positive test sera will join up with those of the positive controls. With weak positive samples the control lines bend toward the antigen and away from the test sample well, but may not form a continuous line between the control and test wells. With negative samples, the precipitin lines will continue into the sample wells without bending toward the antigen. All weak positive samples and other samples that produce questionable results should be repeated or retested using an EHD serogroup-specific assay.

Test Procedure

To prepare the agar plates, obtain borate saline or saline agar plates and punch 7-well patterns into the plate. Each pattern comprises one central well and six peripheral wells. Do not use the central area of the plate. Remove the agar plugs**.** Draw a line down the lip of the plate adjacent to a well to indicate well 1. Peripheral wells in each pattern are identified as 1 – 6, beginning with number 1 closest to the periphery of the plate and progressing clockwise.

Add 50 µL to the wells without spilling and underrunning the agarose, as follows:

- central well – antigen
- wells 1, 3 and 5 – reference serum
- wells 2, 4 and 6 – test sera (including the negative and positive control).

Use a new tip for each test sample. The plates are incubated for 72-96 hours. Ensure that there is no spillage within the plate. Any spillage of liquid from one well to another may lead to false positives, or the immunoprecipitate line may be compromised making the test unreadable. Plates are read at day 2 with a final reading on day 4 of the incubation.

The test results are valid if a precipitin line is formed between the antigen and reference serum wells (equidistant is the aim), **t**he positive control is positive, the negative control is negative and there is no evidence of solutions running between wells, gel bridges, or tears in the agar.

Interpretation of Results

Negative (-): The precipitin line continues into the test serum well without bending.

Weak Positive (1): The precipitin line bends slightly towards the antigen well, forming a complete line across the face of the test well, but still in contact with the well.

Positive (2): The precipitin line is weaker than the reference line but produces a continuous line across the face of the test well. The precipitin line is no longer in contact with the well.

Strong Positive (3): The precipitin line is the same strength as the reference serum. The line is formed half way between the test serum well and the antigen well.

XS (3+): The precipitin line is stronger than the reference line. The line is formed closer to the antigen well than the test serum well.

Non-specific lines: These lines will either cross the precipitin line, or fail to join the line smoothly. These lines are formed by antigen/antibody complex other than the test antigen.

Unreadable (X): The test is unreadable. Occasionally the precipitin line will not form, or the line formed is too faint to accurately read, or is obscured by a haze, non-specific lines, or scratches in the plate.

Antibody detection by VNT

Principle of the Test

This test is used only for testing serum that has previously been screened (by the AGID) with a result that is positive for the presence of EHD antibody. The VNT for EHD virus is carried out using aseptic technique in flat-bottomed 96-well microtitre plates that support the growth of cell monolayers. Two types of tests are performed: a screening test done in triplicate at a serum dilution of 1:4, and a titration of positive sera (determined from the screening test) from a dilution of 1:4 and done in up to four wells (quadruplicate).

Reagents and Materials

The serum for the test is heated at 56°C for 30 minutes before testing. For the screening test, aseptically dispense 50 μL of each serum into a microtitre well using a suitable pipette. Dilute the serum 1:4 in the well by adding 150 μL of appropriate diluent. Dispense 50 μL of each diluted sera into the next three wells, allowing the first well to be a serum control with three test wells. Add diluent to the serum control wells and virus to the test wells. The screening test serum control also acts as the serum control for the titration.

To titrate, make serum-dilutions of each serum in duplicate (minimum) from 1:4 to 1:128, using columns 1-6 or 7-12 with columns 1 or 7 being used for a serum control. Dilute the serum as described above. Add diluent to the next five wells and make serial two-fold dilutions across the plate using a multichannel pipette. Discard 50 μL from the last dilution well (6 or 12). All wells should now contain 50 μL of diluted serum. Up to four sera can be tested per plate.

Cells: Use BSR, Vero or BHK-21 cell lines.

Media/Diluent: For BSR cells, use Eagle's basal medium (BME) or Minimum essential medium (MEM) containing 5-7% inactivated foetal bovine serum and antibiotics as required. For BHK-21 cells, use BME containing 5% inactivated foetal bovine serum (FBS) or foetal calf serum (FCS) and antibiotics as required. For Vero cells, use Medium 199 containing 10% inactivated foetal bovine serum and antibiotics as required.

Virus: The virus to be used is EHDV1-8 diluted to contain 100 $TCID_{50}$ in 50 μL at five days after inoculation. The virus is diluted in 10-fold dilutions from 10° to 10^{-5}

Positive control serum: Use positive field sera of known titre.
Negative control sera: Use field sera negative for antibodies to EHD group viruses.

Test Procedure

Heat the test sera at 56°C for 30 minutes. Using flat-bottomed, 96-well, sterile, cell culture plates, dispense 50 μL of diluent to each well of the plate, except those of column 1 and column 7. Dispense 100 μL of 1:4 diluted test serum in column 1 and column 7 as necessary. Run each serum in quadruplicate (rows A, B, C, D, etc.). Serially dilute test serum across the plate from column 1 to column 6 (dilutions 1:4-1:128) using a multichannel pipette, thoroughly mixing each dilution and discarding 50 μL from column 6, when the dilution series is complete.

