

SECTION 3.8.

CAPRINAE

CHAPTER 3.8.1.

BORDER DISEASE

SUMMARY

Border disease (BD) is a viral disease of sheep and goats first reported in sheep in 1959 from the border region of England and Wales, and since recorded world-wide. Prevalence rates in sheep vary from 5% to 50% between countries and from region to region within countries. Clinical signs include barren ewes, abortions, stillbirths and the birth of small weak lambs. Affected lambs can show a fine tremor, abnormal body conformation and hairy fleeces (so-called 'hairy-shaker' or 'fuzzy' lambs). Consequently, the disease has sometimes been referred to as 'hairy shaker disease'. Vertical transmission plays an important role in the epidemiology of the disease. Infection of fetuses can result in the birth of persistently infected (PI) lambs. These PI lambs are viraemic, antibody negative and constantly excrete virus. The virus spreads from sheep to sheep, with PI animals being the most potent source of infection. Infection in goats is less common with abortion being the main presenting sign.

BD is caused by the Pestivirus border disease virus (BDV), but in some parts of the world, especially where there is close contact between sheep or goats and cattle, the same clinical signs may be caused by infection with bovine viral diarrhoea virus (BVDV). Therefore the genetic and antigenic differences between BDV and BVDV need to be taken into consideration when investigating disease outbreaks or certifying animals or germplasm for international movement. It is important to identify the viraemic PI animals so that they will not be used for breeding or trading purposes. Serological testing is insufficient. However, it is generally considered that serologically positive, nonviraemic sheep do not present a risk as latent infections are not known to occur in recovered animals. Pregnant seropositive, nonviraemic animals may, however, present a risk by carrying a PI fetus that cannot be detected until after parturition.

Identification of the agent: BDV is a species of Pestivirus (*Pestivirus ovis*) in the family Flaviviridae and is closely related to classical swine fever virus (*Pestivirus suis*) and BVDV viruses, which are classified in the distinct species: *Pestivirus bovis* (commonly known as BVDV type 1), *Pestivirus tauri* (formerly BVDV type 2) and *Pestivirus brazilense* (BVDV type 3 or Hobi-like pestivirus). Nearly all isolates of BDV are noncytopathogenic in cell culture. There are no defined serotypes but virus isolates exhibit considerable antigenic diversity. A number of separate genotypes have been identified.

Apparently healthy PI sheep resulting from congenital infection can be identified by direct detection of virus or nucleic acid in blood or tissues or by virus isolation in cell culture followed by immunostaining to detect the noncytopathogenic virus.

Diagnostic methods: The demonstration of virus by culture and antigen detection may be less reliable in lambs younger than 2 months that have received colostral antibody. Acute infection is usually subclinical and viraemia is transient and difficult to detect. The isolation of virus from tissues of aborted or stillborn lambs is often difficult but virus can be detected by sensitive reverse transcriptase polymerase chain reaction methods that are able to detect residual nucleic acid. However, tissues and blood from PI sheep more than a few months old contain high levels of virus, which can be easily identified by isolation and direct methods to detect antigens or nucleic acids. As

sheep may be infected with BVDV, it is preferable to use diagnostic assays that are 'pan-pestivirus' reactive and will readily detect all strains of BDV and BVDV.

Serological tests: Acute infection with BDV is best confirmed by demonstrating seroconversion using paired or sequential samples from several animals in the group. The enzyme-linked immunosorbent assay and virus neutralisation test (VNT) are the most commonly used antibody detection methods. Due to the antigenic differences between BDV and BVDV, assays for the detection of antibodies to BDV, especially by VNT, should preferably be based on a strain of BDV.

Requirements for vaccines: There is no standard vaccine for BDV, but a commercial killed whole-virus vaccine has been produced. Ideally, such a vaccine should be suitable for administration to females before breeding for prevention of transplacental infection. The use of BVDV vaccines has been advocated, but the antigenic diversity of BD viruses must be considered. In many instances, the antigenic diversity of BDV strains is sufficiently different to BVDV that a BVDV vaccine is unlikely to provide protection.

BD viruses have contaminated several modified live veterinary vaccines produced in sheep cells or containing sheep serum. This potential hazard should be recognised by manufacturers of biological products.

