

Infectious Bovine Rhinotracheitis

Xingnian Gu and PD Kirkland

Elizabeth Macarthur Agricultural Institute

PMB 8
 Camden, NSW 2570
 Australia
 peter.kirkland@dpi.nsw.gov.au

Part 1. Diagnostic Overview**Summary**

Bovine herpesvirus1 (BHV-1) virus causes two diseases in cattle: infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis (IPV). These infections occur worldwide, including in Australia and New Zealand. The clinical signs are characterised by fever and involvement of the upper respiratory tract, including conjunctivitis, rhinitis and tracheitis. Secondary bacterial infections may lead to pneumonia, especially in intensively managed livestock, such as beef cattle in feedlots. The venereal forms of the disease result in pustular lesions in the prepuce and penile epithelium of the bull male and vulva and vagina of the cow. These lesions can impair reproduction. Strains of BHV-1 found in many countries can cause abortion but these are not present in Australia and New Zealand. As BHV-1 is excreted in semen and can be spread by artificial insemination, bulls entering artificial breeding centres are screened for freedom from infection. Freedom from infection is most frequently determined by serological methods, using the virus neutralisation test (VNT) or enzyme-linked immunosorbent assay (ELISA). Disease is diagnosed by demonstration of seroconversion with paired sera or by virus isolation from specimens collected during the acute phase of the disease. Semen certification is achieved by virus isolation in cell culture or by use of the polymerase chain reaction (PCR) to detect viral DNA.

1. Aetiology

Bovine herpesvirus1 (BHV-1) virus is a member of the Family *Herpesviridae* (subfamily Alphaherpesvirinae). Three subtypes of BHV-1 are recognised worldwide: BHV-1.1, BHV-1.2a and BHV-1.2b. Subtypes 1.1 and 1.2a are present in North America and parts of Europe but do not appear to be present in Australia¹ and New Zealand². BHV-1.2b strains are less virulent than the other strains. Viruses from the BHV-1.1 subtype cause severe respiratory disease and can be associated with abortion. Viruses isolated from buffaloes and goats and previously identified as BHV-1 by serological tests have a different restriction enzyme profile to subtypes of BHV-1, are now regarded as separate viruses and have been classified as BHV-2 and caprine herpesvirus, respectively. The bovine herpesvirus causing meningoencephalitis (previously BHV1.3) has been classified as BHV-5¹. BHV-4, which was found widespread in Israel, can cause mastitis, pneumonia, metritis, vaginitis, conjunctivitis, interdigital dermatitis and abortion in cattle.^{3,4,5}

2. Clinical Signs

BHV-1 infection causes an acute, contagious disease that affects either the respiratory or reproductive tracts in the following forms:

2.1 Respiratory infection

The respiratory form of the disease is most frequently observed in cattle managed under intensive conditions (for example, feedlots) and is not often noticed in cattle under grazing conditions. The clinical signs and pathological changes of BHV-1 infection of cattle are not characteristic and could easily be confused with

disease produced by a number of other pathogens. Laboratory confirmation is therefore essential. The incubation period ranges from 2-7 days. If the cases are uncomplicated, the disease may be very mild with only slight serous nasal discharge and a modest rise in body temperature over one to two days. Many cases remain unnoticed. In more severe cases, there is pronounced pyrexia of 40-42°C, which may last for several days. Affected animals are depressed with an increased respiratory rate and show a decline in milk production. The initial serous nasal discharge often becomes muco-purulent within a few days. The mucosa of the nares becomes reddened and shallow erosions may be present. Some animals develop excessive salivation. Oral lesions, which are uncommon, consist of shallow erosions of the oral mucosa. Some animals develop unilateral or bilateral conjunctivitis and have a clear ocular discharge, which may later become muco-purulent. In feedlot or other intensively managed cattle, there can be a severe necrotising laryngo-tracheitis and pneumonia that is complicated by secondary bacterial infections. These infections are usually encountered within the first 3-4 weeks of animals entering a feedlot. From time to time, there can be outbreaks of severe pneumonia due to BHV-1 infection at later times after entry to the feedlot.

Abortion as a complication of the respiratory form of BHV-1 infection has been frequently reported in North America and Europe but abortigenic strains of BHV-1 have not been found in Australia^{1,6,7,8,9} or New Zealand².

2.2 Genital infection

Genital infection with BHV-1 occurs in both sexes and is a more frequent manifestation of this herpesvirus infection in cattle on pasture. The infection may result in the development of vesicles, pustules and erosions or ulcers in the mucosa of the vulva and vagina or on the penis and prepuce^{7,8}.

