Cattle of all ages are susceptible to infection with bovine viral diarrhoea virus (BVDV) (see also Chapter 4.3 in the Terrestrial Animal Health Code). Distribution of the virus is world-wide. The clinical signs range from subclinical to the fulminating fatal condition called mucosal disease. Acute infections may result in transient diarrhoea or pneumonia, usually in the form of group outbreaks. Acute forms of the disease associated with high mortality have also been described, often, but not always, associated with a haemorrhagic syndrome. However, most infections in the young calf are mild and go unreco gnised clinically. The virus spreads mainly by contact between cattle. Vertical transmission plays an important role in its epidemiology and pathogenesis.

Infections of the bovine fetus may result in abortions, stillbirths, teratogenic effects or persistent infection in the neonatal calf. Persistently viraemic animals may be born as weak, unthrifty calves or may appear as normal healthy calves and be unreco gnised clinically. Some of these animals may later develop mucosal disease with anorexia, gastrointestinal erosions, and profuse diarrhoea, leading invariably to death. Mucosal disease can arise only in persistently infected animals.

It is important to avoid the trade of viraemic animals. It is generally considered that serologically positive, nonviraemic cattle are ‘safe’, providing that they are not pregnant. Antibody-positive pregnant cattle carrying persistently infected fetuses are important transmitters of the virus between herds. About 15% of persistently viraemic animals have antibody to the NS/2 protein and a lower percentage to the E2 glycoprotein. Therefore, seropositivity cannot be equated with ‘safety’. Latent infections are not generally thought to occur following recovery from acute infection, though semen from acutely infected animals and, very rarely, recovered animals may be suspect.

Identification of the agent: BVDV is a pestivirus in the Flaviviridae and is closely related to classical swine fever and ovine Border disease viruses. BVDV occurs in two forms: noncytopathogenic and cytopathogenic. There are two antigenically distinct genotypes (types 1 and 2), and virus isolates within these groups exhibit considerable biological and antigenic diversity. Persistently viraemic healthy animals resulting from congenital infection can be readily identified by isolation of noncytopathogenic virus in cell cultures from blood or serum. It is necessary to use an immune-labelling method to detect the growth of virus in the cultures. Alternative methods based on direct detection of viral antigen or viral RNA in leukocytes are also available. Persistence of virus should be confirmed by resampling after an interval of at least 3 weeks. These animals will usually have no or low levels of antibodies to BVDV.

Viraemia in acute cases is transient and can be difficult to detect. In fatal cases of haemorrhagic disease, virus can be isolated from tissues post-mortem. Confirmation of mucosal disease can be made by isolation of the cytopathogenic biotype of BVDV, particularly from intestinal tissues. Noncytopathogenic virus may also be detected, especially in blood.

Serological tests: Acute infection with BVDV is best confirmed by demonstrating seroconversion using sequential paired samples from several animals in the group. The testing of paired (acute and convalescent) samples should be done a minimum of 21 days apart and samples should be tested side by side. The enzyme-linked immunosorbent assay for antibody and the virus neutralisation test are the most widely used.

Requirements for vaccines and diagnostic biologicals: There is no standard vaccine for BVD, but a number of commercial preparations are available. Modified live virus vaccine should not be administered to pregnant cattle (or to their sucking calves) due to the risk of transplacental infection. There is also a risk of inducing mucosal disease in persistently infected animals. Killed virus
Chapter 2.4.8. - Bovine viral diarrhoea