A. INTRODUCTION

Border disease virus (BDV) is a *Pestivirus* of the family *Flaviviridae* and is closely related to classical swine fever virus (CSFV) and bovine viral diarrhoea virus (BVDV). There are a number of officially recognised species, namely – BDV (*Pestivirus ovis*) CSFV (*Pestivirus suis*), BVDV types 1 and 2 (taxonomically known as *Pestivirus bovis* and *Pestivirus tauri*, respectively) and BVDV 3 or Hobi-like pestivirus (*Pestivirus brazilense*) (Postler et al., 2023), but a number of other pestiviruses that are considered to be distinct species have been reported. While CSF viruses are predominantly restricted to pigs, there are situations where the other species have been recovered from sheep. While the majority of isolates have been identified as BD viruses in areas where sheep or goats are raised in isolation from other species (Vilcek et al., 1997), in regions where there is close contact between small ruminants and cattle, BVDV may be frequently identified (Carlsson, 1991). Nearly all virus isolates of BDV are noncytopathogenic, although occasional cytopathic viruses have been isolated (Vantsis et al., 1976). BDV spreads naturally among sheep by the oro-nasal route and by vertical transmission. It is principally a cause of congenital disease in sheep and goats, but can also cause acute and persistent infections. Infection is less common in goats, in which persistent infection is rare as abortion is the main presenting sign. Pigs may also be infected by pestiviruses other than CSFV and antibodies to BDV in pigs may interfere with tests for the diagnosis of CSF (Oguzoglu et al., 2001). Several genotypes of BD viruses from sheep, goats and Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) have been described. Phylogenetic analysis using computer-assisted nucleotide sequence analysis suggests that genetic variability among BD viruses is greater than within each of the other *Pestivirus* species. At least eight BDV genotypes have been described (BDV type 1 to BDV type 8). Other ovine pestiviruses have been identified that are responsible for BD-like syndromes such as Tunisian and Tunisian-like, Aydin-like (Pestivirus I, Turkey) pestivirus genotypes from Tunisian sheep and a goat and a new emerging ovine pestivirus (OVPV) that was found to be genetically and antigenically closely related to CSFV (Becher et al., 2003; Righi et al., 2021; Vilcek & Nettleton, 2006). The chamois BDV is similar to isolates from sheep in the Iberian Peninsula (Valdazo-Gonzalez et al., 2007). This chapter describes BDV infection in sheep. Chapter 3.4.7 *Bovine viral diarrhoea* should also be consulted for related diagnostic methods.

1. Acute infections

Healthy newborn and adult sheep exposed to BDV usually experience only mild or inapparent disease. Slight fever and a mild leukopenia are associated with a short-lived viraemia detectable between days 4 and 11 post-infection, after which virus neutralising antibody appears in the serum (Thabti et al., 2002).

Acute infections are best diagnosed serologically using paired sera from a representative number of sheep. Occasional BDV isolates have been shown to produce high fever, profound and prolonged leukopenia, anorexia, conjunctivitis, nasal discharge, dyspnoea and diarrhoea, and 50% mortality in young lambs. One such isolate was recovered from a severe epidemic of BD among dairy sheep in 1984 (Chappuis et al., 1986). A second such isolate was a BDV contaminant of a live CSFV vaccine (Wensvoort & Terpstra, 1988).

2. Fetal infection

The main clinical signs of BD are seen following the infection of pregnant ewes. While the initial maternal infection is subclinical or mild, the consequences for the fetus are serious. Fetal death may occur at any stage of pregnancy, but is more common in fetuses infected early in gestation. Small dead fetuses may be resorbed or their abortion may pass unnoticed as the ewes continue to feed well and show no sign of discomfort. As lambing time approaches, the abortion of larger fetuses, stillbirths and the premature births of small, weak lambs will be seen. Confirmation that an abortion or stillbirth is due to BDV is often difficult to establish, but virus may be isolated from fetal tissues in some cases. The use of an appropriate real-time reverse-transcription polymerase chain reaction (RT-PCR) assay may give a higher level of success because of the advantages of high sensitivity and the ability to detect genome from non-infectious virus. In aborted fetuses, it is also possible to detect virus by immunohistochemistry of brain, thyroid and other tissues (Thur *et al.*, 1997). Samples of fetal fluids or serum should be tested for BDV antibody.

During lambing, an excessive number of barren ewes will become apparent, but it is the diseased live lambs that present the main clinical features characteristic of BD. The clinical signs exhibited by BD lambs are very variable and depend on the breed of sheep, the virulence of the virus and the time at which infection was introduced into the flock. Affected lambs are usually small and weak, many being unable to stand. Nervous signs and fleece changes are often apparent. The nervous signs of BD are its most characteristic feature. The tremor can vary from violent rhythmic contractions of the muscles of the hindlegs and back, to barely detectable fine trembling of the head, ears, and tail. Fleece abnormalities are most obvious in smooth-coated breeds, which develop hairy fleeces, especially on the neck and back. Abnormal brown or black pigmentation of the fleece may also be seen in BD-affected lambs. Blood samples to be tested for the presence of BDV or antibody should be collected into anticoagulant from suspect lambs before they have received colostrum. Once lambs have ingested colostrum, it is difficult to isolate virus until they are 2 months old and maternal antibody levels have waned. However, during this period, it may be possible to detect viral antigen in skin biopsies, by immunohistochemistry, in washed leukocytes by enzyme-linked immunosorbent assay (ELISA) or by real-time RT-PCR. ELISAs directed at detection of the Erns antigen appear to be less prone to interference by maternal antibodies and can often be used to detect antigen in serum.

With careful nursing, a proportion of BD lambs can be reared, although deaths may occur at any age. The nervous signs gradually decline and can disappear by 3–6 months of age. Weakness, and swaying of the hind-quarters, together with fine trembling of the head, may reappear at times of stress. Affected lambs often grow slowly and under normal field conditions many will die before or around weaning time. In cases where losses at lambing time have been low and no lambs with obvious signs of BD have been born, this can be the first presenting sign of disease.

Some fetal infections occurring around mid-gestation can result in lambs with severe nervous signs, locomotor disturbances and abnormal skeletons. Such lambs have lesions of cerebellar hypoplasia and dysplasia, hydranencephaly and porencephaly resulting from necrotising inflammation. The severe destructive lesions appear to be immune mediated, and lambs with such lesions frequently have high titres of serum antibody to BDV. Most lambs infected in late gestation are normal and healthy and are born free from virus but with BDV antibody. Some such lambs can be weak and may die in early life (Barlow & Patterson, 1982).

