AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

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Bluetongue

Virology and Serology

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1. Introduction

Bluetongue virus (BTV) occurs world wide in the tropics and subtropics wherever substantial populations of ruminants are pastured. Twenty-four serotypes of bluetongue (BLU 1–24) are recognised and eight (BLU 1, 3, 9, 15, 16, 20, 21 and 23) have been recovered in Australia. All eight types have been isolated in the Top End of the Northern Territory and BLU 1 and 21 have been recovered from Queensland and New South Wales.

The ecology of this midge-borne virus in Australia is not understood but BTV is considered enzootic in the north of the continent with virus activity in favourable seasons extending into New South Wales as far south as Sydney. BTV has never been recorded in Victoria, South Australia, Tasmania or southern Western Australia.

In Australia BTV is generally endemic in the northern cattle-raising areas and is spread by *Culicoides* midges which preferentially feed on cattle. Infection of cattle is subclinical. Even though Australia's BLU serotypes can cause disease in sheep experimentally, infection of sheep in the field is uncommon, and disease has yet to be recorded, in commercially farmed sheep.

2. The Disease

2.1. Host Range

BTV is a classical arbovirus, with a small number of *Culicoides* species having the capacity to transmit the virus to ruminant vertebrate reservoirs. Cattle appear to be the natural hosts of the virus and although BTV seems to be able to multiply in all species of ruminants, sheep are normally the only species where infection causes disease.

2.2. Clinical Signs

A sheep with classic, acute BLU disease is initially pyrexic and then exhibits the clinical hallmarks of congestion, oedema and haemorrhage, especially of the mouth, nose, conjunctiva and coronets. Sheep may be lame as a result of coronitis in acute cases or of skeletal myopathy in subacute cases. The clinical manifestations of BLU can vary considerably, and the predominant signs in an affected flock may be lameness, rhinitis or ill-thrift.

2.3. Pathology

Lesions of congestion, oedema and haemorrhage may be seen at post mortem. Subcutaneous gelatinous oedema may be observed, especially on the head, neck and thorax. Haemorrhages and ulcers are common in the buccal cavity, especially opposite the molar teeth. Haemorrhages in the wall of the pulmonary artery at its base are considered pathognomonic.

2.4. Differential Diagnosis

Causes of facial and nasal congestion, oedema and haemorrhage, lameness, respiratory disease and depression must be considered. These include photosensitisation, orf, facial eczema, footrot, pneumonia and haemonchosis.

2.5 . Collection of Specimens for the Diagnosis of Bluetongue

The best source of virus, and the diagnostic specimen of choice, is blood from early acute cases. A vacuum tube containing heparin should provide a sterile uncoagulated blood sample. Post mortem samples of spleen, heart and lymph nodes may be collected for virus isolation but heparinised blood from acutely infected in-contacts is preferred. Diagnostic specimens for virus isolation should not be frozen. Samples are best held chilled before attempted virus isolation.

Serum antibody usually develops one to two weeks after infection so chronically infected and convalescent sheep should have detectable antibody.

3. Serology

The four tests used routinely in BTV serology are the complement fixation (CF) test, the agar gel immunodiffusion (AGID) test, the competitive enzyme-linked immunosorbent assay (C-ELISA) test and the serum neutralisation (SN) test. The first three tests detect group specific antibodies, i.e. antibodies shared by all BTV serotypes, whereas the serum neutralization test detects serotype specific antibodies which are usually different for each BTV serotype although cross reactions occur between some serotypes. Antibodies to BTV detected in the CF test are generally only present for four to six months post infection, AGID antibodies are usually detectable for longer, especially in sheep, whereas C-ELISA and SN antibodies normally persist for some years, probably life long.

4. The Bluetongue Complement Fixation Test

4.1. Introduction

The CF test has now fallen out of favour because of its generally low specificity and sensitivity and frequent anticomplementary reactions. It is expected that Office International des Epizooties (OIE) will soon suggest that the internationally recognised serological tests for BLU be AGID and C-ELISA. Because some countries still require the CF test on ruminants for export from Australia, the method is described here.

4.2. Preparation of Standard Reagents

Standard reagents used in the CF test are described by Alton (1977).

4.3. Enhancing Serum

The CF test described is a modification of that used by the Canadian workers

(Boulanger *et al.*, 1967) who employed a supplementary factor of 5% of a pretested negative, unheated, normal calf serum in the complement diluent. This serum was found to increase the sensitivity of the test at least four-fold. The exact nature of the enhancing factor is obscure except that the extra complement contained in the calf serum is added to the test.

Incorporation of 2.5% enhancing serum to the complement has been found most effective. The 'enhancing' serum is selected from unheated normal calf serum which is pretested with a known BTV CF antigen and reference antiserum. A satisfactory enhancing serum is considered to be one which increases the CF titre of the reference antiserum the most (when compared to other possible enhancing sera, and a negative control with no enhancing serum) without showing any non-specific reaction or anticomplementary activity. Many calves (6–12 months old preferably) may have to be screened before a satisfactory serum is located. Once such an animal is found, satisfactory enhancing serum may be recovered for some months from the same animal. The serum which is to be tested as a possible enhancing serum should be separated from the clot on the same day as bleeding and stored immediately. A large batch of enhancing serum can be stored in small aliquots at -70°C or in Richardson's solution (Alton, 1977 for detailed method) at 4°C. Using the former method of storage, sera retained the enhancing activity for up to 30 months; with the latter preservation method sera have been stored successfully for up to six months.

4.4. Performing the Test

The test is performed in disposable plastic microtitre plates with U-shaped wells. All solutions and reagents used in the test are stored at 4°C and where possible the test is performed with all components at 4°C. Eight heat inactivated (60°C for 30 min) sera can be titrated on each plate. Using an automatic syringe, 100 µL of CF diluent (Oxoid Complement Fixation Test Diluent) is added to well No. l; 25 µL of diluent is added to wells 2–11 using a microtitre dropper pipette (25 µL drops). Then 25 µL of the inactivated serum sample is added to well 1; this gives a 1/5 dilution. Using a microdiluter (volume 25 µL) serial two-fold dilutions are made from wells 2–6, using the 1/5 diluted sample from well 1; then the same dilution steps are used from well 1 to wells 7 and 8 and from well 1 to wells 9–11. This gives a series of dilutions as shown in Table 1.

Table 1. Dilution of serum samples

Well No.:	1	2	3	4	5	6	7	8	9	10	11
Dilution of serum sample	5	10	20	40	80	160	10	20	10	20	40

One drop (25 µL) of complement (see 4.6.), containing 2.5% enhancing sera and diluted to give 2 mean haemolytic doses (MHD) units in the presence of antigen, is added to each well except wells 1 and 12. This is followed by one drop (25 µL) of diluted BTV antigen (see 4.7. for preparation) to wells 2–6. To wells 7 and 8 one drop (25 µL) of diluent is added (these wells represent the serum control and will detect anticomplementary activity) and to wells 9–11 one drop (25 µL) of diluted normal tissue culture control antigen (CON AG; see 4.7.) is added (these wells represent the control antigen).

The reagents are mixed by tapping the plate gently. The plates are covered with a layer of 3 inch wide (7.3 cm) cellotape (Vine Brand) and incubated overnight (18 hours) in a refrigerator (4–10°C); cellotape is used in preference to plastic lids as the system is sealed, hence no evaporation occurs. After overnight refrigeration the plates are transferred to a 37°C incubator for 30 min, then one volume (25 µL) of 1.5% sensitised sheep erythrocytes (see 4.8.) is added to wells 2–11. The plates are resealed with the tape and transferred to a shaker, at 37°C, for 30 min. The plates can then be either centrifuged at 1840 *g* for four minutes (International centrifuge equipped with plate carriers) or left at 4°C for three to four hours to allow the erythrocytes to settle. With the latter method pellets may not fully develop due to the presence of a haemagglutinin in some sera; this means more care will be required in reading the percentage of cell lysis. Generally haemagglutination only presents a problem at low serum dilutions (i.e. 1/10).