2.2.1 Vulvovaginitis

This painful condition, which is known as infectious pustular vulvovaginitis (IPV), may be observed within a few days of mating. Frequent micturition and raising of the tail are the first clinical signs. There may be hyperaemia or oedema of the vulva and the posterior third of the vagina. Small red to white ulcers develop into pustules (0.5-3 mm in diameter). There may be a thick yellow or white mucopurulent exudate, especially in cases complicated by secondary bacterial infection.^{7,8}

2.2.2 Balanoposthitis

The disease in bulls is known as infectious pustular balanoposthitis (IPB). After a 2-3 day incubation period, pustules appear on the mucosal surface of the penis and prepuce.¹⁰ These pustules can progress to ulcers with a mucopurulent discharge and may prevent a bull from serving. A proportion of infected bulls will also excrete virus in their semen. In turn, infected semen can infect susceptible females, by natural or artificial insemination.

2.3 Conjunctivitis

The conjunctival form of BHV-1 infection, which resembles 'pink eye', is relatively uncommon. There can be occasional involvement of the cornea, and a panophthalmitis. In some cases the only sign of infection is conjunctivitis.

3. Epidemiology

Infection occurs via the respiratory and genital routes. The virus is spread both within and between herds mainly by horizontal transmission such as direct and indirect contact (fomites) and aerosol droplets, or from infected bulls by coitus and in infected semen either by artificial or natural insemination. Frozen semen is held under optimal conditions for virus survival.

As with other herpesviruses, infection with BHV-1 results in lifelong latent infection. This may occur in the absence of clinical signs and in the absence of detectable serum antibody. Corticosteroid treatment may induce a recrudescence of infection and excretion of virus. Natural excretion may occur following stress but the mechanism of latency and activation has not yet been fully elucidated. BHV-1 isolates vary in virulence in a manner unrelated to subtype. When introducing new animals into a closed herd or importing animals from overseas, those animals with antibody should be rejected, as they will be latently infected.

Seronegative animals should be checked repeatedly for antibody and should preferably be treated with corticosteroid and sampled for virus excretion before being allowed entry to a breeding herd.

4. Occurrence and Distribution

IBR occurs worldwide. In Australia, IBR was first diagnosed in 1962 and the virus was isolated after an outbreak of vaginitis and rhinitis in dairy herds in Victoria and Queensland. The prevalence of antibodies in mature breeding cattle ranges from 25-40%. In Australian beef feedlots, a high proportion (>80%) of young cattle are seronegative at entry and the prevalence of antibody rises to about 60% at slaughter.¹¹ In New Zealand, IBR virus is widely distributed in the cattle population and 19–82% of adult cattle have antibody titres. Infection in young cattle or beef cattle is less common.²

5. Pathogenesis and Latent Infections

Replication of BHV-1 takes place in the mucosal epithelial surfaces of the upper respiratory tract and genital mucosa and virus is shed in nasal and genital secretions. Semen may be contaminated during ejaculation. Local nerve cell endings are infected and the virus is transported to trigeminal and sacral ganglia where it establishes a lifelong latent infection. Once infected, animals become lifelong carriers of the virus. The latent infection may be reactivated periodically, with or without clinical signs; the virus is transported back to the site of entry and is shed with potential transmission to other animals. Most, if not all, seropositive animals are latently infected and virus shedding can be reactivated following stress or corticosteroid treatment. Viremia is rarely detected, but does occur.

6. Gross and Microscopic Lesions

The gross changes associated with an uncomplicated BHV-1 infection usually consist of pustular formation and shallow ulceration of the epithelium of the upper respiratory tract (including larynx and trachea) and the genitalia. There can be severe necrotic ulceration of the epithelium of the larynx and trachea in some cases. When pneumonia occurs, the changes are not pathognomic and are due to a combination of the effects of the virus and secondary bacterial infections. Histological changes that occur in uncomplicated respiratory cases are those of acute catarrhal inflammation. There is destruction of the epithelium with necrotic foci in the laryngeal and epiglottal mucosa. Broncho-pneumonic lesions can result from bacterial complication. Intranuclear inclusions may be found in epithelial cells of the respiratory tract during the early stages of infection.

7. Diagnostic Tests and Specimens

Specimens are likely to be examined for evidence of BHV-1 infection for:

- (a) diagnosis of specific disease incidents;
- (b) certification of the health or virus status of animals or of semen and embryos;

7.1 Disease Diagnosis

BHV-1 infection is commonly diagnosed by detection of the host response to the virus (for example, antibodies in serum) or by direct detection of the agent.

Serological tests are frequently used for the detection of BHV-1 infection. The types of serological tests commonly used for testing for BHV-1 antibody are:

- (a) The virus neutralisation (VN) test; and
- (b) The antibody ELISA.