3. Persistent viraemia

When fetuses survive an infection that occurs before the onset of immune competence, they are born with a persistent viraemia. The ovine fetus can first respond to an antigenic stimulus between approximately 60 and 85 days of its 150-day gestation period. In fetuses infected before the onset of immune competence, viral replication is uncontrolled and 50% fetal death is common. In lambs surviving infection in early gestation, virus is widespread in all organs. Such lambs appear to be tolerant of the virus and have a persistent infection, usually for life. A precolostral blood sample will be virus positive and antibody negative. Typically, there is no inflammatory reaction and the most characteristic pathological changes are in the central nervous system (CNS) and skin. Throughout the CNS, there is a deficiency of myelin, and this causes the nervous signs. In the skin, primary wool follicles increase in size and the number of secondary wool follicles decreases, causing the hairy or coarse fleece.

Persistently viraemic sheep can be identified by the detection of viral antigens, nucleic acids or infectious virus in a blood sample. Viraemia is readily detectable by testing of serum at any time except within the first 2 months of life, when virus may be masked by colostral antibody and, possibly, in animals older than 4 years, some of which develop low levels of anti-BDV antibody (Nettleton *et al.*, 1992). Methods other than virus isolation may be preferred to avoid interference from antibodies. When the presence of colostral antibodies is suspected, the virus may be detected in washed leukocytes and in skin by using sensitive ELISAs. Although virus detection in blood during an acute infection is difficult, persistent viraemia should be confirmed by retesting animals after an interval of at least

3 weeks. The use of real-time RT-PCR should be considered at all times and for any sample type due to its high analytical sensitivity and the lack of interference from antibodies in a sample.

Some viraemic sheep survive to sexual maturity and are used for breeding. Lambs born to these infected dams are always persistently viraemic. Persistently viraemic sheep are a continual source of infectious virus to other animals and their identification is a major factor in any control programme. Sheep being traded should be screened for the absence of BDV viraemia.

Usually persistently infected (PI) rams have poor quality, highly infective semen and reduced fertility. All rams used for breeding should be screened for persistent BDV infection on a blood sample. Semen samples can also be screened for virus, but virus isolation is much less satisfactory than from blood because of the toxicity of semen for cell cultures. Real-time RT-PCR for detection of pestivirus nucleic acid would usually overcome toxicity problems, and thus this assay should be useful for testing semen from rams.

4. Late-onset disease in persistently viraemic sheep

Some PI sheep housed apart from other animals spontaneously develop intractable diarrhoea, wasting, excessive ocular and nasal discharges, sometimes with respiratory distress. At necropsy such sheep have gross thickening of the distal ileum, caecum and colon resulting from focal hyperplastic enteropathy. Cytopathic BDV can be recovered from the gut of these lambs. With no obvious outside source of cytopathic virus, it is most likely that such virus originates from the lamb's own virus pool, similar to what occurs with BVDV. Other PI sheep in the group may not develop the disease. This syndrome, which has been produced experimentally and recognised in occasional field outbreaks of BD, has several similarities with bovine mucosal disease (Nettleton *et al.*, 1992).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of border disease and their purpose

| Method | Purpose | | | | | |
|--|-----------------------------------|--|------------------------------------|--------------------------------|--|---|
| | Population freedom from infection | Individual animal freedom from infection prior to movement | Contribute to eradication policies | Confirmation of clinical cases | Prevalence of infection – surveillance | Immune status in individual animals or populations post-vaccination |
| Identification of the agent ^(a) | | | | | | |
| Virus isolation | + | ++ | ++ | +++ | – | – |
| Antigen detection by ELISA | + | ++ | +++ | +++ | – | – |
| NA detection by real-time RT-PCR | +++ | +++ | +++ | +++ | +++ | – |
| NA detection by ISH | – | – | – | + | – | – |
| Detection of immune response | | | | | | |
| Antibody detection by ELISA | ++ | ++ | ++ | + | ++ | ++ |
| VN | +++ | +++ | ++ | +++ | +++ | +++ |

Key: +++ = recommended for this purpose; ++ recommended but has limitations;

+ = suitable in very limited circumstances; – = not appropriate for this purpose.

ELISA = enzyme-linked immunosorbent assay; NA = nucleic acid; RT-PCR = reverse-transcription polymerase chain reaction; ISH = *in-situ* hybridisation; VN = virus neutralisation.

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

1. Identification of the agent

There is no designated WOAHP Reference Laboratory for BDV, but the reference laboratories for BVDV or CSFV will be able to provide advice¹. One of the most sensitive proven methods for identifying BDV remains virus isolation. However, a broadly reactive real-time RT-PCR assay (preferably pan-pestivirus reactive) will usually provide higher analytical sensitivity than virus isolation, can be used to test samples that are difficult to manage by virus isolation and can be performed in a few hours. Antigen-detection ELISA and immunohistochemical techniques on tissue sections are also valuable methods for identifying BDV-infected animals.