4.5. Reading and Interpretation of Test

The results are read with the aid of a platform with a mirror mounted underneath that reflects the pattern of the wells. The degree of lysis in each well is read from the diameter of the erythrocyte pellet in relation to the red cell control well (three drops of diluent plus one drop of sensitised red blood cells) and the degree of haemolysis present in the supernatant. Titres of the sera are expressed in terms of the highest dilution at which 50% haemolysis or less occurred with the antigen. At no time may the titres read for the anticomplementary and non-specific controls be subtracted from the test sera results.

Controls in the test should include a titration of the working dilution of complement in the presence of:

(a) CF diluent;
(b) the working dilution of BT CF antigen; and
(c) the working dilution of normal tissue culture control antigen by making the dilutions in CF diluent of the complement as shown in Table 2.

Table 2. Dilution of complement

Well:	1	2	3	4
Dilution of complement:	1/1	1/2	1/4	1/8
CF diluent				
BTV CF AG				
CON AG				

This is incubated overnight with the test. The complement in the presence of antigen should show full lysis at the dilution of 1/2, i.e. two full units of complement. The working dilutions of the BTV CF and CON CF antigens are prepared in dilutions of 1/1, 1/2, 1/4 and 1/8 and incubated with a working dilution of the standard BTV antiserum (see 4.7.) and complement. Complete fixation of complement should occur at 1/1 and 1/2 dilutions with the BTV CF antigen (i.e. two full units of antigen) and not with the CON AG.

Examples of test and control plates are given below, accompanied by their interpretation.

4.5.1. Plate 1 (see Table 3)

4.5.1.1. Interpretations — Plate 1

A – The titre of this serum sample cannot be read at a dilution of 1/20 as the sample is anticomplementary and non-specific. Hence the result is AC 1/20, NS 1/20.

B – Serum titre 1/10.

C – Serum titre 1/20, AC 1/10.

D – Once again the titre of this serum cannot be read as it is anticomplementary at ≥1/20. Hence the result is AC ≥1/20, NS 1/20. This sample could be retitrated, titrating the serum control to 1/40 to see if the serum is anticomplementary at this dilution.

E – Serum titre ≥1/160, AC 1/10, NS 1/10.

F – AC 1/10, NS 1/10.

G – AC ≥1/20, NS 1/40.

H – AC ≥1/20, NS ≥1/40. It may be possible to obtain the titre of this serum by retitrating the serum control, the normal tissue culture control antigen, and the BT CF antigen to 1/320 with the test serum to determine whether the CF titre of the serum exceeds the anticomplementary and non-specific titres.

Table 3. Example of test plate

Well No.:	1	2	3	4	5	6	7	8	9	10	11	12
Serum dilution:		10	20	40	80	160	10	20	10	20	40	
Serum sample:												
A	*	4	4	1	—	—	4	2	4	2	—	
B	*	4	tr	—	—	—	—	—	—	—	—	
C	*	4	3	—	—	—	4	—	—	—	—	
D	*	4	4	3	—	—	4	4	4	3	—	
E	*	4	4	4	4	4	2	—	2	—	—	
F	*	2	—	—	—	—	2	—	2	—	—	
G	*	4	4	2	—	—	4	4	4	4	2	
H	*	4	4	4	4	4	4	4	4	4	4	

*Dilution of test sera to 1:5.
Percentage Fixation of Complement: 4, complete fixation of complement; 3, 25% or less of cells lysed; 2, 50% of cell lysis; 1, 75% of cell lysis; tr, trace of complement fixation; — , total cell lysis; NS, non-specific reaction; AC, anticomplementary activity.

4.5.2. Plate 2 — (see Table 4)

4.5.2.1. Interpretations — Plate 2

A – Complement shows greater than two full units at the working dilution.

B – Complement shows two full units at the working dilution in the presence of BTV CF antigen.

C – Complement shows two full units at the working dilution in the presence of control antigen.

D – The BTV CF antigen shows two full units of activity in the presence of the working dilution of bluetongue reference antiserum.

H – The control antigen shows no activity in the presence of the working dilution of the bluetongue reference antiserum.

4.6. Preparation and Testing of Complement

Guinea pig complement is collected and stored according to Richardson's method (Alton, 1977) at 4°C or is stored directly in sealed ampoules frozen at or below -70°C. The titration of complement may be done using a spectrophotometer (Alton, 1977) or as follows:

(a) Dilute 1 mL of preserved complement in 7 mL of cold (4°C) distilled water. This restores the tonicity, being equivalent to a 1/10 dilution of the original guinea pig serum.

(b) Prepare a 1/100 dilution in cold CF diluent by adding 1 mL of the above 1/10 dilution of complement to 9 mL of diluent.

(c) Prepare the following further dilutions in cold diluent (Table 5).

The following titration is then made:
To each in a series of nine CF tubes, 0.5 mL of diluent is added followed by 0.25 mL of one of the above dilutions of complement to eight tubes; an extra 0.25 mL of diluent is added to the last tube which acts as a control. The tubes are incubated in a water bath at 37°C for 30 min after which 0.25 mL of a 1.5% sensitised sheep erythrocyte suspension is added to each tube. The tubes are then incubated a further 30 min at 37°C with shaking every 10 min. Following

Table 4. Example of control plate

Well No.:		1	2	3	4
Complement dilution:		1:1	1:2	1:4	1:8
CF diluent:	A	—	—	1	4
BTV CF antigen:	B	—	—	3	4
Control antigen:	C	—	—	2	4
	D				
	E				
	F				
BTV CF antigen dilution:		1:1	1:2	1:4	1:8
BTV antiserum:	G	4	4	—	—
Control antigen dilution:		1:1	1:2	1:4	1:8
BTV antiserum:	H	—	—	—	—

Table 5. Dilutions of diluent

1/100 Complement dilution							
1	1	1	1	1	1	1	1
Diluent							
1	1.5	2	2.5	3	3.5	4	4.5
Final dilution							
1/200	1/250	1/300	1/350	1/400	1/450	1/500	1/550

incubation the tubes are centrifuged to sediment the cells and the amount of haemolysis estimated to determine the titre of complement, i.e. the quantity of complement required to lyse 50% of optimally sensitised erythrocytes is determined, which is 1 MHD_{50}.

Once a titre for the complement has been established with a particular batch of sheep erythrocytes, a working dilution can be confidently prepared for use each day in the CF test using a diluent with dilutions being made in CF diluent containing 2.5% enhancing serum. It is necessary however, that this dilution be checked and, if necessary, adjusted by a check titration of the complement dilution being used in the test in the presence of the BTV CF antigen and the control antigen. Two MHD of complement in the presence of the antigen has been used and this corresponds to about 5 MHD_{50} units when the complement is diluted in diluent containing 2.5% enhancing serum.

4.7. Production and Titration of Bluetongue Virus Complement Fixation Antigen and Control Antigen

4.7.1. Growth of Bluetongue Virus in Tissue Culture

Vero cells are grown to confluency in roller bottles containing 200 mL of Medium 199 supplemented with 5% heat inactivated foetal calf serum. The bottles are seeded with sufficient cells (5×10^7) to produce a confluent monolayer in about four days of incubation at 37°C on a roller apparatus.

Before inoculation, the cell sheets are washed with about 20 mL of Hank's BSS containing 0.2% bovine serum albumin (BSA). Then 1–5 mL of BTV seed virus (titre 10^6 tissue culture infective dose 50% ($TCID_{50}$)/mL] is added. This is allowed to adsorb while rolling the bottle at 37°C for 30–60 min. The inoculum must be evenly distributed over the monolayer. Following adsorption, the inoculum is removed and the cell sheet washed once with 20 mL of Hank's BSS containing 0.2% BSA and then 50 mL of either minimal essential medium (MEM) with 0.2% BSA or Medium 199 with 0.2% BSA is added to each bottle.

On the third day post infection or when at least 50% of the cell sheet shows cytopathic effects, the medium is harvested, clarified by centrifugation at 10 000 *g* for 10 min at 4°C and the supernatant stored at 4°C in a sealed container. The fluid from uninoculated Vero roller bottle cultures is similarly collected, treated and stored as the control. Stocks of live virus are stored in sealed ampoules at or below -70°C.

