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Bovine Ephemeral Fever
Pathology, Virology and Serology

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1. Introduction

Bovine ephemeral fever (BEF) is enzootic in some of the northern areas of Australia, in particular areas of Queensland and the Northern Territory, north of the latitude of Townsville. At intervals, major epizootics spread from northern Australia down through the eastern States of Australia, and on occasions reach Victoria. There are, however, large areas of Victoria where BEF has never been diagnosed. BEF has not been diagnosed in Tasmania, extensive areas of South Australia, and southern Western Australia. Diagnosis may be achieved by virus isolation and identification or by serological means. The former is too time-consuming and costly to be used routinely and is usually reserved for special purposes. Use of serology generally requires that serum samples be taken to cover the clinical and convalescent periods so that a rise in antibody titre can be related to the clinical disease.

The diagnostic interpretation is complicated by the potential contribution of BEF-related viruses, as infection by at least one, Kimberley, has been associated with a subsequent rise in pre-existing BEF virus neutralising antibody. Australia has valuable export markets for cattle and buffalo in countries to which BEF is an exotic disease. Health certification for trade purposes, in regard to BEF, is based almost entirely on serological tests. Under different agreements either negative or positive serological status may be required, with different implications for standardisation and interpretation. For accurate diagnosis it is important, and for certification it is essential, that there be a standard test, which is uniformly interpreted, throughout Australia. This is facilitated by the recognition of an Australian Reference Laboratory (ARL) for BEF which can issue standard materials, establish criteria for the acceptance and interpretation of tests and maintain quality control and surveillance of routine test performances.

2. Host Range

The only animals clinically affected in Australia are cattle and the only additional species found with antibody in nature are water buffalo and deer. Clinical disease has not been observed in buffalo in Australia, but has been reported from India, Indonesia, Malaysia and China. Though the prevalence of antibody is high in domesticated and feral buffaloes the titres are mostly low (<10) and of uncertain specificity. The epidemiological role of buffaloes and deer as alternative hosts is obscure. However, they do present a problem for export certification as certain countries require that they be free of neutralising antibody to BEF. Their sera can be tested in the same manner as cattle sera.

BEF is insect borne. There is a close association between rainfall and the outbreak of disease and epidemics tend to be influenced by prevailing wind patterns. In addition, BEF virus has been isolated from *Culicoides* spp. in Africa and Australia (Muller and Standfast, 1986) and from at least two species of mosquitoes in Australia. In the laboratory the virus has been recovered from *Culex annulirostris* mosquitoes and *Culicoides marksi* and *C. brevitarsis* biting midges 10 days after experimental infection. A comparison of the known distribution of these species with the limits of BEF outbreaks tends to support the view that more than one vector is responsible for the transmission of BEF virus. It is likely that *Anopheles bancroftii* circulates the virus in the north of Australia, and *Cx. annulirostris* and *C. brevitarsis* are probably involved in the transmission of the disease since they have a wide distribution pattern which matches or fits within the BEF range.

3. Clinical Signs

A monophasic, biphasic or triphasic fever of 24–48 hours duration is characteristic of ephemeral fever. Typically, mild clinical signs are observed during the first temperature rise and these may go unnoticed although milk yield in dairy cattle can fall markedly. The clinical signs of the disease are more usually associated with the second temperature peak and can include anorexia, elevated heart rate, rapid respiration, serous ocular–nasal discharge becoming mucopurulent, salivation, shivering and depression. Most cases show stiffness and lameness in one or more limbs and many cattle may become recumbent, particularly lactating cows and heavy adult steers and bulls. Recovery usually occurs within 24 hours of the onset of clinical signs and is uneventful. Complications occur in less than 1% of cattle and secondary lung infections from prolonged recumbency are common in this group. BEF also has profound long term economic effects on milk production in dairy cattle, spermatogenesis in bulls while spontaneous abortions in pregnant cows have also been reported. There is no evidence of venereal transmission of BEF virus or that infection during pregnancy adversely affects foetal development.