1.1. Virus isolation

It is essential that laboratories undertaking virus isolation have a guaranteed supply of pestivirus-free susceptible cells and bovine serum, or equivalent, that contain no anti-pestivirus activity and no contaminating virus. It is important that a laboratory quality assurance programme be in place. Chapter 3.4.7 provides detailed methods for virus isolation in either culture tubes or microplates for the isolation of pestiviruses from sheep or goat samples, including serum, whole blood, semen and tissues. The principles and precautions outlined in that chapter for the selection of cell cultures, medium components and reagents are equally relevant to this chapter. Provided proven pan-pestivirus reactive reagents (e.g. monoclonal antibodies [MAbs], primers and probes for real-time RT-PCR) are used for antigen or nucleic acid detection, the principal difference is the selection of appropriate cell cultures.

BD virus can be isolated in a number of primary or secondary ovine cell cultures (e.g. kidney, testes, lung). Ovine cell lines for BDV growth are rare. Semicontinuous cell lines derived from fetal lamb muscle (FLM), whole embryo (Thabti *et al.*, 2002) or sheep choroid plexus can be useful, but different lines vary considerably in their susceptibility to the virus. Ovine cells have been used successfully for the isolation and growth of BD viruses and BVDV types 1 and 2 from sheep. In regions where sheep may become infected with BVD viruses from cattle, a virus isolation system using both ovine and bovine cells could be optimal. However, bovine cells have lower sensitivity for the primary isolation and growth of some BD viruses, so reliance on bovine cells alone is inadvisable. Details of suitable bovine cell cultures are provided chapter 3.4.7. The precautions outlined in that chapter for the establishment of cells and medium components that are free from contamination with either pestiviruses or antibodies, and measures to ensure that the cells are susceptible to a wide range of local field strains are equally relevant to systems for detection of BDV.

From live animals, serum is the most frequently used sample to be tested for the presence of infectious virus. However, for difficult cases, the most sensitive way to confirm pestivirus viraemia is to wash leukocytes repeatedly (at least three times) in culture medium before co-cultivating them with susceptible cells in either cell culture tubes or microplates. After culture for 5–7 days, the cultures should be frozen and thawed once and an aliquot of diluted culture fluid passaged onto further susceptible cells grown in microplates or on chamber slides to allow antigen detection by immunocytochemistry. Staining for noncytopathic pestiviruses will usually detect virus at the end of the primary passage, but to detect slow-growing viruses in poorly permissive cells two passages are desirable. It is recommended that the culture supernatant used as inoculum for the second passage is diluted approximately 1/100 in new culture medium because some high titred field isolates will replicate poorly if passaged undiluted (i.e. at high multiplicity of infection – moi).

Tissues should be collected from dead animals in virus transport medium. In the laboratory, the tissues are ground to give a 10–20% (w/v) suspension, centrifuged to remove debris, and the supernatant passed through 0.45 µm filters. Spleen, lung, thyroid, thymus, kidney, brain, lymph nodes and gut lesions are the best organs for virus isolation.

Semen can be examined for the presence of BDV, but raw semen is strongly cytotoxic and must be diluted, usually at least 1/10 in culture medium. As the major threat of BDV-infected semen is from PI rams, blood is a more reliable clinical sample than semen for identifying such animals. There are many variations in virus isolation procedures. All should be optimised for maximum sensitivity using a standard reference virus preparation and, whenever possible, recent BDV field isolates. Most of the limitations of virus isolation for the detection of BDV in serum or blood, tissues or semen can be overcome by the use

1 Please consult the WOAHP Web site: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>

of a proven, sensitive pan-pestivirus reactive real-time RT-PCR. Some laboratories screen samples by real-time RT-PCR and undertake virus isolation on positive samples to collect BDV strains for future reference or research purposes.

For specific technical details of virus isolation procedures, including immunoperoxidase staining, refer to chapter 3.4.7.

1.2. Nucleic acid detection methods

The complete genomic sequences of three BD viruses have been determined and compared with those of other pestiviruses (Becher *et al.*, 1998; Ridpath & Bolin, 1997). Phylogenetic analysis shows BD viruses to be more closely related to CSFV than to BVDV (Becher *et al.*, 2003; Van Rijn *et al.*, 1997; Vilcek & Nettleton, 2006; Vilcek *et al.*, 1997). Real-time RT-PCR for diagnosing pestivirus infection is now used widely and a number of formats have been described. Real-time RT-PCR assays have the advantages of being able to detect both infectious virus and residual nucleic acid, the latter being of value for investigating abortions and lamb deaths. Furthermore, the presence of virus-specific antibodies in a sample will have no adverse effect on the sensitivity of the real-time RT-PCR assay. These assays are also useful for screening semen and, when recommended nucleic acid extraction protocols are followed, are less affected by components of the semen compared with virus isolation. Because of the potential for small ruminants to be infected with genetically different strains of BDV or with strains of BVDV, a pan-pestivirus reactive real-time RT-PCR with proven high sensitivity should be used. To ensure that the genetic spectrum of BDV strains is sufficiently covered, it may be necessary to apply a broadly reactive BDV specific real time RT-PCR in parallel to maximise diagnostic sensitivity. Suitable protocols for both nucleic acid extraction as well as the real-time RT-PCR are described in chapter 3.4.7. All precautions to minimise laboratory contamination should be followed closely.