Antibodies are detected in the serum of most animals within 2–3 weeks of infection. Maternally-derived antibodies may be detected for up to 7 months, but usually disappear in about 4–5 months.^{12,13} There is no known way of distinguishing passively transferred antibodies from those resulting from active infection.

Confirmation of disease caused by BHV-1 infection by serological means depends on the collection of acute and convalescent sera and either the demonstration of seroconversion (no detectable antibodies changing to a significant level of antibody) or a significant rise in antibody levels. The acute phase serum should be collected as soon as possible after clinical signs are observed (and always within 1–4 days) and the convalescent sample about 3–4 weeks later.

All ELISAs in use in Australia and New Zealand are available as commercial kits. The performance and sensitivity of different ELISAs is described in Parts 2 & 3.

Direct identification of agent can be used when the infection is in the acute phase.

Virus isolation is used routinely for diagnostic purposes. Usually virus isolation is attempted on swabs that have been collected from lesions in the respiratory or reproductive tracts as early as possible during the course of the disease. The transport medium into which the swabs have been placed is subsequently used to inoculate susceptible cell cultures. The presence of BHV-1 virus is detected by the development of characteristic changes in the monolayer cell cultures. Any cytopathogenic agent detected is then identified by neutralisation with specific antiserum or by immunoperoxidase or immunofluorescent staining to confirm its identity.

Recently PCR has been used to detect BHV-1 virus in semen. Although this assay was not evaluated for disease diagnosis, the level of sensitivity described should allow its application for diagnostic purposes.

7.2 Health Certification

Health certification may be required for a diversity of commercial purposes. These include screening of bulls for entry to artificial insemination (AI) centres, selection of both donors and recipient cows for embryo transfer, and the sale and introduction of animals to herds of known disease status. It is usual to test animals first to detect specific antibodies to BHV-1 as an indication of past and probable latent infection with BHV-1. For many purposes, the detection of antibodies to BHV-1 will render the animal ineligible for the purpose for which it was being tested. Occasionally, when BHV-1 seropositive bulls are detected, an importing country will accept the semen if it has been tested by virus isolation with negative results.

The VN test is an international standard and is most frequently used for regulatory testing (especially for certification of semen and embryo donors) although the ELISA may be used for the export of individual animals. The selection of VN test or ELISA to detect BHV-1 antibodies will vary depending on the requirement of the country importing the animals or germ plasm.

For virus isolation, it is usual to test semen or perhaps embryos. Due to the low levels of virus that may be present and the likelihood that the biological material (especially semen) has been diluted after collection, it is expected that a specified minimum volume of material will be collected. In most cases this will be 1–2 mL, representing 5–10 straws of commercially collected extended semen. A comparable volume of fluid from an embryo collection may also be tested.

Apart from virus isolation, the polymerase chain reaction (PCR) has been used to directly detect BHV-1 nucleic acid in specimens. A 'real time' PCR assay¹⁴ has recently been approved by OIE for the certification of semen for trade purposes but is not in yet in widespread use.

7.3 Specimen Transport and Storage

Blood and serum samples should be chilled (2–8°C) during transport to the laboratory and samples should be processed in the laboratory as soon as possible after arrival. Freezing sera at -20°C or lower is preferred for long-term storage but repeated freeze-thaw cycles should be avoided. Swabs should be placed in viral transport medium (phosphate buffered gelatine saline containing antibiotics – PBGS) immediately after collection and chilled while being transported. Samples of tissue collected at necropsy should be placed in sterile containers and kept chilled at all times. On receipt at the laboratory, the cotton-tipped swab should be removed from the PBGS and the fluid frozen at -50°C or lower if virus isolation cannot be commenced

within a few days. Freezing and thawing more than once may result in a significant reduction in the virus titre.

Part 2: Diagnostic Test Methods

1. Detection of Bovine Herpesvirus I Virus

Specimens are likely to be examined for evidence of BHV-1 infection for:

- (a) diagnosis of specific disease incidents;
- (b) certification of the health status of animals;
- (c) screening of materials of animal origin used in assays and the preparation of various biologicals (for example, vaccines).

For diagnostic testing, it may be necessary to test either for the virus or for antibodies. Testing for health certification almost exclusively requires detection of latently infected animals using specific antibody detection methods although semen may be screened by virus isolation or PCR. When various biological materials of animal origin are being screened for freedom from BHV-1, on most occasions testing is limited to ensuring freedom from infectious virus but occasionally there may also be a requirement to test for antibodies to BHV-1.

Virus isolation procedures for BHV-1 rely on the use of bovine cells and usually bovine serum. Although Australian strains of BHV-1 virus do not cause foetal infection, serum that is used to supplement cell culture medium should still be screened to confirm that it is free from specific antibody and non-specific inhibitors of BHV-1 virus replication. Due to the highly cytopathogenic nature of BHV-1 virus, it is improbable that cell cultures will be contaminated with this virus. However, when first used, new cell cultures of bovine origin should be routinely passaged at least 3 times in proven BHV-1 free medium to confirm absence of cytopathogenic agents.