4.7.2. Inactivation of Bluetongue Virus in Antigen Preparations

To the clarified live virus antigen a 1/10th volume of 1 mol/L Tris-HCl buffer is added. The buffered, live antigen is transferred to a wide mouthed sealable (screw cap) flask taking care not to contaminate the neck. This container is placed in an ice bath. When the antigen is cooled to 4°C, β-propiolactone (Sigma, >99% pure by saponification) is added slowly with constant stirring to a final concentration of 0.2% v/v (*N.B.* a precipitate will appear immediately, which is normal). The mixture is stirred every minute for 10 min at 4°C and then incubated at 37°C for two hours. The pH drops from about 7.6 to 7.0. After storage for 24 hours at 4°C, the material is then clarified by centrifugation at 10 000 *g* for 10 min at 4°C. The supernatant, inactivated CF antigen, is stored at -20°C in small aliquots (5–10 mL). The supernatant from uninoculated Vero cultures is similarly treated with β–propiolactone and clarification before storage at -20°C as control antigen.

4.7.3. Testing Batches of Antigen

Each batch of CF antigen is titrated using a standard bluetongue CF antiserum prepared by inoculating cattle with BTV grown in BHK-21 cells. A block titration is performed to determine the optimum antigen and antiserum dilutions to use in the test; increasing dilutions of antigen being tested against increasing dilutions of antiserum. One unit represents the highest dilution of antigen that will give complete fixation with the highest dilution of antiserum; it thus represents what is essentially the optimal proportion between antigen and antibody.

The block titration is set out as shown in the example below (Table 6) including also a serum control and an antigen control.

Serial two-fold dilutions are made of the antigen and one volume (25 µL) of complement dilution added followed by one volume of appropriately diluted antiserum and incubated overnight at 4°C. The mixtures are then incubated at 37°C for 30 min, one volume of 1.5% sensitised erythrocytes added, and the plate incubated

Table 6. Block titration

Serum dilutions:	1/5	1/10	1/20	1/40	1/80	1/160
Antigen dilutions:						
1/2	4	4	4	4	2	—
1/4	4	4	4	2	—	—
1/8	4	4	2	—	—	—
1/16	3	tr	—	—	—	—
1/32	—	—	—	—	—	—
1/64	—	—	—	—	—	—

with shaking at 37°C for 30 min. After centrifuging at 1850 *g* for four minutes, or holding at 4°C, for three to four hours the plates are ready to determine the antigen and antiserum titres. In the above test plate the antigen and antiserum titres are 1/16 and 1/80, respectively.

The antigen and antiserum are diluted to give two full units of working antigen and antiserum, respectively. In the above example the antigen diluted 1/4 and the antiserum diluted 1/20 would give two full units of antigen and antisera.

Each dilution of antigen is used in a check titration of the complement used in the test to detect the level of non-specific fixation of complement by the antigen.

Each batch of control antigen is similarly titrated with the standard bluetongue antiserum.

4.8. Preparation and Storage of Sensitised Sheep Erythrocytes

They are prepared as described by Alton (1977).

4.8.1 Collection and Storage of Sheep Blood

Sheep known to produce erythrocytes of a consistently satisfactory level of sensitivity should be chosen and used exclusively. Blood is withdrawn under aseptic conditions into an equal volume of Alsever's solution (Alton, 1977) and thoroughly mixed. The preserved sheep blood is stored aseptically in screw-capped bottles at 4°C and should not be used for at least five days after collection; thereafter, it may be used for one month.

4.8.2. Washing the Erythrocytes

Up to 10 mL of sheep blood stored in Alsever's solution is placed in a 50 mL centrifuge tube which is filled with diluent and the contents thoroughly mixed. The suspension is then centrifuged and the supernatant discarded with the thin layer of white cells that overlays the pellet. The erythrocytes are suspended in fresh diluent and the centrifugation repeated. For the third and final centrifugation the erythrocytes are resuspended in about 15 mL of diluent and centrifuged in a graduated tube at 1000 *g* for 10 min. The deposit is used to prepare the suspension.

4.8.3. Standardisation of the Erythrocyte Suspension

The standardised erythrocyte suspension used should contain 0.95 g haemoglobin per 100 mL as determined by the cyanmethaemoglobin method (Alton, 1977), which is equal to a suspension containing $6x10^5$ erythrocytes as determined in an electronic cell counter. Such a suspension is equal to a 3% suspension of erythrocytes obtained by centrifugation at exactly 1000 *g* for 10 min of a suspension of sheep erythrocytes in a graduated centrifuge tube and resuspending the pellet in 32.3 times its volume of diluent. For a routine preparation of the standardised erythrocyte suspension see Alton (1977).

4.8.4. Haemolysin (Amboceptor)

The haemolysin is titrated to determine the concentration which, when added to an equal volume of standardised erythrocyte suspension, will produce an optimally sensitised erythrocyte suspension. Haemolysin used in this test is one commercially available (CSL Ltd, Poplar Rd, Parkville, Vic. 3052, Australia. Tel. (03) 389 1911; Fax (03) 389 1646], usually in liquid form preserved with an equal quantity of glycerine. In use a 1/100 stock dilution of glycerinated haemolysin may be made in CF diluent and stored in frozen aliquots. The haemolysin is titrated each time a batch of 1/100 dilution is made and each time a new batch of erythrocytes is brought into use.

4.8.5. Method of Titrating Haemolysin

Prepare the standardised erythrocyte suspension as already described. From the 1/100 stock of haemolysin prepare the following range of dilutions in CF diluent: 1 in 500, 1000, 1500, 2000, 3000, 5000 and 10 000. Add 1 mL of each haemolysin dilution to 1 mL of standardised erythrocyte suspension while gently agitating the erythrocyte suspension, then leave the mixtures at 37°C for 15 min to allow sensitisation of the erythrocytes to occur, agitating the tubes every five minutes. The rest of the procedure is done in duplicate, the tubes being kept cold, while the reagents are being dispensed. To each of a duplicate series of seven tubes add 1.0 mL of diluent and 0.5 mL of complement, diluted so that it will produce about 70–80% haemolysis with the more concentrated haemolysin dilutions. From each of the haemolysin erythrocyte mixtures in turn, 0.5 mL is transferred to each of a pair of tubes containing complement and diluent and thoroughly mixed. The tubes are then incubated for 30 min in a 37°C water bath with gentle shaking. After 15 min remove the tubes from the water bath, add 2 mL of cold diluent to each, then centrifuge the tubes to deposit any erythrocytes remaining unlysed. Pour off the supernatant from each tube and read the optical densities (ODs) in a photometer. The OD given by 100% haemolysis is the same as the target OD used in preparing the standardised erythrocyte suspension (Alton, 1977).

Calculating the percentage haemolysis for each tube in the titration, e.g. the OD produced by 100% lysis is 0.5; therefore, a tube with an OD of 0.21 would have:

$[(0.21 \times 100)/0.5] = 42\%$ haemolysis.

Plot the percentage haemolysis given by each dilution versus the haemolysin dilution (Alton, 1977). The optimal dilution of haemolysis for use in the test is decided by determining where the

plateau begins and selecting a dilution about 25% more concentrated. The quantity of haemolysin to use in the test is not critical so long as an ample amount is selected. This quantity forms a fixed point against which the amount of complement to be used is determined accurately.

5. The Bluetongue Agar Gel Immunodiffusion Precipitation Test

5.1. Introduction

An AGID precipitin reaction is recognised to be common to all members of the bluetongue group. The AGID test lacks the sensitivity and specificity of the ELISA and SN tests but has proved a very useful serological procedure. The AGID test is relatively simple to perform and interpret and can be provided by laboratories with basic resources. The test may be used to demonstrate antibody in sera or antigen in infected cell cultures.

5.2. Testing Procedure

5.2.1. Medium

The medium is borate buffered agarose prepared to the following recipe:

Sodium hydroxide, NaOH	0.2 g
Boric acid, H_3BO_3	0.9 g
Sodium azide, NaN_3	0.01 g
Agarose	1.0 g
Distilled water	100 mL

Dissolve salts in water, check pH and adjust to 8.5–8.6 with 1 mol/L sodium hydroxide if necessary. This borate buffer is used as diluent for reference antigen and serum.