After testing samples in a pan-pestivirus reactive assay, samples giving any level of reactivity should be investigated further by the application of a BDV-specific real-time RT-PCR (Willoughby *et al.*, 2006). It is important to note however that different genotypes of BDV may be circulating in some populations, especially wild ruminants such as chamois and deer, and may be transferred to sheep. An assay that is specific for the detection of BDV should be used with some caution as variants or previously unrecognised genotypes may not be detected, hence the value of initially screening samples with a pan-pestivirus reactive real-time RT-PCR. Nevertheless, there are also situations where a pan-pestivirus reactive real-time RT-PCR may have lower analytical sensitivity. Consequently, in any situation where BDV infection is suspected, the application of several diagnostic methods is recommended. Maternal serology can also play an important role as negative results should exclude the potential involvement of a pestivirus.

1.3. Enzyme-linked immunosorbent assay for antigen detection

ELISAs for the direct detection of pestivirus antigen in blood and tissues of infected animals have proven to be extremely useful for the detection of PI animals and the diagnosis of disease. The first ELISA for pestivirus antigen detection was described for detecting viraemic sheep and was later modified into a double MAb capture ELISA for use in sheep and cattle (Entrican *et al.*, 1994). The test is most commonly employed to identify PI viraemic sheep using washed, detergent-lysed blood leukocytes. The sensitivity is close to that of virus isolation and it is a practical method for screening large numbers of blood samples. As with virus isolation, high levels of colostral antibody can mask persistent viraemia. The ELISA is more effective than virus isolation in the presence of antibody, but may give false-negative results in viraemic lambs younger than 2 months old. The ELISA is usually not sensitive enough to detect acute BDV infections on blood samples. As well as for testing leukocytes, the antigen ELISA can also be used on tissue suspensions, especially spleen, from suspected PI sheep and, as an alternative to immunofluorescence and immunoperoxidase methods, on cell cultures. Several pestivirus ELISA methods have been published but there are at present no commercially available kits that have been fully validated for detecting BDV. Prior to use for regulatory purposes, these kits should be validated in the region where they are to be used to ensure that a wide range of field strains of BDV can be detected and that they are suitable for the sample types to be tested.

1.4. Immunohistochemistry

Viral antigen demonstration is possible in most of the tissues of PI animals (Braun *et al.*, 2002; Thur *et al.*, 1997) although this is not a method that is routinely used for diagnostic purposes. This should be done on acetone-fixed frozen tissue sections (cryostat sections) or paraffin wax embedded samples using appropriate antibodies. Pan-pestivirus reactive antibodies with NS2-3 specificity are suitable. Tissues with a high amount of viral antigen are brain, thyroid gland, lung and oral mucosa. Skin biopsies have been shown to be useful for *in-vivo* diagnosis of persistent BDV infection.

2. Serological tests

Antibody to BDV is usually detected in sheep sera using VN or an ELISA. The less sensitive agar gel immunodiffusion test is not recommended. Control positive and negative reference sera must be included in every test. These should give results within predetermined limits for the test to be considered valid. Single sera can be tested to determine the prevalence of BDV in a flock, region or country. For diagnosis, however, acute and convalescent sera are the best samples for confirming acute BDV infection. Repeat sera from one animal should always be tested alongside each other on the same plate to provide a reliable comparison of titres.

2.1. Virus neutralisation test

Due to antigenic diversity among pestiviruses the choice of test virus is difficult (Dekker *et al.*, 1995; Nettleton *et al.*, 1998). No single strain of BDV is ideal. A local strain that gives the highest antibody titre with a range of positive sheep sera should be used.

Because there are few cytopathogenic strains of BDV available, to achieve optimal analytical sensitivity, it is more usual to employ a representative local non-cytopathogenic strain and read the assay after immunoperoxidase staining of the cells. Proven highly sensitive, pestivirus-free sheep cells such as lamb testis or kidney cells are suitable and can be maintained as cryogenically frozen stocks for use over long periods of time. The precautions outlined for selection of pestivirus-free medium components are equally applicable to reagents to be used in VN tests. A recommended procedure follows.

2.1.1. Test procedure

- i) The test sera are heat-inactivated for 30 minutes at 56°C.
- ii) From a starting dilution of 1/4, serial twofold dilutions of the test sera are made in a cell-culture grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each sample, three or four wells are used at each dilution depending on the degree of precision required. Also, for each sample and at each serum dilution, one well is left without virus to monitor for evidence of sample toxicity that could mimic viral cytopathology or interfere with virus replication. Control positive and negative sera should also be included in each batch of tests.
- iii) An equal volume (e.g. 50 µl) of a stock of BDV containing 100 TCID₅₀ (50% tissue culture infective dose) is added to each well. A back titration of virus stock is also done in some spare wells to check the potency of the virus (acceptance limits 30–300 TCID₅₀).
- iv) The plate is incubated for 1 hour at 37°C.
- v) A flask of suitable cells (e.g. ovine testis or kidney cells) is trypsinised and the cell concentration is adjusted to 2×10^5 /ml. 100 µl of the cell suspension is added to each well of the microtitre plate.
- vi) The plate is incubated at 37°C for 4–5 days, either in a 5% CO₂ atmosphere or with the plate sealed.
- vii) The wells are examined microscopically to ensure that there is no evidence of toxicity or cytopathic effect (CPE), then fixed and stained by immunoperoxidase staining using an appropriate MAb. The VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman–Kärber or Reed Muench methods. A seronegative animal will show no neutralisation at the lowest dilution of serum (i.e. 1/4), equivalent to a final dilution of 1/8. For accurate comparison of antibody titres, and

particularly to demonstrate significant (more than fourfold) changes in titre, samples should be tested in parallel in the same test.