Bovine viral diarrhoea virus (BVDV) is a pestivirus in the family Flaviviridae and is closely related to classical swine fever and ovine Border disease viruses (23). Two antigenically distinct genotypes of BVDV exist, types 1 and 2, with further subdivisions discernable by genetic analysis (74). The two genotypes may be differentiated from each other, and from other pestiviruses, by monoclonal antibodies (MAbs) directed against the E2 and E\text{RNS} major glycoproteins, or by genetic analysis (65, 68). Multiplex polymerase chain reaction (PCR) enables virus typing direct from blood samples (33). Type 1 virus is generally more common although the prevalence of type 2 is reported to be almost as high as type 1 in North America. BVDV of both genotypes may occur in noncytopathogenic and cytopathogenic forms (biotypes), classified according to whether or not it produces visible change in cell cultures. Usually, it is the noncytopathogenic biotype that circulates in cattle populations. Each biotype has a specific role in a variety of clinical syndromes – acute, congenital and chronic infections (5, 11). Type 2 viruses are usually noncytopathogenic and have been associated with outbreaks of severe acute infection and a haemorrhagic syndrome (16). However recent type 2 viruses isolated in the United Kingdom have been associated with a disease indistinguishable from that seen with the more frequently isolated type 1 viruses. Some type 1 isolates have been associated with particularly severe and fatal disease outbreaks in adult cattle (20) clinically mild and inapparent infections are common with both genotypes.

Although ubiquitous, control of BVDV can be achieved at the herd level, and even at the national level, as evidenced by the progress towards eradication made in many European countries (56).

B. DIAGNOSTIC TECHNIQUES

a) Acute infections

Acute infections of cattle occur particularly in young animals, and may be clinically inapparent or associated with diarrhoea (1). Affected animals may be predisposed to secondary infections, for example those leading to shipping disease, perhaps due to an immunosuppressive effect of the virus. Bulls may suffer a temporary depression of fertility and can show transient shedding of virus in the semen (62). Cows may also suffer from infertility, likely associated with changes in ovarian function (35) and secretions of gonadotrophin and progesterone (30). During acute infections, a brief viraemia may be detectable and nasal shedding of virus may occur. There may also be a transient leukopenia, thrombocytopenia or temperature response, but these can vary greatly among animals. A serological response is the most certain means of diagnosing a previous infection. The clinical picture is generally one of high morbidity and low mortality, though more severe disease is sometimes seen (12). In particular, outbreaks of a severe form of acute disease with haemorrhagic lesions, thrombocytopenia and high mortality have been reported sporadically from some countries (1, 6) and infection with Type 2 viruses in particular has been demonstrated to cause altered platelet function (76). Other acute outbreaks may show fever, pneumonia, diarrhoea and sudden death in any age group, with haemorrhagic signs (16).

b) Congenital infection

If noncytopathogenic virus infects the bovine fetus, this may result in abortion, stillbirth, teratogenic effects or a congenital infection that persists in the neonatal calf (1, 11, 26, 55). Confirmation that an abortion is caused by BVDV is often difficult to establish (69), but virus may be isolated from fetal tissue in some cases, or viral antigen or genome may be demonstrated. An virulent strain should also be made to detect specific antibody in samples of fetal fluids or serum, or in the supernatant fluid from a tissue suspension. Stillbirths or teratogenic effects may be associated with an active fetal immune response to the virus during mid-to-late gestation. The dam will often have high antibody titres (>1/2000) to BVDV, which is suggestive of fetal infection and is probably due to the fetus providing the dam an extended challenge of virus (47).

Although congenital infection with BVDV often leads to abortion, it is not always recognised in the field. Infection during the first third of the gestation period can result in the abortion of a conceptus that is small and goes unnoticed by the farmer. The cow would return to service and the failure to maintain pregnancy would be classified as an example of early embryonic death. Another possible outcome of infection is the death and subsequent resorption of fluids from the fetus that results in mummification. It is frequently
observed that aborted fetuses have subcutaneous oedema and copious pleural and peritoneal effusions. There may also be congenital abnormalities that result in growth retardation and in selective central nervous system (CNS) defects, such as cerebellar hypoplasia and dysmyelination (70), and eye defects, such as cataracts and retinal atrophy. Sometimes there are skeletal defects, the most advanced of which is arthrogryposis.