1.1 Virus Isolation Procedures

The reliable diagnosis of BHV-1 infection by virus isolation depends on the availability of cell cultures that have proven susceptibility to BHV-1 virus. Different batches of primary bovine cells and cells of different types vary in their susceptibility to virus infection and should be tested for their susceptibility before use for virus isolation. Contamination of the virus isolation system with antibody can result in false negative results as well as reducing the sensitivity of the procedure.

1.1.1 Selection of Cell Type

A range of bovine cells are suitable for BHV-1 isolation and serology. When a new stock of cells is being produced, after passing quality control criteria for freedom from adventitious agents, they should be tested for susceptibility to infection with BHV-1. For this testing, it is necessary to have proven cells and a virus stock of known titre. The test cells are then used to titrate the virus stock to confirm that they give the titre obtained in the cell standard. If the titre is more than 0.5 log₁₀ lower than in the standard cells, the test cells are rejected. A similar procedure can be used to determine the yield of virus from a new batch of cells.

Cell types routinely used for BHV-1 testing include the following:

1.1.1.1 Primary cells

Primary cells are usually derived from testis, lung or kidney tissue. These are generally obtained from a foetus or neonate. The tissues are diced, trypsinised and then held frozen for subsequent end-use without previous culture.

1.1.1.2 Secondary cells

Secondary cells are commonly obtained from testis, kidney or lung. As these cells are subcultured three to six times prior to use, there is the advantage of being able to produce a much larger (and hence longer lasting) batch of cells than primary cells. Generally these cells are derived from the same source as primary cells. Limitations may be required on the number of subcultures permitted.

1.1.1.3 Continuous cells

Continuous bovine cells have been derived from turbinate tissue (commercially available bovine turbinate line) and bovine kidney (Madin Darby Bovine Kidney – MDBK) or lung (not available commercially). When obtained from commercial sources, extreme care should be taken to ensure freedom from adventitious viruses.

1.2 Virus Isolation Methods

As BHV-1 is readily cytopathogenic in cell culture, replication of the virus is usually monitored by observation of cells for a cytopathic effect (CPE) characteristic of a herpesvirus. The isolation of BHV-1 in cell culture can be usually considered in two stages:

- (a) inoculation and passage of the specimen in susceptible cell cultures; and
- (b) monitoring of cultures for the presence of cytopathology and identification of any cytopathogenic agent.

1.2.1 Reagents for virus isolation***Antibiotics – cell culture grade:***

Penicillin
Streptomycin
Amphotericin B

Phosphate buffered saline (PBS)

NaCl	8.0 g
Potassium chloride, KCl	0.2 g
Disodium hydrogen phosphate, Na ₂ HPO ₄	2.9 g
Potassium dihydrogen phosphate, KH ₂ PO ₄	0.2 g

Make up to 1 L with water and adjust pH to 7.4.

Phosphate buffered gelatin saline (PBGS)

Phenol red	0.4 mg
Gelatin	5.0 g
PBS (pH 7.4)	1 L

Dissolve gelatin in PBS at 56°C, add phenol red, dispense in 5 mL volumes and autoclave at 121°C for 20 min.

1.2.2 Preparation of Specimens

Swabs – Swabs are usually taken from the upper respiratory or reproductive tracts. After removal of the swab from the transport medium (PBGS is preferred), centrifuge at 800 g at approximately 4°C for 10 min. Discard the cell pellet.

Tissues – Fresh lung or trachea are the preferred tissues. Prepare a 10% (w/v) homogenate of the tissue or tracheal epithelium in cell culture growth medium (no serum) or PBGS containing penicillin (1000 units/mL), streptomycin (50,000 units/mL) and amphotericin B (4 ug/mL). Centrifuge at 1500 g at approximately 4°C for 10 min, discard the cell pellet. The supernatant is then ready to be cultured.

Semen – For each individual batch of semen that has been submitted, a minimum of 5 straws of extended semen is required. If there are multiple collections from the same animal on different days, these requirements still apply: 5 straws are needed from each semen batch. Just prior to examination, thaw each

straw of semen from the batch(es) to be examined and remove the contents of each of the 5 straws. Pool all of the semen from a single collection batch and estimate the volume (this should be approximately 1 mL).

