**SECTION 2.7.**

**OVIDAE AND CAPRIDAЕ**

**CHAPTER 2.7.1.**

**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. Distribution of the virus is world-wide. Prevalence rates vary in sheep 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 tremor, abnormal body conformation and hairy fleeces (so-called ‘hairy-shaker’ or ‘fuzzy’ lambs) and the disease has 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. In many regions the commonest cause of BD is the pestivirus border disease virus (BDV), but in some parts of the world, bovine viral diarrhoea virus (BVDV) may be a more common cause of BD. The source of BVDV for sheep often is close contact with cattle.

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. It is generally considered that serologically positive, nonviraemic sheep are ‘safe’, as latent infections are not known to occur in recovered animals.

**Identification of the agent:** BD virus (BDV) is a Pestivirus in the family Flaviviridae and is closely related to classical swine fever virus and BVDV. Nearly all isolates of BDV are noncytopathogenic in cell culture. There are no defined serotypes but virus isolates exhibit considerable diversity. Three distinct antigenic groups, plus two further separate genotypes, have been identified.

Apparently healthy PI sheep resulting from congenital infection can be identified by isolation and immunostaining of noncytopathogenic virus from blood or sera in laboratory cell cultures. Rapid direct methods to identify PI sheep include detection of viral antigen or viral RNA in leukocytes and immunohistochemical demonstration of viral antigen in skin biopsies. The demonstration of virus is 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. From dead animals, the isolation of virus from tissues of aborted or stillborn lambs is difficult, but tissues from PI sheep contain high levels of virus, which can be easily detected by isolation and direct methods.

**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 are the most commonly used antibody detection methods.

**Requirements for vaccines and diagnostic biologicals:** 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.
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). Pestivirus taxonomy is quite problematic at present. There are four recognised species, namely – CSFV, BVDV types 1 and 2 and BDV (20). While CSF viruses are predominantly restricted to pigs, examples of the other three species have all been recovered from sheep, with the majority of isolates being BD viruses (29). Nearly all virus isolates of BDV are noncytopathogenic, although occasional cytopathic viruses have been isolated (24). 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. Sheep can become infected with BVDV from cattle (6), and in some countries, BVDV can be a more common cause of BD than BDV. 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 (15). Several new BD viruses from sheep, goats and Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) have been described recently. 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. Four distinguishable genogroups of BDV have been described as well as putative novel pestivirus genotypes from Tunisian sheep and a goat (2, 28). The chamois BD virus is similar to isolates from sheep in the Iberian Peninsula (22). This chapter describes BDV infection in sheep.

a) Acute infections

Healthy newborn and adult sheep exposed to BDV 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 (19).

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 (7). A second such isolate was a BDV contaminant of a live CSFV vaccine (31).

b) 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. In aborted fetuses, it is also possible to detect virus by immunohistochemistry of brain, thyroid and other tissues (21). 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 and/or antibody should be collected into anticoagulant from suspect lambs before they have received colostrum. Once lambs have ingested colostrum, it is difficult to detect 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 reverse-transcription polymerase chain reaction (RT-PCR).
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 (1).

c) 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 diagnosed by virus isolation/detection in a blood sample. Viraemia is readily detectable at any time except within the first 2 months of life, when virus is masked by colostral antibody; however, the virus may be detected in washed leukocytes during this period, and in animals older than 4 years, some of which develop low levels of anti-BDV antibody (13). 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.

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 due to the toxicity of semen for cell cultures. RT-PCR for detecting pestivirus nucleic acid may be justifiable on semen from some rams.

d) 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. Other PI sheep in the group do 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 (13).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent (the prescribed test for international trade)

There is no designated OIE reference laboratory for BDV, but the reference laboratories for BVDV or CSFV will be able to provide advice (see Table given in Part 3 of this Terrestrial Manual). One of the most sensitive proven methods for identifying BDV remains virus isolation. Direct immunofluorescence or other immunohistochemical techniques on frozen tissue sections as well as antigen-detecting ELISA and conventional and real-time RT-PCR are also valuable methods for identifying BDV-infected animals.
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a) Virus isolation

It is essential that laboratories undertaking virus isolation have a guaranteed supply of pestivirus-free susceptible cells and fetal bovine serum (FBS), or equivalent, that contain no anti-pestivirus activity and no contaminating virus. It is important that a laboratory quality assurance programme be in place.