Add agarose to borate buffer and stir to disperse before heating. Heat in a boiling water bath, with stirring, for 30 min or till completely dissolved. Cool and pour 15 mL per 9 cm Petri dish.

Store plates at 4°C in sealed humidified containers.

5.2.2. The Test

Wells of 5 mm diameter are cut in the agar at 7 mm centre to centre distances, in patterns of seven wells which form the points and centre of a regular hexagon. Six patterns may be cut around the periphery of a plate. The area in the centre should *not* be used.

Peripheral wells in each pattern are identified as 1– 6, beginning with No. 1 closest to the periphery of the plate and progressing clockwise fashion. When wells are charged as indicated, this arrangement minimises interference between adjacent patterns, i.e. wells 3 and 5 are closest and contain the same material throughout.

Wells are loaded, each with 50 µL, as follows:

(a) Central — antigen;
(b) 1, 3 and 5 — reference serum;
(c) 2, 4 and 6 — test samples.

It is very important that reference and test reagents be arranged as above. The AGID test is far more sensitive when test samples are allowed to exert an influence on standard reference lines, so three reference lines per pattern are required for maximum sensitivity and specificity.

Plates are incubated in a humid atmosphere at ambient temperatures of 15–25°C.

5.2.3 Reading and Interpretation

The test is read at one and two or three days over transmitted illumination at an angle of about 45°.

Antibody activity in a test material turns the reference lines within the peripheral hexagon and antigenic activity turns the lines outwards between peripheral wells. In either case the strength of the reaction is recorded by comparison with the reference line (Table 7).

5.3. Source of Reference Serum

Reference serum should ideally be obtained from an animal, preferably bovine, which has recovered from BLV infection and has been shown, in an established standard test, to have developed adequately strong antibody of the required specificity. Reference serum may be diluted for use after assay as described under standardisation (see 5.5).

An alternative, less acceptable, source of reference serum is an animal with naturally acquired antibody. Such a serum is satisfactory in a test used to detect antibody but should not be used in a test to detect antigen.

5.4. Antigen

Cell cultures, usually in rolling bottles, of baby hamster kidney (BHK), Vero or McCoy cells are infected with BTV. A high dose of virus is an advantage and an inoculum of 1 mL containing 10^6 $TCID_{50}$, from a working stock held at 2–4°C, to 100 mL cell culture medium on about 1500 cm^2 of monolayer is acceptable.

Table 7. Interpretation of the agar gel immunodiffusion precipitation test

Antibody	*Antigen*	
(3) or xs	(+++) or xs	Test stronger than reference.
3	+++	Test equivalent to reference.
2	++	Test weaker than reference but producing a continuous line across the face of the test well.
1	+	A distinct turn at the end of the reference line.
?	?	Doubt whether a turn can be recognised (as distinct from doubt whether a minor degree of reaction is significant or specific).

Note that some experience should be gained in reading negative tests before weak reactions are identified, as a common characteristic of the BTV reference line is for a very slight turn, which might be regarded as a suspicious reaction in other AGID tests, to occur on the end of the line.

When the monolayer shows advanced cytopathic effect (CPE) (75–100%) the medium is harvested and clarified by centrifugation (1500 *g* x 60). The supernatant fluid is inactivated by β–propiolactone, as described for CF antigen, and concentrated by dialysis against polyethylene glycol (m.w. 20 000) or by hollow-fibre ultrafiltration (nominal 10 000 m.w. retention). A 10–50-fold concentration may be necessary. Antigen is usually concentrated 50 times and then diluted as required.

5.5. Standardisation

Reference serum and antigen must be balanced, for optimum proportions, and at the minimum strength that allows for the formation of a reference line of adequate extent, i.e. just reaching the test wells.

A standard serum which satisfies this criterion should be established and freeze-dried.

Each batch of antigen is balanced against the standard serum and its specificity at that standard dilution is established in a standard test.

Each batch of reference serum is similarly balanced against an antigen at standard strength and its specificity, at that balancing dilution, established in a standard test.

Reference reactants are normally available from the Virology Laboratory, Elizabeth Macarthur Agricultural Institute, Woodbridge Rd, Menangle, NSW 2570, Australia.

6. Bluetongue Competition Enzyme-linked Immunosorbent Assay

6.1. Introduction

The bluetongue C-ELISA has been developed to measure BTV specific antibody, without detecting cross-reacting antibody to other orbiviruses (Lunt *et al.*, 1988). The specificity results from the use of a monoclonal antibody (Mab) which binds to the group reactive protein (VP7) of the Australian BTV 1, and competes for this site with antibodies in the test serum. The binding site for this Mab has been reproduced in a yeast expressed antigen, which is used as the coating antigen reagent. This antigen is non-infectious.

6.2. Reagents

6.2.1. Coating Buffer

The coating buffer is Tris-HCl 0.05 mol/L, NaCl 0.15 mol/L, pH 9.0.

Solution 1:	Tris-base	6.05 g
	NaCl	8.78 g
	H_2O	1 L
Solution 2:	Tris-HCl	1.58 g
	NaCl	1.76 g
	H_2O	200 mL

The coating buffer is prepared by mixing 90 mL of solution 1 with 10 mL of solution 2.

6.2.2. Phosphate Buffered Saline (PBS) Tween pH 7.2 (PBST) Concentrate (10 Times)

Disodium hydrogen phosphate, Na_2HPO_4 anhydrous	10.7 g
Sodium dihydrogen phosphate, $NaH_2PO_4.2H_2O$	3.9 g
NaCl	85.0 g
H_2O	1 L
Tween 20	5 mL

Dissolve PBS prior to adding Tween 20. To make up working strength wash buffer, make a 1:9 (1/10) dilution of the 10 times stock in water. The 10 times concentrate can be stored at room temperature. The working strength solution should be prepared freshly for each test.

6.2.3. Skim Milk Serum Dilution Buffer

Skim milk serum dilution buffer (SMSDB) is PBST + 1% skim milk powder.

To prepare 50 mL of SMSDB, add:

PBS-Tween, 10 times	5 mL
H_2O	45 mL
Skim milk powder	0.5 g

This should be prepared freshly for each test.

6.2.4. Conjugate Dilution Buffer

A working-strength solution of PBST is used (see 6.2.2.).

6.2.5. Citrate–Acetate Buffer

Prepare 100 mL of 1 mol/L sodium acetate (CH_3CO_2Na) and 5 mL of 1 mol/L citric acid $C_6H_8O_7$). Adjust the 100 mL of sodium acetate to pH 5.9 by the addition of citric acid (about 1.5 mL).

6.2.6. Tetramethylbenzadine Substrate

Dissolve 3,3 5,5 tetramethylbenzidine (TMB) (Sigma T-2885) in dimethyl sulfoxide [$(CH_3)_2SO$), DMSO] to 42 mmol/L (Bos *et al.*, 1981).

TMB	0.101 g
DMSO	10 mL

(Caution: DMSO aids the penetration of TMB through skin.)

This 10 times stock should be stored at 4°C in small aliquots (0.05 mL).

6.2.7. Sulfuric Acid

Prepare a sulfuric acid (H_2SO_4) solution of 1 mol/L.

6.3. Methodology

6.3.1. Precoating Microplates with Antigen

The plates used are 96-well U-bottom micro-ELISA plates (Dynatech Cat. No. 001-010-2401).

Antigen is wet frozen and should be kept at -20°C. If the stocks of antigen are frozen in small aliquots, the working antigen aliquot can be kept at 4°C for up to two weeks.

The antigen is diluted 1/200 and the plates are coated with 50 µL of diluted antigen per well and incubated on a microplate shaker at 37°C for one hour.

The plates are next washed thoroughly with working strength PBST. If a plate washer is available, five rinses using a five second interval and 80–90 µL per well filling is recommended. If plates are washed with a squeeze bottle, three rinses of one minute should be given.

After washing, plates should be inverted and tapped on tissue or blotter to remove excess liquid and left on this paper (no longer than 10 min) until ready to transfer serial dilutions.