- viii) Occasionally there may be a need to determine whether antibody in a flock is against a virus belonging to a particular *Pestivirus* serogroup. A differential VN test can be used in which sera are titrated out against representative viruses from each of the four *Pestivirus* groups, i.e. BDV, BVDV types 1 and 2, and CSFV. Maximum titre will identify the infecting serotype and the spectrum of cross-reactivity with the other serotypes will also be revealed.

2.2. Enzyme-linked immunosorbent assay

An MAb-capture ELISA for measuring BDV antibodies has been described. Two pan-pestivirus MAbs that detect different epitopes on the immunodominant nonstructural protein NS 2/3 are used to capture detergent-lysed cell-culture grown antigen. The results correlate qualitatively with the VN test (Fenton *et al.*, 1991).

2.2.1. Antigen preparation

Use eight 225 cm² flasks of newly confluent FLM cells; four flasks will be controls and four will be infected. Wash the flasks and infect four with a 0.01–0.1 m.o.i. of Moredun cytopathic BDV. Allow the virus to adsorb for 2 hours at 37°C. Add maintenance media containing 2% FBS (free from BDV antibody), and incubate cultures for 4–5 days until CPE is obvious. Pool four control flask supernatants and separately pool four infected flask supernatants. Centrifuge at 3000 *g* for 15 minutes to pellet cells. Discard the supernatants. Retain the cell pellets. Wash the flasks with 50 ml of PBS and repeat the centrifugation step as above. Pool all the control cell pellets in 8 ml PBS containing 1% Nonidet P40 and return 2 ml to each control flask to lyse the remaining attached cells. Repeat for infected cells. Keep the flasks at 4°C for at least 2 hours agitating the small volume of fluid on the cells vigorously every 30 minutes to ensure total cell detachment. Centrifuge the control and infected antigen at 12,000 *g* for 5 minutes to remove the cell debris. Supernatant antigens are stored at –70°C in small aliquots.

2.2.2. Test procedure

- i) The two MAbs are diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6. All wells of a suitable ELISA-grade microtitre plate (e.g. Nunc maxisorb, Greiner 129b) are coated overnight at 4°C.
- ii) After washing three times in PBST, a blocking solution of PBST containing 10% horse serum (PBSTH) is added to all wells, which are incubated at 37°C for 1 hour.
- iii) The antigen is diluted to a predetermined dilution in PBSTH and alternate rows of wells are coated with virus and control antigens for 1 hour at 37°C. The plates are then washed three times in PBST before addition of test sera.
- iv) Test sera are diluted 1/50 in PBSTH and added to duplicate virus and duplicate control wells for 1 hour at 37°C. The plates are then washed three times in PBST.
- v) Anti-ovine IgG peroxidase conjugate is diluted to a predetermined dilution in PBSTH and added to all wells for 1 hour at 37°C. The plates are washed three times in PBST.
- vi) A suitable activated enzyme substrate/chromogen, such as ortho-phenylene diamine (OPD) or tetramethyl benzidine (TMB), is added. After colour development, the reaction is stopped with sulphuric acid and the absorbance read on an ELISA plate reader. The mean value of the two control wells is subtracted from the mean value of the two virus wells to give the corrected absorbance for each serum. Results are expressed as corrected absorbance with reference to the corrected absorbance of known positive and negative sera. Alternatively, ELISA titres can be extrapolated from a standard curve of a dilution series of a known positive reference serum.

If antigens of sufficient potency can be produced the MAb capture stage can be omitted. In this case alternate rows of wells are coated with virus and control antigen diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6, overnight at +4°C. The plates are washed and blocked as in step ii above. After washing, diluted test sera are added and the test proceeds from step iv as above.

C. REQUIREMENTS FOR VACCINES

1. Background

To be useful, a BDV vaccine should be effective when administered to female sheep before breeding to prevent transplacental infection. Experimental and commercial inactivated whole virus BDV vaccines have been produced in Europe (Brun *et al.*, 1993; Vantsis *et al.*, 1980). Unlike vaccines for BVDV, there is limited demand for vaccines against BDV and those produced have only been inactivated products. No live attenuated or recombinant subunit vaccines for BDV have been produced commercially.

Pestivirus contaminants of modified live virus vaccines have been found to be a cause of serious disease following their use in pigs, cattle, sheep and goats. Contaminated vaccines have included those used for the control of Aujeszky's disease, CSF, rotavirus, coronavirus, rinderpest, sheep pox and contagious pustular dermatitis. The insidious ability of pestiviruses to cross the placenta, and thus establish PI animals, gives them the potential to contaminate vaccines through cells, serum used as medium supplement, or seed stock virus. As nearly all isolates of pestiviruses are noncytopathic, they will remain undetected unless specific tests are carried out. Although such contamination should be less likely to be a problem with an inactivated vaccine, nevertheless steps should be taken to ensure that materials used in production are not contaminated.

1.1. Characteristics of a target product profile

Traditionally, pestivirus vaccines fall into two classes: modified live or inactivated virus vaccines. The essential requirement for both types is to provide a high level of fetal infection. Only inactivated vaccines have been produced for BDV. Properly formulated inactivated vaccines are very safe to use but, to obtain satisfactory levels of immunity, they usually require booster vaccinations, which may be inconvenient. Because of the propensity for antigenic variability, the vaccine should contain strains of BDV that are closely matched to viruses found in the area in which they are used. This may present particular challenges with BDV in regions where several antigenic types have been found. Due to the need to customise vaccines for the most commonly encountered strains within a country or region, it is not feasible to produce a vaccine antigen bank that can be drawn upon globally.