4. Clinical Pathology

A marked neutrophilic leucocytosis coinciding with the onset of fever is characteristic of ephemeral fever and could almost be considered pathognomonic of the disease. The neutrophilia, often with up to 30% immature forms, is accompanied by lymphopaenia and eosinopaenia. Plasma fibrinogen can rise to three to four times the resting value, returning to normal levels within one to two weeks. A consistent observation in both natural and experimental cases is a

transient drop in total serum calcium during the febrile phase (Uren and Murphy, 1985). Falls of 20–25% are common and frank hypocalcaemia can occur in a small percentage of affected cattle. However, the underlying mechanisms responsible for this aberration are not clear. The effect of hypocalcaemia on the expression, course and duration of clinical signs of ephemeral fever is not known although it is interesting to observe that many of the signs displayed by affected animals closely resemble those seen in hypocalcaemic cows during milk fever. Plasma zinc and iron levels fall during experimental infection and serum non-esterified fatty acid levels are significantly elevated. In severely affected cattle, plasma alkaline phosphatase may be depressed and is often accompanied by elevated creatine kinase levels particularly in recumbent cattle.

5. Pathology

The outstanding features of BEF at post mortem are a polyserositis accompanied by elevated numbers of neutrophils in the tissues and synovial fluids. Increased fibrinous fluid is found in pleural, pericardial and peritoneal cavities as well as in joint capsules, particularly the lower limb joints. Fibrin clots containing sheets of synovial cells intertwined with neutrophils may be present in joint fluids. Changes to the musculoskeletal system include periarthritis, tenosynovitis, fasciculitis and cellulitis accompanied by localised focal necrosis of skeletal muscle. Pulmonary emphysema, both vesicular and interstitial with lobular consolidation have been reported. Histological changes are consistent with the inflammatory nature of the disease. Swollen and hyperplastic endothelium accompanied by perivascular neutrophil infiltration and perivascular oedema is characteristic of BEF. Focal necrosis of skeletal muscle and skin, together with fibrinoid necrosis of small vessels in synovial membranes and tendon sheaths has been observed. Lesions in the lungs include peribronchiolar haemorrhage, oedema, emphysema, tracheitis, bronchiolitis and pulmonary and subcutaneous hyperaemia.

6. Differential Diagnosis

Apart from the broad field of transient systemic malaise, specific conditions which should be considered include:
(a) Botulism;
(b) Babesiosis;
(c) Infectious Bovine Rhinotracheitis;
(d) Mucosal Disease;
(e) Bluetongue;
(f) Sporadic Bovine Encephalomyelitis;
(g) Leptospirosis;
(h) Laminitis (Vesicular Diseases); and
(i) Vitamin A Deficiency.

7. Collection and Preparation of Blood for the Isolation of Virus

BEF virus is only circulating in affected animals for two to three days, from about 24 hours before the temperature response to the end of the temperature response. There is little value in attempting virus isolation if the animal's temperature has returned to normal.

Whole blood is collected aseptically into heparin [a final concentration of 100 International Units (IU) of heparin per 10 mL of blood] or EDTA (final concentration of 20 mg of the sodium or potassium salt of EDTA per 10 mL of blood). The specimen should preferably comprise at least 10 mL. It should be chilled during holding or transport and prepared for storage or inoculation for virus isolation as soon as possible. The whole uncoagulated blood may be used as inoculum for virus isolation or, preferably, it is centrifuged and buffy coat separated, washed and resuspended in diluent.

8. Virus Isolation and Animal Transmission

The laboratory systems of choice for the isolation of BEF virus are the inoculation of suckling mice and insect or mammalian tissue cultures. No system has yet proven to be highly efficient. The most sensitive system for the demonstration of BEF virus in affected animals is the intravenous inoculation of susceptible cattle with the suspect material. This procedure should be required only in very unusual circumstances now that alternatives are available.