6.3.2. Serum Incubation Stage

Test sera are tested in duplicate at a 1/10 dilution. Serum (25 µL) is thoroughly mixed in 225 µL of SMSDB in a polystyrene microtitre plate and 50 µL of the diluted serum is transferred to each of two wells of the antigen-coated micro-ELISA plate.

Three control sera are included in each test plate: a high titred + control; a low titred + control; and a negative control serum. Foetal calf serum (FCS) is recommended for a negative control.

Antigen-coated plates must be put through a wash cycle immediately (up to 10 min) prior to the serum transfer stage. The use of a multichannel pipette allows rapid transfer of diluted sera to the test plate. Tips used for transferring diluted sera need not be changed between sera if rinsed three times in distilled water.

Incubation is for one hour at 37°C. Plates are covered with a plate cover and secured to a plate shaker with rubber bands. The shaker is shaken at about 400 rpm (speed 6 on the Flow plate shaker). Shaking at other speeds may cause variable results due to inadequate mixing or spillage.

6.3.3. Addition of Monoclonal Antibody

The plate is not washed after the serum incubation stage, as the next step requires Mab to be co-incubated with the serum for a further 30 min.

The Mab (20E9/B7/G2) is supplied freeze dried and should be reconstituted in 1 mL of distilled water on receipt. On reconstitution, an equal volume of glycerol is added and 100 or 200 µL aliquots are stored at -20°C.

For use, the reconstituted Mab-glycerol is diluted 1/250 in PBST (without skim milk powder) and 50 µL added to all wells of the plate.

The plate is covered and shaken at 37°C at 300 rpm (speed 5 on the Flow plate shaker) for 30 min.

6.3.4. Addition of Conjugate

Sheep antimouse horseradish peroxidase (HRP) conjugate (Silenus code DAH) is diluted 1/5000 in PBST and 50 µL is added to each well. There is no need to wash the plates before addition of the conjugate. The plate is covered and shaken at 400 rpm at 37°C for 30 min. Plates are next washed as described in 6.3.1.

6.3.5. Addition of Substrate

The TMB should be removed from 4°C, 30 min prior to use. If not fully melted, warm at 37°C for five minutes.

Substrate solution, sufficient for four plates, is prepared as follows: to 18 mL of distilled water add 0.2 mL of TMB (10 times in DMSO), 2 mL of citrate–acetate buffer and 0.1% bovine serum albumin. Add 25 µL of 30% H_2O_2 to 225 µL of distilled water and 25 µL of this 3% H_2O_2 is transferred to the diluted TMB solution and mixed well.

Diluted substrate solution (50 µL) is added to all wells of the test plates and to a column of eight wells in an additional plate, to be used as a blanking plate. Plates are incubated at room temperature for 10 min. The reaction is stopped by the addition of 50 µL of 1 mol/L sulfuric acid to all wells of the test plates and to the column of substrate wells in the blanking plate.

6.3.6. Reading the Plates

Plates are read at 450 nm on a microplate photometer. The photometer must be blanked prior to reading test plates using the column of substrate-acid on the blanking plate.

Results of tested sera can be determined by expressing them as percentage inhibition. Calculation of percentage inhibition:

$$100 - [100 \times \text{OD test serum mean}) / (\text{OD negative control serum mean})]$$

Where this value is >50%, the serum is positive. The test should be repeated if the low titre positive control shows <40% inhibition or if the negative controls fall outside of the 1.1–1.6 OD range.

7. Bluetongue Virus Neutralisation Tests

Normally, virus neutralisation tests are carried out either as a conventional microtitre neutralisation test or as a plaque reduction test.

7.1. Microneutralisation Test

The virus microneutralisation test for bluetongue is carried out with sterile techniques in flat-bottomed 96-well microtitre plates, which have been treated to allow for the growth of cell culture.

7.1.1. Equipment

(a) Pipette droppers (25 µL and 50 µL), eight- and 12-tip multichannel pipettors or automatic dispensing machines.
(b) Sterile 96-well microplates — flat bottomed for tissue culture. Sterilise by radiation, not gas.
(c) Pipettors, 25–200 µL.
(d) Sterile tips for pipettors.
(e) Stirrer.

7.1.2. Serum for Test

Prior to testing, the test serum is heated at 56°C for 30 min. This can be achieved in tubes in a waterbath or in the test plate in an incubator, before adding the diluent and virus. Sera are usually added to each of four wells of a 96-well microplate. The sera in three wells are mixed with virus to test for antibody and the extra well is kept as a serum control (with no virus added) to check for toxicity of the test serum for the cell cultures.

For screening tests, 50 µL of serum is dispensed to one well using a sterile disposable tip on a pipettor. The serum is then diluted 1/4 by adding 150 µL of the diluent (see 7.1.3.). The diluted serum is then dispensed to three other wells (50 µL per well). An extra 50 µL of diluent is added to the serum control well and 50 µL of virus is added to the other three wells.

To titrate a serum, 25 µL of serum is added to each of four wells. To each of these wells, 75 µL of diluent is added and 50 µL of diluent is added to the remaining wells. The serum in the first four wells is then mixed with a pipettor fitted with sterile tips and 50 µL transferred to the next set of four wells containing 50 µL of diluent, resulting in a two-fold dilution series. Normally, two sera (in adjacent wells down the first column of the 8 x 12 plate) would be diluted from 1/4 to 1/8192 concurrently, allowing the use of an eight-tip multichannel pipettor. The tips are then changed and the dilution process repeated on the next set of sera. Dilution loops may be used, but the use of a pipettor with disposable tips avoids rinsing, blotting and flaming of the loops and also avoids the problem of occasional damage to the microplate.

7.1.3. Virus

The virus to be used is plaque purified BTV, which is calculated to contain 100 $TCID_{50}$/50 µL. The titre of the test virus should be determined by incubation for 10 days at 37°C. Add 50 µL of the diluted virus to all test wells, but do not add virus to the serum control wells. The virus suspension used in the test is then diluted in 10-fold steps from 10^{0} to 10^{-4} and then added to the microtitre wells, 50 µL per well, eight wells per dilution, using a 50 µL Cooke pipette dropper.

7.1.4. Diluent

The diluent is M199+10% FCS. The FCS is heated at 56°C for 30 min prior to use and must be free of inhibitors to BTV and non-toxic to Vero cells.

7.1.5. Cells

The tissue culture cells are Vero 121 (Green Monkey kidney) at a concentration of 1.5 x 10^5 cells/mL. The cells are gently stirred in a flask to prevent settling, while being added to the wells at a rate of 100 µL per well. The cells can be added with a range of devices, including automatic pipetting machines with an autoclavable head and 12-tip multichannel pipettors.

7.1.6. Controls

7.1.6.1. Cell control

Four wells receive 100 µL cell suspension and 100 µL diluent only.

7.1.6.2. Foetal calf serum, negative control

Four wells receive 100 µL cell suspension, 50 µL diluent and 50 µL of a 1/4 dilution of FCS.

7.1.6.3. Foetal calf serum, positive control

Four wells receive 100 µL cell suspension, 50 µL of a 1/4 dilution of FCS and 50 µL virus suspension.

7.1.6.4. Virus control

The virus is diluted and dispensed in sets of eight wells/dilution in 10-fold steps, 10^{0} to 10^{-4}; the concentration of virus used in the test being 10^{0}. Each well receives 50 µL of virus, 50 µL of diluent and 100 µL of virus suspension.

7.1.6.5. Positive serum control

A serum with a known neutralising antibody titre is diluted in two fold steps using four wells/dilution, from 1/2 to two steps beyond its expected titre. Sheep serum of known antibody titre, prepared against plaque purified virus, is available in limited stocks from Oonoonba Veterinary Laboratory, Townsville.

7.1.6.6. Individual serum control

One well is inoculated with each individual serum being tested, but without virus, to check for serum toxicity.