Guidance for the production of veterinary vaccines is given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

An ideal vaccine should contain a strain or strains of virus that give protection against all sheep pestiviruses. This may be challenging however, because of the range of pestiviruses with which sheep can be infected. There is considerable antigenic variation across these viruses – both between viruses that have been classified in the BDV genogroup as well as between viruses in the BVDV1 and BVDV2 genotypes (Becher *et al.*, 2003; Vilcek & Nettleton, 2006; Wensvoort *et al.*, 1989). Infection of sheep with the putative BVDV-3 genotype has also been described (Decaro *et al.*, 2012). It is likely that the antigenic composition of a vaccine will vary from region to region to provide an adequate antigenic match with dominant virus strains. Cross-neutralisation studies are required to establish optimal combinations. Nevertheless, it would appear that any BDV vaccine should contain at least a representative of the BDV and BVDV (type 1) groups. Characterisation of the biologically cloned vaccine viruses should include typing with MAbs and genotyping (Paton *et al.*, 1995).

2.1.1. Quality criteria (sterility, purity, freedom from extraneous agents)

It is crucial to ensure that all materials used in the preparation of the bulk antigens have been extensively screened to ensure freedom from extraneous agents. This should include master and working seeds, the cell cultures and all medium supplements such as bovine serum. Some bovine viruses and particularly BVDV can readily infect small ruminants such as sheep. Therefore, it is particularly important to ensure that any serum used that is of bovine origin is free of both adventitious BVDV and antibodies against BVDV strains because low levels of either virus or

antibody can mask the presence of the other. Materials and vaccine seeds should be tested for sterility and freedom from contamination with other agents, especially viruses as described in the chapter 1.1.8 and Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

If a vaccine passes basic tests, the efficacy of vaccination should ultimately be measured by the capacity to prevent transplacental transmission. Effective challenge of vaccinated pregnant ewes at 50–60 days gestation has been achieved by intranasal installation of virus or by mixing with PI sheep (Brun *et al.*, 1993). Usually this reliably produces persistently viraemic offspring in non-immune ewes. In regions where multiple genotypes of BDV viruses are commonly encountered, efficacy in protecting against multiple strains should be measured.

2.2. Method of manufacture

2.2.1. Procedure

Inactivated vaccines have been prepared using conventional laboratory techniques with stationary or rolled cell cultures. Inactivants have included formalin and beta-propiolactone. Adjuvants have included aluminium hydroxide and oil (Brun *et al.*, 1993; Vantsis *et al.*, 1980). Optimal yields depend on the cell type and isolate used. A commercial BDV vaccine containing two strains of virus has been prepared on ovine cell lines (Brun *et al.*, 1993). Cells must be produced according to a seed-lot system from a master cell seed (MCS) that has been shown to be free from all contaminating microorganisms. Vaccine should only be produced in cells fewer than 20 passages from the MCS. Control cells from every passage should be checked for pestivirus contamination. Standard procedures may be used, with the expectation for harvesting noncytopathic virus on days 4–7 after inoculation of cultures. The optimal yield of infectious virus will depend on several factors, including the cell culture, isolate used and the initial seeding rate of virus. These factors should be taken into consideration and virus replication kinetics investigated to establish the optimal conditions for large-scale virus production. Whether a live or inactivated vaccine, the essential aim will be to produce a high-titred virus stock. This bulk antigen preparation can subsequently be prepared according to the type of vaccine being considered.

2.2.2. Requirements for ingredients

BDV vaccines have usually been grown in cell cultures of ovine origin that are frequently supplemented with medium components of animal origin. The material of greatest concern is bovine serum due to the potential for contamination with BVD viruses and antibodies to these viruses. These adventitious contaminants not only affect the efficiency of production but also may mask the presence of low levels of infectious BVDV that may have undesirable characteristics. In addition to the virus seeds, all materials should be tested for sterility and freedom from contamination with other agents, especially viruses as described in chapters 1.1.8 and 1.1.9. Furthermore, materials of bovine or ovine origin should originate from a country with negligible risk for transmissible spongiform encephalopathies (see chapter 1.1.9).

2.2.3. In-process controls

In-process controls are part of the manufacturing process. Cultures should be inspected regularly to ensure that they remain free from gross bacterial contamination, and to monitor the health of the cells and the development or absence of CPE, as appropriate. While the basic requirement for efficacy is the capacity to induce an acceptable neutralising antibody response, during production, target concentrations of antigen required to achieve an acceptable response may be monitored indirectly by assessment of the quantity of infectious virus or antigen mass that is produced. Rapid diagnostic assays such as the ELISA are useful for monitoring BVDV antigen production. Alternatively, the quality of a batch of antigen may be determined by titration of the quantity of infectious virus present, although this may underestimate the quantity of antigen. For inactivated vaccines, infectivity is evaluated before inactivation. For inactivated vaccines the inactivation kinetics should be established so that a suitable safety margin can be determined and incorporated into the routine production processes. At the end of production, *in-vitro* cell culture assays should be undertaken to confirm that inactivation has been complete. These

innocuity tests should include a sufficient number of passages and volume of inoculum to ensure that very low levels of infectious virus would be detected if present.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Identity

Identity tests should demonstrate that no other strain of BDV is present when several strains are propagated in a facility producing multivalent vaccines.

iii) Safety

Samples from inactivated vaccines should be tested rigorously for viable virus. Samples of the product should be passaged for a minimum of three passages in sensitive cell cultures to ensure absence of live BDV. This *in-vitro* monitoring can be augmented by injecting two BDV-seronegative sheep with 20 doses of unformulated antigen as part of a standard safety test. Presence of live virus will result in the development of a more convincing serological response than will occur with inactivated virus alone. The sheep sera can also be examined for antibody to other prescribed agents.