8.1. Insect Cell Cultures

A mosquito tissue culture cell line (C6/36) developed from *Aedes albopictus* is preferred to mammalian cell cultures or mice. Cell cultures are grown in 25 cm^2, flat tissue culture flasks. L15 medium (5 mL) containing 10×10^6 cells/mL are used per flask. About 0.1 mL of washed leucocytes from a clinically ill animal are inoculated into each flask. The medium is changed 24 hours later. After incubation for 7–14 days at 20–28°C, cells and medium may be subinoculated into BHK cell cultures which are then observed for cytopathic effect (CPE). Alternatively, after 14 days incubation subcultures may be made into 96-well plates or chamber slides. These are stained and examined for immunofluorescence as described by Cybinski and Zakrzewski (1983) using specific rabbit antiserum prepared against the BB7721 strain of BEF virus (Doherty *et al.*, 1968).

8.2. Mammalian Cell Cultures

It is possible to isolate virus by direct inoculation of mammalian cell cultures, but this method is less sensitive than is the use of other hosts described (cattle, suckling mice or insect cell cultures).

It is commonly used as a final step so that other activities, such as identification or serological comparisons, can be made more conveniently. BHK21 and Vero are the cell cultures of choice. Material prepared from ox blood, mouse brain or insect cell culture is inoculated, both undiluted and at a dilution of 1:10, in 0.1 mL volumes to four tubes and a chamber slide culture. The chamber slide culture is examined by immunofluorescent (IF) staining on post-inoculation (p.i.) day 1. Tube cultures are rolled, receive a change of medium on p.i. day 1, and are then examined daily for CPE. At seven days p.i., or earlier if CPE develops, medium and scraped cells are harvested and passed to similar cell cultures. As not all strains of BEF virus are cytopathogenic, chamber slide cultures are included in each pass. Examination of the material is usually concluded after a total of three passes. Virus isolates, recognised by IF or CPE, are specifically identified (see 9.).

8.3. Mice

Suckling mice one to three days of age may be used for the isolation of BEF virus. Litters of mice (six to eight per litter) are inoculated with 10–30 µL of whole blood or white cell suspension by the intracerebral route. Whole blood should be inoculated undiluted and at a dilution of 1:10 into separate litters, as on occasions undiluted blood appears to contain an interfering factor that delays the onset of sickness when compared with mice inoculated with the diluted blood. Inoculated mice are inspected daily for signs of ill health. Any mice dying within 48 hours are discarded. Mice that become sick or die after 48 hours have their brains removed and, individually, either stored below -70°C or passed as 10% suspensions by inoculation into further litters or mammalian cell cultures. Litters of mice showing no evidence of ill health 10–14 days after inoculation are killed and pooled brain suspensions prepared and inoculated into further litters. The original material is considered to be free of BEF virus if, on three passes in mice, no disease has been induced.

8.4. Cattle

The successful transmission of BEF to cattle is recognised by the production of a clinical response and the demonstration of antibody to BEF virus following inoculation with suspect material. Animals should be six months of age or older, and should have no detectable serum neutralising antibody prior to use. Whole blood (10 mL) or the leucocytes from this amount of blood, are inoculated intravenously into one or more experimental cattle. Temperatures are taken twice daily for 10 days and animals examined for clinical signs of disease. The incubation period from the time of inoculation until a temperature rise is usually between two and 10 days. The ani-

mal is bled into heparin or ethylenediamminetetraacetic acid (EDTA) on p.i. days five and seven, or when fever or other clinical signs are detected. Portions of these samples are stored at or below -70°C, while portions are used directly, or after storage if necessary, for virus isolation in other host systems. Serum samples should be collected 14 and 21 days after inoculation and tested for neutralising antibody to BEF virus. The transmission is successful if neutralising antibody develops in the serum of the recipient animal, whether or not clinical signs characteristic of ephemeral fever are also noted.