Stillborn calves has been reported to be sequel to congenital infection before 150 days of gestation and the calves usually appear to be fully developed at parturition, but fail to survive. However, it has been reported, that in many cases, BVD virus cannot be isolated from these animals and they are PCR negative. If infection occurs after day 150 of pregnancy, the immune system of the fetus will be developed and infection of the fetus will usually result in an antibody response and the birth of a normal calf.

c) Persistent infection

When infections of the fetus occur before approximately 110 days of gestation and before immunocompetence, the calf may be born with a persistent infection. Identification of these animals is readily made by detection of noncytopathogenic BVDV in blood. The virus can also be identified in the skin by immunohistochemistry. Furthermore, animals with a persistent infection will also lack specific antibody, but diagnosis in the young calf, up to approximately 3 months of age, may be confused by the presence of maternal antibody to BVDV. Maternal antibody may also interfere with virus isolation. In older animals with persistent viraemia infection, low levels of antibody may be present due to their ability to seroconvert to strains of BVDV (including vaccines) ‘heterologous’ (antigenically different) from the persisting virus (12). To confirm a diagnosis of persistent infection, animals should be retested after an interval of at least 3 weeks.

There are no pathognomonic lesions in the viraemic calf. Depending on the gestational age at infection, lesions may be mediated entirely by the effects of the virus on the differentiating cells of the fetus, they may be mediated by the maturing immune system of the developing fetus, or both. The clinical signs vary from the apparently normal healthy animal to the weak, unthrifty calf that has difficulty in standing and sucking. These latter calves can show CNS defects, such as muscular tremors, incoordination and blindness. They often die within days of birth, thus contributing to the ‘weak calf syndrome’.

Approximately 1–2% of cattle within a population are persistently infected, with many viraemic animals surviving to sexual maturity and retained for breeding. Calves born to these infected dams are always persistently viraemic, and are often weak at birth and fail to thrive. Persistently viraemic animals are a continual source of infective virus to other cattle, and thus their rapid identification and removal from the herd are required. Animals being traded should first be screened for the absence of persistent BVD viraemia.

Bulls that are persistently infected usually have poor quality, highly infective semen and, as a result, reduced fertility (45, 67). All bulls used for natural or artificial insemination should be screened for persistent BVD infection. A rare event, possibly brought about by acute infection during pubescence, can result in persistent infection of the testes and thus strongly seropositive bulls (59, 75). This phenomenon has also been observed following vaccination with an attenuated virus (34). Female cattle used as embryo recipients should always test negative for BVD viraemia before first use. Donor cows that are persistently infected with BVDV also represent a potential source of infection, as oocysts without an intact zona pellucida are shown to be susceptible to infection in vitro (73). However, a limited study of two persistently infected animals revealed that the majority of oocytes were BVDV-negative (71). Embryos may also become contaminated following acute infection of the donor (3). Biological materials used for in-vitro fertilisation techniques (bovine serum, bovine cell cultures) have a high risk of contamination and should be screened for BVDV (9). Recent incidents of apparent introduction of virus via such techniques (24, 48) have highlighted this risk. It is considered essential that serum supplements used in media should be sterilised as detailed in chapter 4.4, Article 4.4.5 of the OIE Terrestrial Animal Health Code (Terrestrial Code) and outlined in Section B.1.a of this chapter. Importing countries may consider requesting additional tests to confirm sterilisation, detailed in Article 4.4.6 of the Terrestrial Code.

d) Mucosal disease

It is well established that persistently viraemic animals may later succumb to mucosal disease (11); however, cases are rare. This syndrome has been shown to be associated with the presence of the cytopathogenic biotype, which can arise either through superinfection (5, 14), recombination between noncytopathogenic biotypes, or mutation of the persistent biotype (50). Consequently, confirmatory diagnosis of mucosal disease should include the isolation of cytopathogenic virus from affected cattle. This biotype may sometimes be isolated from blood, but it can be recovered more consistently from a variety of other tissues, in particular intestinal and Peyer’s patch tissue (17). Virus isolation is also readily accomplished from spleen. This is easy to collect and is seldom toxic for cell culture after preparation for viral isolation. Isolation from gut samples may be difficult if autolysis has occurred; in this case suspensions from lymph nodes or tonsil should then be tested. Noncytopathogenic virus can also be detected, particularly from blood or blood-
associated organs. Cryostat tissue sections from mucosal disease cases can be stained for viral antigen by immunofluorescence or immunoperoxidase labelling.