7.1.7. Sequence of test

The reagents are added to microtitre plates in the following order:

(a) 50 µL serum (at one in four for preliminary testing);
(b) 50 µL virus (100 $TCID_{50}$);
(c) shake;
(d) incubate 60 min at 37°C;
(e) 100 µL Vero cells (1.5 x 10^5 cells/mL);
(f) the plates should be incubated in a carbon dioxide incubator (5% CO_2) at 37°C for 10 days.

7.1.8. Reading the Test

The test is read with an inverted microscope after 10 days incubation. All wells to which virus has been added are scored positive if any cytopathic effect is observed. When partial CPE (1+) is seen, those wells should be noted and scored accordingly.

7.1.9. Interpretation of Test

The cell culture, negative FCS, and individual serum controls without virus should show no CPE and can be recorded as (–). Foetal calf serum plus virus should show evidence of CPE and be scored as (+) (i.e. absence of antibody to virus). The virus control should ideally record four wells with CPE (+) and four wells without CPE (–) at a

virus dilution of 10^{-2}. Wells containing serum under test in which there is any evidence of CPE (+) are regarded as containing no antibody and those without CPE (–) are regarded as containing antibody. This result is valid if the control of the individual serum under test also shows no evidence of CPE (–). Titres are calculated by standard methods. With some serotypes it may be necessary to interpret as antibody positive, wells in which there is a 75% (or greater) reduction of CPE [i.e., those wells showing (1+) CPE may be interpreted as having low levels of antibody).

Many variables are involved in the bluetongue microneutralisation test and modifications of the procedures described here exist. These variables include cell line, seeding rate, virus serotype and strain, day of reading and CPE endpoint.

7.2. Plaque Reduction Test

This is essentially as described in 9.2.3., the difference being that the serum under test is incubated with BTV of known serotype. The test is normally carried out on Vero monolayers in 24-well plates, but other cell types and other laboratory ware may be used.

Sera can be screened at 1/10 or can be diluted and titrated through two-fold steps from 1/10 to 1/320 or greater. The diluted sera are incubated with virus diluted to contain 50 plaque forming units. The test must include appropriate controls, including antibody positives and negatives. If field sera are under test, the best antibody positive control is the serum of an animal from which the BTV serotype being tested against has been recovered or the sera of a convalescent animal which has been experimentally infected with that BTV serotype. Preinoculation serum makes a good antibody negative control, otherwise FCS.

The plaque reduction test is generally easily read and this, and an increased sensitivity, are the main advantages compared with microneutralisation. Homologous sera at low dilution usually neutralise all, or nearly all, of the virus. When sera are diluted, an end point has to be decided on and is usually 50% plaque reduction. The end point is normally easily determined although some BTV serotypes, such as BLU 21, are more difficult to read with incomplete neutralisation occuring at low serum dilutions and the end point being drawn out over several higher serum dilutions.

As is the case with all serological procedures, the result of a BTV plaque reduction test is interpreted in conjunction with the readings obtained with a well selected range of appropriate controls.

8. Virus Isolation

8.1. Introduction

Field strains of BTV readily replicate in ruminants and embryonated eggs. Clinical disease in animals and death of the embryo may, or may not, occur. Field strains replicate less readily in mosquito cell cultures, uncommonly in mammalian cell cultures and very rarely in baby mice. Laboratory adapted strains of BTV are different biologically from unmanipulated field strains and readily multiply in ruminants, eggs, mice and a wide variety of cell cultures.

8.2. Animal Inoculation

The inoculation of BTV antibody negative animals is probably the most sensitive system for the detection of virus and is especially useful when specimens are low-titred or may be toxic for eggs. Most species of ruminant are probably suitable but sheep are preferred. Intravenous and subcutaneous inoculation seem equally efficient.

Inoculated sheep can be clinically monitored but disease, or even fever, may not result from virus multiplication. The unequivocal indicator for the presence of BTV in the inoculum is the development of BLU antibody 14–28 days post inoculation. Heparinised blood should be collected from inoculated animals five, six and seven days after inoculation because any virus in the inoculum would be greatly amplified and these heparinised bloods will provide a high-titred source of virus for further studies if the animals subsequently seroconvert.

8.3. Egg Inoculation

Embryonated eggs provide a convenient *in vivo* isolation system for the recovery of field strains of BTV in diagnostic and research programs. Laboratories which use eggs for this purpose tend to have local modifications of a core method. The method in use at the Northern Territory Department of Primary Industry and Fisheries Laboratory at Darwin is as follows.

8.3.1. Preparation

(a) Fertile hens eggs with good quality white shells are used after incubation for 9–12 days at 39°C. If fewer than nine days the embryos are rather fragile and the veins small. If older than 12 days the embryos are too large and feathery at harvesting for efficient processing.

(b) The eggs are candled and the shell is marked with a pencil over a straight, prominent, superficial blood vessel.

(c) The shell about the pencil mark is cleaned with *tincture iodine mitis.*

(d) A cut in the shell about 10 mm long and 1–2 mm either side of the marked blood vessel is made with an abrasive cutting wheel fixed to a dental drill or an electric engraving tool.

(e) The shell over the selected vessel between the two horizontal cuts is carefully broken off with a finger nail or the tip of a scalpel blade.

(f) The eggs can be left at room temperature for several hours during the procedures above.

Some laboratories deliberately cool the eggs to ambient temperature before inoculation in the expectation that cooler eggs will bleed less on inoculation. This is not considered vital if the eggs are carefully inoculated and the eggs are normally returned to the incubator if undue delay occurs between any of the steps during preparation or before inoculation.

8.3.2. Inoculation

(a) Undiluted inoculum is normally toxic for eggs and the lowest dilution should be 10^{-1}. Heparinised blood (50 µL) is diluted in 450 µL of sterile distilled water (pH 7.2–7.4) to lyse blood cells and facilitate the availability of cell associated BTV. Further dilutions can then be made in isotonic medium. Normal saline, PBS, laboratory standard virus diluent or cell culture medium can all be used.
Some laboratories prefer to sonicate the blood to lyse the cells and release cell associated virus but the use of a mechanical sonicator when multiple samples are to be examined for BTV can easily result in cross-contamination and the lysis of blood cells by water avoids this risk.
Critical samples of uncoagulated blood for virus isolation which may contain antibody are best washed several times in isotonic medium to remove serum antibody before cell lysis.

(b) The eggs are returned to the candling room for inoculation.

(c) A drop of sterile (by filtration, not autoclaved) paraffin oil is placed on the exposed shell membrane over the vessel to be inoculated to enhance the visibility of the vessel.

(d) Several eggs per inoculum are inoculated intravenously with 0.1 mL of inoculum using a 0.5–1.0 mL syringe and a 26G needle (disposable insulin syringe-needle combinations are ideal). Slow, gentle inoculation and careful removal from the vessel will minimise bleeding.

(e) The inoculation site is covered with adhesive tape.

(f) The eggs are returned to an incubator or hot room. Maximum specific embryo mortality is achieved if eggs are incubated at 33.5°C after inoculation but this mortality results from the additive insults to the embryo contributed by virulence of BTV inoculum (depending on virus strain and history), toxicity of the inoculum and general vitality of the embryonated eggs as well as an abnormal temperature of incubation after inoculation. Highly embryo-lethal strains will kill all inoculated eggs at all virus dilutions whereas many field strains have no obvious effect on inoculated eggs. If the eggs are incubated at 33.5°C after inoculation and have a low vitality due to poor handling before inoculation or if the inoculum is slightly toxic, an inconvenient high non-specific mortality may be experienced. In the Darwin Laboratory, embryo mortality post inoculation is not considered a key index of the presence of BTV in the inoculum and the eggs are placed in a hot room after inoculation where the temperature basically varies with shelf level from 35 to 38°C.

(g) Eggs are candled for deaths daily. Even the most embryo-lethal strains of BTV will not kill the embryo in the first 24 hours after inoculation so eggs dying during this period are discarded. Eggs dying one to five days post inoculation are regarded as possible specific deaths and are held at 4°C. BTV stability in unopened eggs at 4°C is similar to that in heparinised blood where the virus remains viable for weeks or months.

(h) Although specific mortalities are reported to occur up to seven days after inoculation, in Darwin embryos alive five days post inoculation are killed by chilling at 4°C for a minimum of three hours before harvesting. All embryos dying 24 hours or more after inoculation or still alive five days post inoculation should be processed.