9. Virus Identification

Infective agents producing sickness and death in suckling mice, or detected by CPE or IF staining in tissue cultures, require specific identification. Specific antisera prepared in cattle, rabbits or any other suitable animal may be used to identify the virus in neutralisation tests in either tissue cultures or mice. Ideally, identification should be achieved by using a reference antiserum provided for the purpose by the ARL. Preferably a constant serum/varying virus method should be used to determine a neutralising index (NI) which can be interpreted according to advice provided with it. For control purposes, standard BEF virus should be identified concurrently.

10. Diagnosis by Serology

Diagnosis by serology requires the collection of one serum sample during clinical disease and a second sample two to three weeks later during the convalescent stage. The established serological technique is the virus neutralisation (VN) test and it is essential that the paired sera be examined concurrently. A diagnosis of BEF is deemed positive if there is a four-fold rise in antibody titre in the convalescent stage sample. Other serological tests such as complement fixation (CF) and enzyme-linked immunoassay (ELISA) can be used for diagnosis. The CF test is laborious to perform and is not specific for BEF and will detect antibody to all BEF-related viruses. A highly specific blocking ELISA test utilising purified antigen and monoclonal antibodies has been developed but is not in general use. When available this test will be capable of distinguishing BEF antibodies from the related viruses and also exclude the minor cross-reactions that can be detected by the VN test. The VN test is a sterile technique in cell culture so blood samples should be collected as cleanly as possible. The serum should be allowed to separate from the clot at room temperature. Following centrifugation to remove any red blood cells the sera should be heated at 56°C for 30 min and then stored at -20°C. Unless care is taken in the collection, preparation and storage of the sera, some of the sera are likely to be toxic for cell cultures. This will prevent testing the sera in low dilutions.

11. Neutralisation Test

11.1. Equipment

11.1.1. Plates

Flat bottomed microtitre plates of 96 x 0.3 mL wells, suitable for cell culture.

11.1.2. Droppers

Volume 25 and 50 µL. These are sterilised with cotton wool filter insert, by autoclaving. Various eight- or 12-place manifold repeating droppers may be used for distributing diluent and virus.

11.1.3. Diluters

Diluters of 25 µL are used for two-fold dilution series. Loop diluters (microdiluters) are cleaned between samples by rotating in three changes of sterile distilled water blotted to remove water and sterilised by dipping in alcohol and flaming. They are allowed to cool before proceeding to the next sample.

'Pipette' diluters, either single, e.g. Oxford Sampler, or multiple, single-dose, full-delivery units, e.g. Costar Octa 8-tip pipettes, with sterile tips may be used instead of microdiluters.

11.2. Materials

11.2.1. Diluent

Growth medium is used for all dilutions of serum or virus, and for substitution volumes in controls. The type of diluent is recommended by the ARL.

11.2.2. Cells

Vero or BHK cells may be used. Cell suspensions are obtained from either current bulk monolayer cultures or from frozen stocks.

Bulk monolayers are removed from the growth surface by trypsin or a trypsin-versene mixture and resuspended in growth medium to a concentration of 3×10^5 cells/mL.

If cells available are found to be unsuitable then parent stocks of suitable cells, and advice regarding their culture and use, should be obtained from the ARL.

11.2.3. Virus Working Stock

Frozen working stocks of virus, of known titre for use in VN tests, are supplied by the ARL. This material must be held at -70°C or lower prior to use.

The virus should be titrated on the cells to be used in the test to determine that the cells support the growth of the virus. The virus is titrated in 10-fold steps from 10^0 to 10^{-6} at the dilution recommended by the ARL. Each dilution (25 µL) are inoculated in quadruplicate wells with 100 µL of cells at 3×10^5 cells/mL. The plate is then sealed, incubated and read (see 11.3.2.). The virus titre should be about 100 $TCID_{50}$, i.e. wells should be positive for CPE at 10^0, 10^{-1} and two wells of 10^{-2}, and negative for the remainder (see 11.3.4.). If not, advice should be obtained from the ARL.