Safety tests shall also consist of detecting any abnormal local or systemic adverse reactions to the vaccine by all vaccination route(s). Batch-to-batch safety tests are required unless safety of the product is demonstrated and approved in the registration dossier and production is consistent with that described in chapter 1.1.8. Vaccines must either be demonstrated to be safe in pregnant sheep (i.e. no transmission to the fetus), or should be licensed with a warning not to use them in pregnant animals.

iv) Batch potency

Vaccine potency is best tested in seronegative sheep in which the development and level of antibody is measured. BVD vaccines must be demonstrated to produce adequate immune responses when used in their final formulation according to the manufacturer's published instructions. The minimum quantity of infectious virus or antigen required to produce an acceptable immune response should be determined. An indirect measure of potency is given by the level of virus infectivity prior to inactivation. *In-vitro* assays should be used to monitor individual batches during production. The antigen content following inactivation can be assayed by MAb-capture ELISA and related to the results of established *in-vivo* potency results. It should be demonstrated that the lowest recommended dose of vaccine can prevent transplacental transmission of BDV in pregnant sheep.

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For registration of a vaccine, all relevant details concerning manufacture of the vaccine and quality control testing should be submitted to the relevant authorities. Unless otherwise specified by the authorities, information should be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

There is no standard method for the manufacture of a BDV vaccine, but conventional laboratory techniques with stationary, rolled or suspension (micro-carriers) cell cultures may be used. Inactivated vaccines can be prepared by conventional methods, such as binary ethylenimine, formalin or beta-propiolactone inactivation (Park & Bolin, 1987). A variety of adjuvants may be used.

2.3.2. Safety requirements

In-vivo tests should be undertaken using repeat doses (taking into account the maximum number of doses for primary vaccination and, if appropriate, the first revaccination/booster vaccination) and contain the maximum permitted antigen load and, depending on the formulation of the vaccine, the maximum number of vaccine strains.

i) Target and non-target animal safety

The safety of the final product formulation of inactivated vaccines should be assessed in susceptible young sheep that are free of maternally derived antibodies and in pregnant ewes. They should be checked for any local reactions following administration, and, in pregnant ewes, for any effects on the unborn lamb.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

In the event that a live virus vaccine was developed for BDV, virus seeds that have been passaged at least up to and preferably beyond the passage limit specified for the seed should be inoculated into young lambs to confirm that there is no evidence of disease. If a live attenuated vaccine has been registered for use in pregnant animals, reversion to virulence tests should also include pregnant animals. Live attenuated vaccines should not be transmissible to unvaccinated 'in-contact' animals.

iii) Precautions (hazards)

BDV is not considered to be a human health hazard. Standard good microbiological practice should be adequate for handling the virus in the laboratory. While the inactivated virus in a vaccine should be identified as harmless for people administering the product, adjuvants included in the vaccine may cause injury to people. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

2.3.3. Efficacy requirements

The potency of the vaccine should be determined by inoculation into seronegative and virus negative lambs, followed by monitoring of the antibody response. Antigen content can be assayed by infectivity titration prior to inactivation and subsequently by ELISA and adjusted as required to a standard level for the particular vaccine. Standardised assay protocols applicable to all vaccines do not exist. Live vaccine batches may be assayed by infectivity titration. Each production batch of vaccine should undergo potency and safety testing as batch release criteria. BVD vaccines must be demonstrated to produce adequate immune responses, as outlined above, when used in their final formulation according to the manufacturer's published instructions.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

To date, there are no commercially available vaccines for BDV that support use of a true DIVA strategy.

2.3.5. Duration of immunity

Inactivated vaccines are unlikely to provide sustained levels of immunity and it is likely that after an initial course of two or three injections annual booster doses may be required. Insufficient information is available to determine any correlation between vaccinal antibody titres in the dam and fetal protection. As there are likely to be different commercial formulations and these involve a range of adjuvants, there are likely to be different periods of efficacy. Consequently, duration of immunity data must be generated separately for each commercially available product by undertaking challenge tests at the end of the period for which immunity has been claimed.

2.3.6. Stability

There are no accepted guidelines for the stability of BDV vaccines, but it can be assumed that an inactivated virus vaccine should remain potent for at least 1 year if kept at 4°C and probably longer. Lower

temperatures could prolong shelf life but adjuvants in a killed vaccine may preclude this. Bulk antigens that have not been formulated into finished vaccine can be reliably stored frozen at low temperatures, but the antigen quality should be monitored with *in-vitro* assays prior to incorporation into a batch of vaccine.

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NB: At the time of publication (2017) there were no WOA Reference Laboratories for border disease (please consult the WOA Web site:

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

NB: FIRST ADOPTED IN 1996. MOST RECENT UPDATES ADOPTED IN 2024.