8.3.3. Harvesting

(a) Sound laboratory techniques need to be employed when harvesting embryos, otherwise the cross-contamination of uninfected embryos with BTV infected material is readily achieved. Embryos are harvested in a biohazard cabinet using a separate set of sterile instruments for each inoculum egg lot. If multiple cabinets are unavailable, day five-alive embryos are harvested first, day one-to-five dead embryos next and known infected embryos (if any) last.

(b The shell over the air space is cleaned with *tincture iodine mitis*.

(c) The shell over the air space is cracked with rat-toothed forceps and removed.

(d) Using fresh forceps the head of the embryo is pinched off and discarded as the hard cranial bones and beak are difficult to process and are not considered essential for virus recovery. BTV is thought to be distributed generally through the embryo. Some laboratories harvest the embryo heart only, considering that this organ will contain virus if any is present in the embryo, is easily processed and is less toxic than other embryo tissues.

(e) The decapitated embryos are pooled by inoculum, dilution, day of death and alive at day five. Embryos dying of specific BTV infection may have reddened or haemorrhagic skins.

(f) The embryos are coarsely ground (in a tissue stomacher, mechanical blender or pestle and mortar) in 5 mL of diluent (heart brain broth pH 7.2–7.4 containing 7500 U penicillin G, 25 mg streptomycin sulfate and 5 mg kanamycin sulfate) per embryo.
(g) The coarsely ground embryos are lightly centrifuged and the supernatant is held at 4°C.

8.4. Cell Culture Inoculation

Few field isolates of BTV will multiply in mosquito or mammalian cell cultures before passage in eggs. However, if animals or insects have been inoculated with laboratory manipulated virus, this can usually be directly reisolated in mammalian cell cultures.

In Darwin, the clarified supernatants of homogenised embryos inoculated with field material are next passaged in *Aedes albopictus* mosquito cell (C6/36) cultures before several passes in mammalian cell monolayers. The Darwin laboratory considers that the mosquito cell passage improves the overall isolation rate and reduces problems with toxicity as frequently occurs when mammalian cultures are inoculated with egg harvest material.

Cell cultures may be grown in disposable or reusable glass or plastic tubes with screw caps or rubber stoppers. However, the experience of many laboratories is that disposable plastic screw-capped tubes generally prove the most reliable.

(a) The C6/36 line of *Aedes albopictus* cells are grown in culture tubes at room temperature (25°C) in Eagle's MEM with 10% FCS. When the monolayers are confluent, the medium is changed to MEM and 2% heat-inactivated FCS and the culture tubes are inoculated with 100 µL of egg harvest and maintained, stationary, at room temperature for seven days. Each egg harvest is inoculated to at least 2xC6/36 tubes.Tubes are not examined for CPE as this is variable and irregular. Lines of C6/36 cells can vary in their culture requirements; some require flat surfaces, others L15 medium.
After seven days maintenance inoculated mosquito cells are passed to mammalian cell cultures.
(b) BHK21 and hamster lung (HmLu-1) monolayers have been used routinely. Either would suffice for recovering field strains of BTV after initial passes in embryonated eggs and C6/36 cells but the use of both lines of mammalian cells offers some advantages. A mammalian cell culture system is available if the second is growing or maintaining poorly and the breadth of arbovirus groups recoverable from diagnostic or research ruminant blood samples is increased [bovine ephemeral fever (BEF) group viruses grow poorly in HmLu-1 monolayers but HmLu-1 cells seem more sensitive than BHK21 for the isolation of some Simbu group members]. BHK21 monolayers are grown in BME with 5% FCS and maintained in BME and 2% FCS. HmLu-1 cells are grown in Eagles MEM and 10% FCS and maintained in M199 with 2% FCS.
(c) Mammalian cell cultures are inoculated with supernatant and cells of inoculated mosquito monolayers which have been maintained for seven days. When large numbers of tubes are being handled, the C6/36 cells can be removed from the surface of the plastic tube and suspended in the maintenance medium by mechanical agitation in a water-bath sonicator, otherwise the cells can be scrapped off the plastic with a Pasteur pipette. The C6/36 tubes which received common inoculum are pooled and 100 µL of supernatant containing suspended cells inoculated to each of two BHK21 and two HmLu-1 culture tubes. These tubes are rolled at 37°C and inspected regularly for CPE for seven days.
(d) After seven days, maintenance supernatant and cells of BHK21 and HmLu-1 tubes not showing CPE are passaged to fresh cultures and examined regularly for CPE.
(e) No further passes are undertaken. No additional isolations have ever been made after the second mammalian cell passage.
(f) Any cultures showing CPE during the first or second mammalian passage are held at 4°C for virus identification.

9. Virus Identification

9.1. Introduction

Isolates need to be identified because ruminants in Australia may be infected with around 30 arboviruses. Even though most of these are unlikely to occur in sheep naturally, all isolates, including those from sick sheep, need to be characterised to virus group and type.

9.2. Virus Grouping

Isolates are serologically defined as BLU group viruses by antigen detection methods using immunofluorescence or immunoperoxidase and employing group-specific antibody (preferably monoclonal) or by plaque reduction serological tests.

9.2.1. Immunolabelling

Conventional immunofluorescent or immunoperoxidase methods are used.

(a) Vero cells are grown on eight-chambered glass slides in M199 plus 10% FCS in a 5% carbon dioxide atmosphere. Confluent, or near-confluent, monolayers are infected with the unknown isolate and maintained in M199 with 2% FCS. Cultures are held for 48 hours or until 25–50% CPE before fixing in acetone.

For peroxidase staining, cells can be grown in 96-well microplates and the infected cells fixed by the addition of 200 µL of 3% formaldehyde solution to the medium.

(b) Group-reactive polyvalent immune rabbit serum or monoclonal antibody is added to the fixed, infected Vero cells followed by labelled antirabbit or antimouse conjugate. If group-reactive immune fluids to other arbovirus groups known to infect Australian ruminants are included in the immunolabelling tests, isolates not identified as BTV may be identified as members of the epizootic haemorrhagic disease (EHD), Palyam, Simbu or BEF arbovirus groups.

9.2.2. Immunospot (Dot Blot) Test

This test is used to identify cytopathic agents directly from cell culture tubes as BTV. The test is based on the Mab (20E9/B7/G2) to the VP7 of BTV1 and detects all Australian BTVs except BLU15. The test is robust and repeatable and exhibits no problems with background colour development or cross reactions with the Australian EHD virus types 2, 5, 6, 7 or 8.

9.2.2.1. Reagents for BTV serogroup immunospot

9.2.2.1.1. Tris buffered saline (TBS).

Tris	2.42 g
NaCl	8.5 g
Distilled or deionised water	1 L

Add the Tris (20 mmol/L) to the NaCl (150 mmol/L) then add to the water, and mix well to dissolve. Adjust pH to 7.5 with HCl.

9.2.2.1.2. Tween 20-Tris buffered saline (TTBS).

Add 0.5 mL of Tween 20 detergent (0.05%) to 1 L of TBS (see 9.2.2.1.1.).

9.2.2.1.3. 2% Skim milk (blocking solution).

Add 1 g skim milk (Carnation brand) to 50 mL TBS (see 9.2.2.1.1.).

9.2.2.1.4. Monoclonal antibody (20E9/G7/B2) to BLU1 VP7.

Dilute to appropriate dilution in TTBS (see 9.2.2.1.2.).

9.2.2.1.5. Horseradish peroxidase (HRP) conjugated goat antimouse IgG (L+H chains).

Dilute to appropriate dilution in TTBS (see 9.2.2.1.2.).

9.2.2.1.6. Colour development solution.

Methanol (-20°C)	10 mL
DMSO	1 mL
4-chloro-1-napthol (4CN)	30 mg
3'-3'-diaminobenzidine tetrahydrochloride (DAB)	5 mg
30% H_2O_2	30 µL
TBS	50 mL

Prepare just prior to use. Dissolve the DAB in the DMSO (may require repeated aspiration with a pipette). Dissolve the 4CN in the chilled methanol. Mix the two solutions. Immediately prior to use, add 30 µL hydrogen peroxide to the TBS and then mix with the DAB– DMSO–4CN–methanol solution.