11.2.4. Positive Control Sera

One or more positive sera of known titre are provided by the ARL as freeze-dried 'use once' ampoules.

11.3. Method

Test serum is tested at a range of two-fold dilutions from 1/2. The end point at the titration is a matter of judgement, acute phase serum should normally have a low titre; therefore, 1/2–1/16 should suffice, but the convalescent serum should be titrated higher. Titres of >256 have been found. It is normal practice to test each dilution in quadruplicate; however, a minimum of two wells per dilution can be used. Testing can also be conducted by three-fold dilutions of 1/3, 1/9, 1/27, etc., but the volumes of serum and virus used must be altered from the method described below; the ARL can advise on appropriate alterations.

11.3.1. Preparation

11.3.1.1. Test serum samples

To each well of the first row is added 25 µL of diluent. In the top row of wells is then added 25 µL of serum, to give a dilution of 1/2. After mixing, 25 µL of this dilution is transferred to the next row to give a dilution of 1/4, this process is repeated down the plate to the end point, e.g. 1/2–1/256. It is important to change sterile tips or wash loops between each step to prevent transfer of extra serum into the dilutions.

11.3.1.2. Controls

11.3.1.2.1. Cell control. To four wells add 50 µL of diluent.

(a) Positive control serum — the supplied positive control(s) are titrated the same as the test serum samples to the end point suggested by the ARL.

(b) Foetal calf serum (FCS) control — (to test for antibodies in the FCS used in the test diluent) — to four wells add 25 µL of FCS and 25 µL of diluent (negative control) and to four wells add 25 µL of FCS only (positive control). A positive control is included to confirm the integrity of the FCS.

11.3.1.2.2. Virus control titration. In quadruplicate add 25 µL diluent for dilutions of virus 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}.

11.3.2. Procedure

The virus should be pretested to determine the appropriate dilution to achieve 100 $TCID_{50}$/well [see 11(ii)(c)].

(a) Virus dilution (25 µL) is added to all test sample wells, the FCS positive wells and the positive serum control(s). Do not add virus to the cell control, FCS negative, the virus control titration (or the toxicity controls if included). An aliquot of the virus dilution is retained in a separate bottle.

(b) The plates and the aliquot of virus dilution are incubated at room temperature 20–25°C for one hour.

(c) After one hour, the separate aliquot of virus dilution is titrated 10 fold from 10^0 to 10^{-4} (see 11.2.3.) and 25 µL of each dilution is added to the corresponding row of wells in the virus control titration.

(d) Then 100 µL of cell suspension at 3×10^5 cells/mL is dispensed into every well.

(e) The plates are then sealed by adding about 100 µL of sterile liquid paraffin to each well and then placed at 37°C and placed into a 37°C/5% carbon dioxide/85% relative humidity incubator.

(f) The plates are read at five days.

11.3.3. Toxicity of Test Samples

Some serum can be toxic to cell cultures. To test for toxicity a control can be included for each test serum. This control is a single well of each serum dilution tested and is the same as normal test wells except virus is not included. Toxic serum will cause cellular destruction within 24–48 hours.

11.4. Reading the Test

Plates are read with an inverted microscope. As a marginal amount of neutralising antibody is still present in wells just beyond the point of complete neutralisation, it may impede the progression of foci of infection that were initially established. CPE which develops just beyond the end-point of neutralisation may be less in degree and extent than is that seen with similar marginal doses of virus just before the end point of a virus titration. Therefore, any convincing CPE, however limited in extent, should be read as positive for virus.