The 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 (19) 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. Several bovine cell cultures may be suggested, including testicular, embryonic tracheal or turbinate cells, or a susceptible continuous kidney cell line. However, bovine cells are insensitive for the primary isolation and growth of some BD viruses, so reliance on bovine cells alone is inadvisable.

From live animals, serum can be tested for the presence of infectious virus, but 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 for 5–7 days. Cells are frozen and thawed once and an aliquot passaged onto further susceptible cells grown on cover-slips, chamber slides or plastic plates. The cells are stained, 3–4 days later, for the presence of pestivirus using an immunofluorescence or immunoperoxidase test. Tissues should be collected from dead animals in virus transport medium (10% [w/v]). In the laboratory, the tissues are ground, centrifuged to remove debris, and the supernatant passed through 0.45 µm filters. Spleen, 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. A practical sensitive isolation procedure is outlined below:

i) Cultures with subconfluent or newly confluent monolayers of susceptible ovine cells are washed at least twice with Hanks balanced salt solution to remove growth medium before being inoculated with approximately 0.2 ml of sample, which is allowed to adsorb for 2 hours at 37°C.

ii) Cultures are washed with at least 2 ml medium. This is discarded and an appropriate volume of culture maintenance medium is added.

iii) Cultures are incubated for 5–7 days at 37°C. They are examined microscopically on a daily basis and evidence of cytopathic effect (CPE) is recorded.

iv) Cultures are frozen at –70°C and then thawed for passage, as before, on to fresh cultures.

v) 3–4 days later, cells growing on glass are fixed in cold acetone for 15 minutes while cells growing on plastic are fixed as described in the virus neutralisation test section below. Fixed cells are stained using an indirect or direct immunofluorescence method. Essential controls must include known negative cells and cells growing standard cytopathic and noncytopathic BDV strains.

vi) The cells are examined under a UV microscope for the diffuse cytoplasmic fluorescence that is characteristic of pestiviruses.

Immunoperoxidase staining can also be used on cover-slips, chamber slides as well as microtitre plates (see method under virus neutralisation [VN] test below). Frozen and thawed cultures can also be tested in an antigen detection ELISA system that employs monoclonal antibodies (MAbs) against epitopes on the conserved nonstructural NS 2-3 protein. Staining for noncytopathic pestiviruses will usually detect virus at the end of the first passage, but in order to detect slow-growing viruses in poorly permissive cells two passages are desirable.

b) Immunohistochemistry

Viral antigen demonstration is possible in most of the tissues of PI animals (4, 21). This should be done on acetone-fixed frozen tissue sections (cryostat sections) or paraffin wax embedded samples using appropriate antibodies. Panpestivirus-specific antibodies with NS2-3 specificity are suitable. Tissues with a high amount of viral antigen are brain, thyroid gland and oral mucosa. Skin biopsies have been shown to be useful for in-vivo diagnosis of persistent BDV infection.

c) Enzyme-linked immunosorbent assay for antigen detection
The first ELISA for pestivirus antigen detection was described for detecting viraemic sheep. This has now been modified into a double MAb capture ELISA for use in sheep and cattle. Two capture MAbs are bound to wells in microwell plates, and two other MAbs, conjugated to peroxidase, serve as detector MAbs (9). 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 high 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 and commercial kits are now available for detecting BDV. ELISAs employing MAbs recognising epitopes on the conserved non-structural NS2-3 should recognise all strains of BDV. ELISAs relying on MAbs recognising epitopes on structural proteins, such as E\textsuperscript{ms}, that are used for BVDV detection in cattle, are unsuitable for the diagnosis of BDV viraemia in sheep.

d) Nucleic acid detection methods

The complete genomic sequences of three BD viruses have been determined and compared with those of other pestiviruses (3, 17). Phylogenetic analysis shows BD viruses to be more closely related to CSFV than to BVDV (2, 23, 28, 29). RT-PCR for diagnosing pestivirus infection is now used widely. Various formats are described. Basic RT-PCR protocols involve the following stages:

i) Total RNA is isolated by phenol-chloroform, TRIZOL, guanidine isothiocyanate (GITC), or a commercially available spin column or magnetic bead separation methods. NOTE: many of these chemicals are highly toxic; adhere to the manufacturers’ safety procedures.

ii) RT-PCR is performed. This is a two-stage reaction that consists of:
   a) Reverse transcription to produce single-stranded cDNA from viral RNA;
   b) Subsequent PCR amplification of the cDNA to produce readily detectable amounts of double-stranded DNA.