Alternatively, if sera are titrated directly rather than undergoing an initial screening test: add 150 µL of media to row 1 of the labelled tissue culture plates. Add 50 µL of media to rows 3-8 of the tissue culture plates. In duplicate, add 50 µL of respective test serum to row 1 (1:4 serum dilution) of the tissue culture plates. Mix well, then transfer 50 µL diluted serum from row 1 to row 2 (1:4 serum dilution) and row 3. Serially dilute each serum from row 3 (1:8 serum dilution) to row 8 (1:256 serum dilution) using a 50 µL single or multichannel pipettor. Discard 50 µL from row 8. Row 1 is a serum control (1:4 serum dilution) and tests for cytotoxicity of the test sera.

To prepare a Control Plate: A known positive serum is titrated; a known negative serum is tested (at least in quadruplicate at a 1:4 dilution, but this may be titrated. Alternatively, a known negative serum can be included on one of the standard test plates and treated in the same manner as a test serum); a cell control (containing only culture medium to a volume to replace all diluent, test serum and virus) (i.e. 100 µL per well) to which 100 µL of cells are added; and a 'back' titration of the stock virus dilution used in the test.

Dilute the virus using the appropriate diluent to give 100 $TCID_{50}$/50 µL. Add 50 µL of diluted virus (100 $TCID_{50}$/50 µL at day 5 after inoculation) to each well excluding the cell control, serum control and back titration wells.

Prepare a back titration by making 6 ten-fold dilutions of the test virus (100 $TCID_{50}$/50 µL). The dilutions are made using the test diluent. Each dilution is inoculated in quadruplicate across the plate. A virus titration of 2 $\log_{10} \pm 0.5 \log_{10}$ is an acceptable result.

Incubate all test and control plates at 37°C in 5% CO_2 for 1 hour.

Add 100 µL of cells to all wells at a seeding rate of $2x10^5$ cells per mL ($2x10^4$ cells/well) using a multistepper pipette. Incubate at 37°C in 5% CO_2 for up to 10 days (ideally 5 days after inoculation). The test must be read when the titre of virus in the virus titration wells indicates 100 $TCID_{50}$ of virus present in the test. When a titre of 100 $TCID_{50}$ is present in the virus titration, the antibody titre for the test sera can be calculated.

For the test to be valid, the cell controls on the control plate should contain a confluent monolayer of healthy cells, and the virus titre employed in the test should not vary by more than $\pm 0.5 \log_{10}$ (i.e. 1.5-2.5 $\log_{10}$/50 µL). If the virus titre is low, negative results may be accepted, but positives should be re-tested. When the virus titre is high, positive results will give a guide to titre, but all negatives should be re-tested.

The positive serum control should not vary more than two dilutions from its known titre in any one test. The test serum controls from the screening test will indicate when a serum is toxic to the cells. If toxicity is encountered, a result cannot be reported for that serum.

Interpretation of Results

If the test is valid, then a titre of 1:10 or greater is considered to be positive. Sera with titres of 1:4–1:10 could be considered equivocal to account for cross-reactions between various serotypes as detailed previously.

The VNT can be difficult to standardise due to fluctuations of the titre of the virus. Titres may vary depending on the growth rate, passage level of the BSR cells, or strain of EHD virus being used. The BSR cell line is used in preference to the Vero cell line as it consistently yields a higher virus titre than Vero cells.

Each batch of FCS should be tested for specific neutralising antibodies to the target virus. FCS found to contain antibody should not be used for that test or rejected outright in

preference to a more compatible batch of FCS. It is preferable to have several technicians able to do EHD VNTs.

If the media becomes too alkaline (pink/purple), EHDV replication is affected. This should not be a problem if plates are incubated in a well-maintained and calibrated, humidified CO_2 incubator.

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Part 3. Reagents and Test Kits

The list of suppliers provided below may not be exhaustive but includes all materials (complete kits and other reagents) that have been evaluated and found to be suitable for use under Australian and New Zealand conditions. These reagents and kits have been approved only for use in Australia and New Zealand for the purposes, and within the limits, described below. Other suppliers who have materials that may be used for the purposes described below are welcome to submit reagents and kits for evaluation by contacting the Executive Officer, SCAHLS <www.scahls.org.au>.

EHDV AGID Reagents

All key reagents (Antigen and control sera) for the EHDV AGID are available from:

Virology Laboratory, Berrimah Veterinary Laboratory, DPIF, Makagon Road, Berrimah, Northern Territory 0828

Australian Animal Health Laboratory, CSIRO, 5 Portarlington Road, East Geelong, Victoria 3219

Virology Laboratory, Elizabeth Macarthur Agricultural Institute, Woodbridge Road, Menangle, New South Wales 2568.

Other reagents and consumables can be obtained from a variety of commercial suppliers

VNT reagents

Reference viruses and control sera for the EHD VNT are available from Virology Laboratory, DPIF, Darwin. Other reagents and consumables can be obtained from a variety of commercial suppliers

Virus typing test

Reference viruses and control sera for the EHD VNT are available from (Virology Laboratory, DPIF, Darwin). Other reagents and consumables can be obtained from a variety of commercial suppliers