Some sera when used undiluted (i.e. one volume of undiluted serum + one volume of virus) are toxic to the cell cultures. This appears to be related to the method of collection, preparation and storage of the sera. Thus it is necessary to distinguish between cellular degeneration caused by toxicity and that caused by virus CPE. This can be achieved by including a serum toxicity control as described in the method above or examining the plates at 24 and 48 hours for evidence of toxic effects. If the serum toxicity control shows no evidence of toxicity, then the other wells are read.

11.5. Toxic Effect on Vero Cells by 'Post Vaccination' Serum

Following two vaccinations, many animals develop a serological response to Vero cells. In the first or second dilutions of serum in the BEF neutralisation test, the Vero cell monolayer will be damaged and patchy. Unlike CPE due to BEF virus, this cellular damage is apparent before the establishment of the monolayer. The Vero cells are retarded in growth and the monolayer does not firm. Instead, small islands of cells grow but are premature in losing adherence and lifting off the plate.

If this effect interferes with reading the neutralisation test, BHK cells should be used in the test instead of Vero cells.

11.6. Interpretations

11.6.1. Interpretation of Controls

The titre of the positive serum control(s) should be within the limits advised by the ARL (see 11.7.). The cell control should show good cell growth with a confluent monolayer and no sign of cell destruction. The FCS controls should show CPE in the positive control with no sign of inhibition of virus growth, and a confluent monolayer in the negative control. If there are signs of inhibition of virus growth or toxicity to the cells in the controls a new batch of FCS should be used. The virus control titration should give a virus titre of 100 $TCID_{50}$/well (see 11.7.).

11.6.2. Interpretation of Test Sera

The titre of a test serum is the reciprocal of the highest dilution showing a 50% or greater reduction in the number of wells exhibiting CPE, e.g. in two-fold dilutions in quadruplicate (Table 1).

Titres should be expressed as a single value, negative serum should be expressed as less than the minimum dilution i.e. <4.

11.7. Acceptance Criteria

The test as a whole will be accepted if the virus content was not less than 50 $TCID_{50}$ nor greater than 150 $TCID_{50}$ and all control sera have reacted within the limits advised by the ARL.

If any of the controls indicate that the test has been too sensitive, i.e. virus titre was not sufficient or the control serum reacted to a titre greater than the limit set for it, then only negative results will be accepted without qualification. Positive results may be reported as titres equal to or less than stated values.

If any of the controls indicate that the test has been too insensitive, i.e. virus titre was too high or a control serum did not react to the required titre, then all negative results are rejected. Positive results may be reported as titres equal to or greater than stated values.

Table 1. Interpretation of test sera

Dilution	Wells showing CPE	Comment
1/4	----	
1/8	----	
1/16	----	
1/32	--++ ←	A titration as illustrated
1/64	++++	gives a titre of 32
1/256	++++	

If there are conflicting indications, in regard to the sensitivity of the test, from two of these controls, including the virus content, then the whole test is rejected.

Acceptance of any reading is dependent on there being no evidence of CPE, cytotoxicity or other departure from normality by the serum control well at the same dilution.

11.8. Use of the Virus Neutralisation Test for Diagnosis or Certification

There is no absolute criterion for the interpretation of the test for the purpose of diagnosis or certification. Conventionally, a four-fold rise in titre between two sera collected from one animal is taken as indicating infection at about the time of the first sampling. Where sera are tested at three-fold dilutions a two-step, or nine-fold, difference should be required. More importantly, the results should be interpreted in the light of other information, particularly the temporal relationship between clinical events, other diagnostic indicators and the collection of the samples. Ideally, several animals from an affected group will have been tested and, particularly in regard to diagnosis of disease, as opposed to the diagnosis of infection, negative results may, providing that the samples were apt, carry as much weight as do positive results. Serological evidence of BEF virus activity may also indicate, indirectly, a period of activity by other arboviruses.

The interpretation of test results for certification purposes is outside the scope of this paper. It will be negotiated by representatives of parties and countries affected by the purpose for which certification is required, separately for each purpose, and may require that antibody be either absent or present, either at nominated titres.

12. References

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