Dilute the pooled semen 1/10 in serum-free cell culture medium (suitable for the cell type to be used). If there is a requirement to test 'raw' (undiluted) semen, it should be diluted 1/100 in suitable medium. The cell culture medium should contain double-strength antibiotics and an antifungal agent. The total volume of diluted semen should be approximately 10 mL. Centrifuge the diluted semen at 1500 g at 4°C for 20 min. Collect the supernatant and discard any pelleted material. Filter through a 0.45 µm filter.

The supernatant can be stored at 4°C for up to 48 hours. Once the cell cultures have been inoculated (see below) the residual supernatant should be stored frozen at about -70°C.

1.2.3 Inoculation of Cultures

Generally, specimens are inoculated onto slightly subconfluent monolayers of the cell type offering maximum sensitivity to BHV-1 infection. It is preferable to grow these cells in sealed tubes or small (12-25 cm²) flasks rather than microplates. Tubes offer the advantages of specimen biosecurity, minimising the potential for cross-contamination between specimens, and permit easy manipulations of the culture especially facilitating medium changing after inoculation with potentially cytotoxic specimens. If tubes are not used, 24-well plates should be used rather than 96-well plates because of the quantity of specimen that has to be examined.

All cultures should be incubated for 5 to 7 days after inoculation, frozen and thawed, and then passaged to new cell cultures for a further 2 passages. Known positive (virus infected) and uninfected specimens should be run as controls with each batch of specimens.

1.2.3.1 Inoculation of Tube Cultures

- (a) Inoculate 0.2 mL of sample (transport medium, semen or tissue homogenate) to duplicate tubes. Tube cultures should be slightly sub-confluent, with tissue culture medium freshly changed to maintenance medium prior to inoculation.
- (b) After rolling the tubes for two hours, the medium is again changed, decanting tissue culture fluid into separate containers for each specimen to prevent cross-contamination of tubes. Add new medium and incubate tubes at 37°C.
- (c) At 5–6 days after inoculation, freeze tube cultures at -50°C or lower prior to sub-culturing.
- (d) Pool the contents of all tubes inoculated with the same specimen. Passage 200 µL of pooled culture fluid to new tubes of confluent cells that have just had maintenance medium added. Incubate the tubes at 37°C for a further 5-6 days and passage once more if no cytopathology is detected.

1.2.4 Identification of BHV-1 virus

For the identification of a cytopathogenic agent in cell cultures that is considered to be a herpesvirus, screening usually employs a BHV-1 specific antiserum or monoclonal antibody to identify infected cells. The usual techniques are an indirect detection method with a second antispecies antiserum that is either coupled to a fluorescent dye or is enzyme-labelled to react with a chromogenic substrate that will stain the infected cells. The more common method is the use of peroxidase labelling, which often can be read macroscopically or microscopically with a conventional light microscope. In addition, a permanent record, in the form of the stained cultures, can be kept for future reference. The greatest problems with immunofluorescence are the need to use a fluorescent microscope and the lability of the fluorescent dye. Further, immunoperoxidase staining is ideally suited if there is a need to examine cells *in situ* in a larger vessel such as a cell culture flask.

1.2.4.1 Equipment

- 96-well tissue culture microplates
- Automatic plate washer

1.2.4.2 Reagents

- Polyclonal antiserum and/or monoclonal antibodies

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These are available commercially (see Part 3). The supplier will usually indicate an approximate working dilution. However, new batches of antibodies should be titrated by checkerboard titration with known infected and uninfected cells to determine optimal dilutions before use.

- Peroxidase conjugated second antibody

Use any commercially available, good quality horseradish peroxidase conjugated anti-serum directed against the immunoglobulin of the species in which the primary antibodies were produced (for example, antimouse IgG antiserum with mouse monoclonal antibodies). New batches of conjugate should be titrated by checkerboard titration with known infected and uninfected cells to determine optimal dilutions before use.

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- Formaldehyde solution

- Skim milk powder

- Gelatin

- Phosphate buffered saline (PBS) – see 1.2.1

- Phosphate buffered gelatin saline (PBGS) – see 1.2.1

- 3-Amino-9-ethylcarbazole substrate

3-Amino-9-ethylcarbazole (AEC)	10 mg
Dimethylsulfoxide (DMSO)	3 mL
Sodium acetate (20 mmol/L), pH 5	50 mL
H ₂ O ₂ , 30%	40 uL

Dissolve AEC in the DMSO. Immediately before use add the AEC/DMSO solution to the acetate buffer and then add hydrogen peroxide.