   This process may be done as two separate reactions, each done in a separate PCR tube (two-step RT-PCR), or as two stages in a single PCR tube (one-step RT-PCR). In a two-step format, either random hexamers or specific primers may be used to prime the RT stage; in the one-step format only specific primers may be used.

   iii) Specific product is detected by one of the following methods:
      a) Use of BD-specific primers in the RT-PCR, with visualisation by agarose gel electrophoresis, ethidium bromide staining and UV transillumination to demonstrate the correct sized amplicon. NOTE: ethidium bromide is highly toxic; adhere to manufacturer’s recommendations for handling. NOTE: UV transillumination must be carried out taking appropriate precautions to minimise skin exposure.
      b) Nested PCR, using pan-pestivirus primers (usually directed to the 5’UTR region) in a primary PCR, followed by specific BD virus primers in a secondary (nested) PCR. Such assays typically employ approximately 25 cycles in the primary PCR and 30–35 cycles in the nested PCR. Amplicons are detected by visualisation by agarose gel electrophoresis as above. These assays increase specificity and sensitivity but are more susceptible to contamination.
      c) Real-time RT-PCR, using specific BD primers and/or fluorophore-labelled oligonucleotide probe to detect BD. This method has advantages in specificity and prevention of contamination. It is also possible to carry out a nested form of real-time RT-PCR.

Oligonucleotide primer/probe design is critical to the success of these assays, due to the genetic variability of BDV isolates. Panpestivirus primers are valuable for detecting and typing all species of Pestivirus (18, 27), and can be combined with sequencing if required for specificity or epidemiological investigation. Specific primers for the specific recognition of BD viruses have also been described (11, 30, 33). Using a closed one-tube RT-PCR with fluorescent probes reduces the potential for cross-contamination of diagnostic samples (12, 33). The development of a real-time RT-PCR allows the rapid simultaneous detection and typing of ovine pestiviruses (33). Important applications of RT-PCR methods include the detection of viral RNA in fetal tissues and in cell culture constituents or vaccines (26); it may also prove valuable for detecting virus when BDV-specific antibodies are present. Validation of the RT-PCR is in process. The precautions to be taken with RT-PCR have been covered in Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases.

2. Serological tests
Antibody to BDV is usually detected in sheep sera using VN or an ELISA. The less sensitive agar gel immunodiffusion (AGID) test may also be used. 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.

a) Virus neutralisation test

A standard cytopathic strain of BDV (e.g. Moredun strain) can be used for the VN test with semicontinuous cells such as FLM. An outline protocol is given below.

i) The culture medium is removed and the cells are washed gently with warm phosphate buffered saline (PBS), air-dried and cooled to 4°C.

ii) The acetone is removed and the plates are dried quickly in a cool environment.

iii) The acetic acid is added to all wells 95% acetone (in water) previously cooled to –20°C. The plates are held at –20°C for 30 minutes and should not be stacked or allowed to warm as etching of the plastic may occur.

iv) 50 µl of BDV antiserum is added to all wells at a predetermined dilution in PBS with 1% Tween 80 (PBST). The plates are incubated at 37°C for 30 minutes in a humid atmosphere.

v) The plates are emptied and washed three times with PBST.

vi) The acetic acid is added to all wells 95% acetone (in water) previously cooled to –20°C. The plates are held at –20°C for 30 minutes and should not be stacked or allowed to warm as etching of the plastic may occur.

vii) The plates are drained and an appropriate anti-species serum conjugated to peroxidase is added to the predetermined dilution is added, and the plates are left for 30 minutes at 37°C in a humid atmosphere. The plates are emptied and washed three times with PBST.

viii) The acetic acid is added to all wells 95% acetone (in water) previously cooled to –20°C. The plates are held at –20°C for 30 minutes and should not be stacked or allowed to warm as etching of the plastic may occur.

ix) The plates are drained and 50 µl of activated substrate, e.g. 3-aminophenol (AEC) is added. AEC stock solution is: AEC (0.1 g) dissolved in dimethyl formamide (15 ml). For use add stock (0.3 ml) to membrane-filtered 0.05 M acetate buffer, pH 5.0, (4.7 ml), and then add 30% H2O2 (5 µl). Note. This solution is toxic and should be handled with adequate precautions.

x) The plates are incubated at room temperature and known virus-positive control wells are monitored for development of specific red-brown cytoplasmic staining. When staining is complete the substrate is removed carefully and the wells are washed thoroughly with tap water. Leaving the tap water in the wells, the plates are examined microscopically for virus-containing wells.