Mucosal disease is invariably fatal. Its onset may be so rapid that the first signs seen are dead or moribund animals. However, it is more common for animals to become anorexic over a period of several days, to be disinclined to move and to show signs of abdominal pain. They can develop a profuse diarrhoea and rapidly lose bodily condition. Erosions can often be seen in the mouth, particularly along the gingival margin. Lacrimation and excessive salivation occur. Generally, cases of mucosal disease are sporadic and rare.

Post-mortem examination reveals erosions in the mucosa at various sites along the gastrointestinal tract. The most noticeable are those overlying the lymphoid Peyer’s patches in the small intestine and in the ileocaecal lymph nodes. On histological examination, there is a clear demonstration of destruction of the lymphoid tissue within the gut-associated lymphoid tissue. Most of the Peyer’s patch lymphoid cells have been lysed and replaced by inflammatory cells, debris and cells from the overlying collapsed epithelium.

Severe acute BVD infection can be clinically similar to mucosal disease and confusion can arise, particularly when a number of animals are so affected. Mucosal disease can occur among cohorts of persistently infected animals when oestrus synchronisation has been carried out. Differentiation requires a careful examination of case histories and testing for antibody as well as antigen or virus among infected and any recovered animals. Seroconversion among recovered animals is indicative of acute infection, whereas two antigen or virus positive results on samples from an affected animal, taken 3 weeks apart, is diagnostic of mucosal disease. Generally, animals with mucosal disease are antibody negative, though low levels of antibody can sometimes be detected.

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

All test methods must be validated by testing on known noninfected and infected populations of cattle, including animals with low- and high-titre viraemias. Methods based on MAb-binding assays or on nucleic acid recognition must be shown to detect the full range of antigenic and genetic diversity found among BVD viruses. There are two designated OIE Reference Laboratories for BVD (see Table given in Part 3 of this Terrestrial Manual); the reference laboratories for classical swine fever could also be approached to offer advice.

a) Virus isolation

The virus may be isolated in a number of bovine monolayer cell cultures (e.g. kidney, lung, testis or turbinate). Growth of both biotypes is usually satisfactory. Noncytopathogenic BVDV is a common contaminant of fresh bovine tissue, and cell cultures must be checked for freedom from adventitious virus by regular testing (8, 28). Primary or secondary cultures can be frozen as cell suspensions in liquid nitrogen. These can then be tested over a series of passages, or seeded to other susceptible cells and checked before routine use. Such problems may be overcome by the use of continuous cell lines, which can be obtained BVD-free (8).

The fetal bovine serum that is selected for use in cell culture must also be free not only from virus, but also and of equal importance, from BVDV neutralising antibody (28). Heat treatment (56°C for 30–45 minutes) is inadequate for the destruction of BVDV in contaminated serum; irradiation at 25 kiloGrays (2.5 Mrad) is more certain. Commercial batches of fetal bovine serum mostly test positive by PCR even after the virus has been inactivated by irradiation. Where appropriate, horse serum can be substituted for bovine fetal serum, although it is often found to have poorer cell-growth-promoting characteristics.

Buffy coat cells, whole blood, washed leukocytes or serum are suitable for isolation of the virus from live animals. Maternal antibody may interfere with isolation from serum in young calves. Tissue suspensions from post-mortem cases should be prepared by standard methods. Semen can also be examined, but a blood sample from the donor bull is preferable if it can be obtained. There is a report of an atypical persistent shedding of BVDV in semen from a bull that was not viraemic (75). Raw semen is cytotoxic and must be diluted in culture medium. Extended semen can usually be inoculated directly on to cell monolayers, but may occasionally cause cytotoxicity. For these reasons, it is important to monitor the health of the cells by microscopic examination at intervals during the incubation.