1.2.4.3 Inoculation of Microplates

- (a) Prepare 96-well microplate cultures of the appropriate cells. Seed plates at a sufficient density that cells are almost confluent at the time of use (24 hours after seeding).
- (b) Add 50 uL of specimen (culture fluid for virus identification) to each of 4 wells.
- (c) Add 50 uL of new cell culture medium to each of 4 wells to serve as a negative control and, lastly, 50 uL of a BHV-1 (containing about 10-50 TCID₅₀) to each of 4 wells to serve as a positive control. Add the positive control carefully to avoid any splashing or aerosol spread to adjacent wells.
- (d) Incubate plates in a CO₂ incubator at 37°C. Examine the plates after 24-48 hours to check for any cytotoxicity or microbial contamination.
- (e) After 3-4 days incubation, prepare the monolayers for antigen detection (see 1.2.4.4 below). Check the monolayers periodically during the incubation period to ensure that there is cytopathology present but it is not advanced, ensuring that there is at least 50% of the monolayer still present.

1.2.4.4 Immunoperoxidase Staining Cell Cultures

The method described below is for the staining of cells in a 96-well microplate but can be adapted for the *in situ* staining of cells in any vessel.

- (a) Fix the microplates for 10 min by adding 200 μ L of 3% formaldehyde (1:10 dilution of formaldehyde stock solution (approximately 30%) in PBS) to all wells. Add this fixative to the plate without removing the tissue culture medium from the wells.
(Note: if there is any need to passage the specimens further, or to retain any potential virus isolates, an appropriate quantity of culture supernatant should be removed before addition of the formaldehyde solution).
Discard the fixative/culture supernatant after 10 min. Although 20-50% (v/v) acetone (CH_3COCH_3) may be used as an alternative fixative to formaldehyde, it should not be used if plates are to be washed in an automated plate washer as the cells are often washed off the plate.
- (b) Wash the fixed monolayers 3 times using an automatic microplate washer with 0.05% Tween 20/water.
- (c) Block the monolayers with 5% skim milk powder in PBS (150 μ L/well) at 37°C in a humidified chamber for one hour. (Note that when monoclonal antibodies are used for staining, this step may be omitted if there is minimal non-specific binding).
- (d) Wash five times as in (b).
- (e) To each well add 50 μ L of BHV-1 specific antibody (polyclonal or mAb) (at an appropriate dilution in 1% gelatin/PBS) and incubate at 37°C in a humidified chamber for 90 min. The optimum concentration should be determined for each batch of BHV-1 antibody by 'checker-board' titration against reference positive and negative controls.
- (f) Wash five times as in (b).
- (g) Add 50 μ L/well of peroxidase conjugated antisppecies immunoglobulin at the optimum dilution in 1% gelatin/PBS and incubate at 37°C in a humidified chamber for 90 min. The optimum concentration should be determined for each batch of conjugate by 'checker-board' titration against reference positive and negative controls.
- (h) Wash five times as in (b).
- (i) Develop with 3-amino-9-ethylcarbazole (AEC) substrate (100 μ L/well) and read after 30 min by examination with an inverted microscope. Infected cells have reddish-brown staining and uninfected cells are unstained.

1.2.4.5 Interpretation

Specific staining of cells in the culture confirms that the virus is BHV-1 but will not confirm the subtype.

1.3 Detection of virus by PCR

A 'real time' PCR (qPCR) assay has been developed¹⁴ and evaluated for the detection of BHV-1 DNA in bovine semen. This method has been accepted by OIE as an approved test for the detection of BHV-1 in semen. Full details of the method for extraction of viral DNA from semen samples, specifications for the primers and fluorogenic probe and the thermocycler conditions are described in the published document. The primers, probe and cycling conditions should be followed precisely. If an alternative DNA extraction method is used it should be thoroughly evaluated in parallel with the published method. Further evaluation in comparison with virus isolation is required before this assay is used for other diagnostic applications. A laboratory may choose to use a different 'internal control' systems to the one described in the published method, especially if the assay is validated for samples other than semen.

It should be noted that this assay¹⁴ is not specific to BHV-1 and will detect a number of other types of ruminant herpesviruses. This should not be considered a disadvantage when the test is used for certification purposes but should be taken into account when interpreting results in a diagnostic context.

2. Serology

The serological tests commonly used for testing for BHV-1 antibody are:

- (a) The virus neutralisation test (VNT);
- (b) The enzyme-linked immunosorbent assay (ELISA) for antibody detection.

Each of these tests has a role in the diagnosis of BHV-1 infection. The choice of one or other test depends to a large degree on the purpose for the testing. The VNT is often requested for regulatory purposes for the

certification of semen and embryo donors. The indirect ELISA (section 2.2) has been used more extensively in recent years than the VNT due to the short time needed to obtain a result and its suitability for screening large numbers of samples (for example, live animal exports). When testing for diagnostic purposes, it is usually necessary to test 'paired' serum samples collected during the acute and convalescent phases of suspected BHV1 virus infection. It is essential that the acute sample is collected within the first 3-4 days of observable signs of disease and the convalescence sample at least 2 and preferably 3-4 weeks later. These 'paired' samples should be tested concurrently in the same test so that antibody titres can be accurately determined and compared. Usually the VNT is more suited for the comparison of antibody titres and demonstration of rising titres, although seroconversions can be determined readily by ELISA.