The VN titre is calculated as above using the Spearman–Kärber method.
Alternatively, the test can be performed using direct fluorescein-isothiocyanate conjugate staining. 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.

b) Enzyme-linked immunosorbent assay

An MAb-capture ELISA for measuring BDV antibodies has been described. Two panpestivirus 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 (10).

Antigen is prepared as follows: 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. (multiplicity of infection) 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. Retain the supernatants. 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.

o 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, such as ortho-phenylene diamine (OPD) or tetramethyl blue (TMB), is added noting the manufacturer’s toxicity warning. 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) Agar gel immunodiffusion test

The AGID test was first used to demonstrate an immunological relationship between BD, BVD and CSF viruses.

The Oregon C24V strain of BVDV grown on calf testis cells has been used to detect antibody in sheep. Suitable antigen can be prepared using medium harvested from cells showing early CPE. Concentration of the medium approximately 100-fold by dialysis against polyethylene glycol (PEG) is required. Alternatively, PEG 6000 can be added to sonicated virus/cell suspensions at the rate of 5% (w/v). After constant stirring overnight at 4°C, the precipitate is removed by centrifugation at 1800 g for 1 hour. The supernatant is decanted thoroughly and the precipitate resuspended to 1% of the original virus/cell culture volume in distilled water. The resuspended precipitate is centrifuged at 286,000 g for 2 hours and the supernatant withdrawn for use as antigen. The precipitate is discarded.
C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

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 (5, 25).

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 Aujesky’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.

1. Seed management
   a) Characterisation of the seed
      An ideal vaccine should contain a strain or strains of virus that give protection against all sheep pestiviruses. Recent evidence is that three antigenically distinguishable groups of pestiviruses infect sheep. One group is represented by the Moredun reference strain of BDV; the second group contains viruses similar to the majority of cattle BVDV strains (BVDV type 1); and the third group contains the less common BVDV (type 2) strains (32). More recently, ovine pestivirus isolates have been divided on the basis of phylogenetic and antigenic analysis into BDV-1, BDV-2 and BDV-3 genotypes (2). Phylogenetic analysis alone suggests that a BDV Italian caprine isolate and the chamois/Iberian sheep isolates represent two further genotypes (28). Further cross-neutralisation studies are required to determine the significance of these findings. 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 (16).

   b) Culture
      A variety of ruminant cell cultures can be used. Optimal yields depend on the cell type and isolate used. A commercial BDV vaccine containing two strains of virus is prepared on ovine cell lines (5). 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.

   c) Validation as a vaccine
      All vaccines should pass standard tests for safety and efficacy. Safety testing of inactivated BDV vaccines should include monitoring of all vaccine components for contaminating pestiviruses.
      Efficacy tests of BDV vaccines should demonstrate their ability to prevent transplacental spread of virus. 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 (5).

2. Method of manufacture
   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 (5, 25).

3. In-process control
   Cultures should be inspected daily to ensure they are free from gross bacterial contamination and that any CPE observed is appropriate to the cytopathic virus being grown. No CPE should be observed in cultures being used to grow noncytopathic strains of virus.

4. Batch control
   a) Sterility
      Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

   b) Safety
Samples from inactivated vaccines should be tested rigorously for viable virus. Samples of the product should be passaged several times 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.

c) Potency

Vaccine potency is also best tested in seronegative sheep in which the development and level of antibody is measured. An indirect measure of potency is given by the level of virus infectivity prior to inactivation. The antigen content following inactivation can be assayed by MAb-capture ELISA and related to the results of established in-vivo potency results. As recommended for potency testing of BVDV vaccine in cattle it should be demonstrated that the vaccine can prevent transplacental transmission of BDV in pregnant sheep.

d) Duration of immunity

No information is available on duration of immunity following vaccination. 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.

e) Stability

There is little information on the stability of BDV vaccines. Inactivated vaccines could be expected to have at least a 1 year shelf life when protected from light and stored at 4°C.

f) Preservatives

Preservatives may be added to multidose vaccine containers subject to the approval of the Control Authority.

g) Precautions (hazards)

BDV is not considered to be a hazard to human health. Standard good microbiological practice should be used when handling the virus.

5. Tests on the final product

a) Safety

In-vitro test only.

b) Potency

In-vitro antigen content test.

REFERENCES


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