9.2.2.2. Immunospot Procedure

(a) Cut 0.2 µm or 0.45 µm nitrocellulose membrane to appropriate size (0.2 µm membrane is less fragile). The membrane can be labelled with a black pen.

(b) Centrifuge (1500 *g* for 5 min) infected cell cultures showing 50–100% CPE.

(c) Spot 2 µL of cell culture supernatant [or cell pellet; see (i) below] onto the membrane and allow to air dry (5–20 min depending on humidity).
BTV positive and negative controls should be included and can be BLU1, 3, 9, 16, 20, 21 or 23 infected and uninfected culture tubes stored at 4°C or -20°C.

(d) Block the non-specific binding sites on the membrane by placing it into blocking solution (in a small Petri dish) for 30 min. Discard the blocking solution then wash the membrane twice for one minute each with TTBS while rocking (on a Rose-Bengal plate rocker).

(e) Remove the TTBS and add the diluted Mab and rock for one hour.

(f) Remove diluted Mab and wash three times for two minutes each with TTBS (see 9.2.2.1.2.).

(g) Remove the TTBS and add the diluted HRP conjugated goat antimouse IgG. Rock for one hour.

(h) Remove the diluted conjugate and wash twice for two minutes each with TTBS and once for two minutes with TBS (see 9.2.2.1.1.).

(i) Add the colour developer solution which should have been made during the previous wash procedure. Rock for three to five minutes using the colour of the positive control as a guide to progress of colour development. If there is poor colour development with the BTV positive control, this indicates that the test lacks sensitivity, and repeat using the cell pellet from step (b), lysed with water, or by freeze-thawing or sonication.

(j) Remove the colour development solution and wash twice with tap water. The colour fades rapidly so a record of the results can be made by photocopying the membranes.

9.2.3. Plaque Reduction

Infected monolayers can be overlaid with agarose or carboxymethyl-cellulose (CMC).

9.2.3.1. Agarose method

(a) Vero or BHK21 monolayers are grown in 24-well disposable plastic plates in a 5% carbon dioxide–95% air incubator. BTV isolates will produce CPE in both cell lines with discrete plaques in Vero monolayers and diffuse, spindly plaques in BHK21 monolayers. EHD2, Akabane and Aino viruses also plaque in Vero monolayers but

most other Australian ruminant isolates will not and BHK21 cells should be used if the identities of isolates are uncertain. When confluent, or near confluent, the growth medium is removed and the monolayers are washed.
Serum-free M199 (Vero) or BME (BHK21) medium is used for washing monolayers, for the dilution of viruses, and antisera and in agar overlay.

(b) High titred rabbit sera, immune to each of the eight Australian BTV types, are each diluted 10^{-2} in a common diluent to give a BTV polytypic immune fluid. Similarly, polytypic immune grouping fluids can be prepared against the five EHD, five Palyam, five Simbu and four BEF viruses which may be isolated from ruminants in Australia.

(c) This grouping fluid (0.25 mL) is added to each well followed by an equal volume of the unknown isolate, diluted 10^{-1} to 10^{-2}. Four isolates can be identified to virus group on each 24-well plate by dispensing the five separate grouping fluids in the first five wells of each of the four rows of the plate and adding 0.25 mL of serum-free diluent to the sixth row of wells for a virus control. Diluted virus (0.25 mL) is then added to each of the six wells in the row.

(d) After at least one hour incubation, 0.5 mL of serum-free medium containing 2% agarose is added to each well and mixed with the virus–serum mixture by gentle agitation resulting in the monolayer in each well being overlaid with 1.0 mL of medium containing 1% agarose. This agarose overlay is prepared by dissolving 4% agarose (seaplaque) in distilled water by boiling or microwaving and then maintaining at 56°C. Before overlaying, the 4% agarose in water is mixed with an equal volume of two times serum-free medium which has been warmed to 37°C.
The overlaid plates are left on the bench until the agarose has set and then are returned to the gassed incubator. Unused Vero overlay is held at 4°C for staining when plaques appear.

(e) Virus plaques may be observed after two days incubation but, depending on virus group and type, normally take longer. Because of the whorled pattern of BHK21 monolayers, BTV plaques are difficult to discern macroscopically after staining and are best examined microscopically.
Vero monolayers are stained by the addition of 0.25 mL of 1% agarose-199 overlay containing 1:30000 neutral red. The neutral red is dispensed from a 0.1% aqueous stock solution.

(f) Plates are examined daily for plaques which are usually quicker growing in BHK21 monolayers than in Vero. Providing the titre of the immune rabbit sera is sufficient, plaque inhibition is generally readily observed unless the unknown virus is a dual isolate or is a virus different from those covered by the polytypic grouping fluids.

9.2.3.2. Carboxymethyl-cellulose method

The Elizabeth Macarthur Agricultural Institute, Menangle, favours the use of CMC overlay for the plaquing of BTV. This method has the advantage that cultures can be infected and overlaid when first attached, three to four hours after seeding and the operator does not have to wait one to two days until the monolayer is confluent, as is the requirement when agarose overlay is used.

Low viscosity (3%) CMC (BDH Cat. No. 27649) is stirred until dissolved in distilled water at 60–70°C and sterilised by autoclaving. The sterilised solution can be stored at room temperature.

Sterile CMC (3%) is mixed with an equal volume of two times growth medium and overlaid onto cell cultures one hour after infection and four to five hours after seeding.

The monolayers can be stained with 0.1% naphthalene black when countable plaques appear. The stain is prepared by stirring until dissolved: 1 g of naphthalene black in 60 mL of glacial acetic acid (CH_3CO_2H), 13.6 g of anhydrous sodium acetate (CH_3CO_2Na) and distilled water to 1 L.

To stain, the overlay is aspirated off, the monolayer washed with PBS and the PBS aspirated off. The monolayer is then well covered with 0.1% naphthalene black which is applied by a wash bottle and left to stain for two to three hours at room temperature. The stain is then aspirated off, the monolayer washed with water and left to dry when the plaques can be counted. The stained, fixed monolayers will keep indefinitely at room temperature.

9.3. Virus Typing

Once an isolate has been identified to virus group, type-specific neutralisation tests characterise the virus further. Microneutralisation or plaque reduction serology is used.

9.3.1. Microneutralisation

This method is essentially the same as the microneutralisation test described in 6.1. for the detection of BTV antibody, but is conducted in reverse with reference BTV type specific antisera (of known neutralising titre) and viruses of unknown type.

The unknown virus is diluted 10^{-1} to 10^{-6} and each dilution is incubated with a constant dilution of reference serum and with known BTV antibody negative serum (FCS may be used). The test is read at 5–10 days and significant

reduction is deemed to have occured if the virus is neutralised 100-fold or greater. The unknown virus is considered serologically identical to a standard reference BTV type if the known virus is run in the test in parallel with the unknown and is similarly neutralised.

9.3.2. Plaque Reduction

Plaque reduction tests to type an isolate are undertaken essentially as per the virus grouping tests described previously (see 9.2.3.).

Isolates grouped as BLU are best typed using Vero monolayers. The isolate is incubated separately with antisera to each of the eight Australian BLU viruses and three isolates can be identified on each 24-well plate. The Darwin Laboratory uses polyvalent immune rabbit sera diluted 1/50, whereas some other laboratories use immune guinea pig or sheep sera. It is essential to be familiar with homologous and related neutralisation properties of the sera used.

Strong cross-neutralisations can occur between BTU types 3 and 16, 6 and 21, 9 and 5, 20 and 4 and 20 and 17. Of the BTU types exotic to Australia, significant cross-neutralisation has been detected between 4 and 17, 7 and 19 and 8 and 18.

Isolates identified as belonging to the EHD, Palyam, Simbu or BEF groups can similarly be typed. Vero monolayers can be used to plaque and identify EHD2, Akabane and Aino viruses but BHK21 cells have a broader host-plaquing susceptibility.

10. References

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11. Acknowledgments

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