2.1. Virus Neutralisation Test (VNT)

The VNT is similar in principle to that used for many viruses. It depends on the reduction or elimination of viral infectivity as measured in a living assay system. Antibody interacts with ('neutralises') infectious virus so that it is unable to enter susceptible cells and multiply. Residual viral infectivity is detected in cell cultures by the development of visible morphological changes or cytopathic effect (CPE) in the monolayer. The test is carried out under defined conditions of time and temperature. The amount of antibody in a sample is determined by the reaction of a range of dilutions of serum, each with the same amount of virus. Controls are included to verify the virus dose in the test and the reactivity of a known positive serum. The test may be conducted either as a 'screening' test (one dilution of test serum only being tested) or as a full test, testing a range of dilutions of serum to achieve an endpoint (a titre).

There are several different methods for the VNT but the OIE standard and the most sensitive method involves incubation of the virus and test serum at 37°C for 24 hours¹⁵. This method has also been adopted as the standard for Australia and New Zealand.

2.1.1. Virus Neutralisation Test

- (a) Inactivate sera to be tested by heating at 56°C for 30 minutes to remove possible 'non-specific' inhibitors of virus infectivity and to inactivate microbial contaminants (including viruses) in the sample. Dilute sera in 96-well tissue culture microplates. Sera are tested in duplicate starting with undiluted serum. For titration, a 2-fold dilution series is usually employed. Allow 2 sets of wells for the neutralisation test and a third set of wells as a 'serum control' to monitor for toxicity of the test samples. Add 75 µL of diluent (complete growth medium) to the first row of wells and 50 µL to all other wells. Make doubling dilutions, using a multichannel pipette, by adding 25 µL of test serum to the first row, discarding tips and transferring 50 µL to the next row to finally give 50 µL of diluted serum per well. Discard 50 µL from the last row of wells. A positive serum with a known neutralising titre should also be included in each test for standardisation purposes.
- (b) Add the BHV-1 virus (50 µL/well), diluted in tissue culture medium to contain 100 TCID₅₀/50 µL. The final dilution will vary depending on batch of virus stock and cell type used. The titre of the stock virus should be measured in the same cell type used for the VNT.
- (c) In each test, titrate the virus used to check the amount of challenge virus added to the test wells. Leave four to eight wells empty wells for later use for the cell culture control.
- (d) Gently mix the contents of each plate (cover on) on a plate shaker or by tapping. Incubate in a 5% CO₂ incubator at 37°C for 24 hours (or at least overnight – 16 hours).
- (e) At the end of the virus-serum incubation period (24 hours), add cells in growth medium (100 µL/well) at the appropriate seeding rate to achieve confluence in about 24 hours. Add cells to all wells including the empty wells reserved for the cell culture control.
- (f) Incubate plates at 37°C in 5% CO₂, for 5 days.
- (g) Examine the plates microscopically, and record observations (presence or absence of CPE, presence of contamination or toxicity). Cytopathology (CPE) is recorded as an absolute - any evidence of CPE at all, or absent.

2.1.2. Acceptance Criteria

- (a) The control virus titration should fall within 0.5 log₁₀ (preferably within 0.3 log₁₀) of the preferred challenge dose (2.0 log₁₀) for the results to be accepted.
- (b) Test samples should show no evidence of cytotoxicity, or if there is evidence of toxicity, it should not affect the ability to read the test result. For example, toxicity at a very low dilution should not affect the ability to detect a high titred, positive serum but would prevent the determination of a negative result.
- (d) The positive reference serum should not vary by more than four-fold.

2.1.3 Interpretation of Results

The titre of a serum sample is the reciprocal of the last dilution at which no CPE is observed. A negative sample is one in which there is evidence of CPE at the specified cut-off dilution (usually no higher than 1/4). In some circumstances, for export of live animals, semen and embryos, an importing country may specify a negative result at a dilution of 1/2 or even with undiluted serum.

2.2 Indirect Enzyme-linked Immunosorbent Assay for Antibody

A number of ELISAs have been developed and are now used extensively for certification where the antibody status of a large number of animals needs to be determined. A number of commercial ELISA kits are available. Most have very similar sensitivity and specificity when testing samples from individual animals.

In general, the following conditions should apply to the use of commercial ELISA kits:

- (i) All kits must be used strictly in accordance with the manufacturer's instructions;
- (ii) The relative sensitivity and specificity of kits from different suppliers should be determined by extensive testing of a large collection (at least 1-200) of sera of known status, both positive and negative, that have been tested in the VNT in an accredited laboratory.

Some kits are designed to be used in a screening test format whereby any positive samples should be retested in a confirmatory test of a different format. The screening tests have all wells coated with viral antigen while the confirmatory tests have alternate wells coated with a 'negative control' antigen (usually an uninfected cell culture extract) or a viral antigen. An alternative confirmatory test can be a monoclonal antibody based blocking ELISA. Some of the blocking ELISAs are glycoprotein specific, in that they will allow the detection of antibodies to only one of the viral glycoproteins. In Europe some of these tests have been used to discriminate between naturally infected animals and animals that have been vaccinated with a modified deletion mutant vaccine.

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Part 3: Reagents and Test Kits for IBR Diagnosis in Australia and New Zealand

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

1. ELISA Kits for IBR Antibody

The kits that have been evaluated for use in Australia and New Zealand are listed below:

1.1 Institute Pourquier

a) Serological diagnosis of Infectious Bovine Rhinotracheitis – Screening kit.

This test is an indirect ELISA that is suitable for testing of individual serum sample or milk samples. The test is designed as a screening test as there are only test wells coated with viral antigens. Both ultrapurified BHV-1 envelope and capsid antigens are included. Positive results of the tests of both individual and milk samples should be confirmed in a confirmatory test, such as the Bovine Infectious Bovine Rhinotracheitis Serodiagnosis – Verification kit.

b) Bovine Infectious Bovine Rhinotracheitis Serodiagnosis – Verification kit.

This kit is identical to the kit above, except that only the even-numbered columns are specifically coated with BHV-1 viral antigen. The samples to be tested are diluted and incubated in the wells. Any antibody specific to BHV-1 present in the serum will form a BHV-1 antibody immune-complex and remains bound in the wells. After washing, an anti-bovine antibody immunoglobulin coupled to an enzyme is added to the wells and incubated. This conjugate will bind to the immune-complex. After washing, the enzyme substrate (TMB) is added to the wells. If the conjugate is fixed in the wells, the enzyme will transform the substrate into a blue compound becoming yellow after stopping. The intensity of the colour is a measure of the rate of antibodies present in the sample to be tested.

The limit of positivity is defined by using a control serum supplied with the kit ("positive control"), which has to be added to each microplate.

IDEXX

IBR ELISA Screen/Verification kits

There are two ELISA kits available from IDEXX: Infectious bovine rhinotracheitis Virus gE and gB antibody kits. The gB kit was designed for detection of glycoprotein-B specific antibodies in bovine serum, plasma and milk samples using IBR gB-specific monoclonal antibodies. The gE kit was designed for detection of bovine herpesvirus-1 specific gE antibodies in bovine serum, plasma and milk samples using IBR gE-specific monoclonal antibodies. This kit can differentiate between animals that have been vaccinated with a marker-vaccine or have been naturally infected.

Svanovir

SVANOVIR IBR- Ab ELISA is designed to detect IBR specific antibodies (IgG₁) in bovine serum and milk samples.

2. Anti BHV-1 antibodies

2.1 Polyclonal antiserum and Monoclonal Antibody

Specific antisera and monoclonal antibodies are available from the American Type Culture Collection, the USDA National Veterinary Services Laboratory, Ames Iowa, or VLA Weybridge, New Haw, Addlestone, Surrey, UK.

2. Suppliers of Reagents and Kits.

The commercial distributors of the above ELISA kits and reagents for BHV-1 diagnosis are:

2.1 Australia:

IDEXX kits – Antibody ELISA kits

IDEXX Laboratories Pty Ltd
Unit 20/38-46 South St.,
Rydalmere NSW 2116
Tel: 02 9898 7300 or 1800 655 978
Fax: 02 9898 7302 or 1800 634 409

Institute Pourquier – Antibody ELISA kits

Laboratory Diagnostics
Unit 8, 106 Canterbury Rd.,
Bankstown NSW 2200
Tel: 02 8707 4222 or 1800 023 623
Fax: 02 8707 4200

Svanovir – Antibody ELISA

Australian Laboratory Services
PO Box 328,
Sydney Markets NSW 2129
Tel: 02 9764 4055 or 1800 252 286
Fax: 02 9764 3533

2.2 New Zealand:

IDEXX kits – Antibody ELISA kits

IDEXX Laboratories Pty Ltd
Unit 20/38-46 South St.,
Rydalmere NSW 2116
Tel: NZ Freecall 0800 102 084
Fax: NZ Freecall 0800 448 443

Institute Pourquier – Antibody ELISAs

Diagnostic Bioserve
PO Box 5246
Mt Maunganui, NZ.

Tel: 64 7 542 2325
Fax: 64 7 542 2326