There are many variations of procedure in use for virus isolation. All should be optimised to give maximum sensitivity of detection of a standard virus preparation. This may include one or more in-vitro passage(s). Conventional methods for virus isolation are used, with the addition of a final immune-labelling step (fluorescence or enzymatic) to detect growth of noncytopathogenic virus. Thus tube cultures should include flying cover-slips, while plate cultures can be fixed and labelled directly in the plate. Examples are given below.
o Microplate immunoperoxidase method for mass screening for virus detection in serum samples (54)

i) 10 µl of the serum sample is placed into each of four wells of a 96-well tissue-culture grade microplate. This is repeated for each sample. Known positive and negative controls are included.

ii) 100 µl of a cell suspension of 150,000 cells/ml in medium without fetal calf serum (FCS) is added to all wells. NB: the sample itself acts as the cell-growth supplement. If testing samples other than serum, use medium with 10% FCS that is free of antibodies to ruminant pestiviruses.

iii) The plate is incubated at 37°C for 4 days, either in a 5% CO₂ atmosphere or with the plate sealed.

iv) Each well is examined microscopically for evidence of cytopathic effect (CPE), or signs of cytotoxicity.

v) The plate is emptied by gentle inversion and rinsed in phosphate buffered saline (PBS).

vi) The plate is fixed as follows: the plate is dipped into a bath of 20% acetone in PBS, emptied immediately, and then transferred to a fresh bath of 20% acetone in PBS for 10 minutes. The plate is drained thoroughly and as much fluid as possible is removed by tapping and blotting. The plate is dried thoroughly for at least 3 hours at a temperature of 25–30°C (e.g. using radiant heat from a bench lamp). NB: the drying is part of the fixation process.

Alternative fixation methods include paraformaldehyde or heat (see Chapter 2.8.3 Classical swine fever, Section B.2.b.viii).

vii) The fixed cells are rinsed by adding PBS to all wells.

viii) The wells are drained and the BVD antibody (50 µl) is added to all wells at a predetermined dilution in PBS containing 1% Tween 80 (PBST) and 5% horse serum. (Horse serum may be added to reduce nonspecific staining.) The plate is incubated at 37°C for 15 minutes.

ix) The plate is emptied and washed three times in PBST.

x) The plate is then drained and appropriate antispecies serum conjugated to peroxidase is added at a predetermined dilution in PBST (50 µl per well) for 15 minutes at 37°C.

xi) The plate is emptied and washed three times in PBST.

xii) The plate is rinsed in distilled water. All fluid is tapped out from the plate.

xiii) Freshly prepared hydrogen peroxide substrate with a suitable chromogen, e.g. 3-amino-9-ethyl carbazole (AEC) is added. The stock solution is: AEC (0.1 g) dissolved in dimethyl formamide (15 ml). For use, the stock (0.3 ml) is added to 0.05 M acetate buffer (5 ml, pH 5.0), and then 30% H₂O₂ (5 µl is added). An alternative substrate can be made, consisting of 9 mg diaminobenzidine tetrahydrochloride and 6 mg sodium perborate tetrahydrate dissolved in 15 ml of PBS. Though the staining is not quite so intense, these chemicals have the advantage that they can be shipped by air.

xiv) The plate is examined microscopically. Virus-positive cells show red-brown cytoplasmic staining.

o Tube method for tissue or buffy coat suspensions, or semen samples

NB: this method can also be conveniently adapted to 24-well plastic dishes.

i) Tissue samples are ground up and a 10% suspension in culture medium is made. This is then centrifuged to remove the debris. Raw semen is diluted 1/10 in culture medium.

ii) Test tube cultures (with cover-slips) with newly confluent or subconfluent monolayers of susceptible bovine cells are inoculated with 0.1 ml of the sample. The culture is left to adsorb for 1 hour at 37°C.

iii) The culture is washed with 1 ml of medium; this is then discarded and 1 ml of culture maintenance medium is added.

iv) The culture is incubated for 4–5 days at 37°C, and examined microscopically for evidence of CPE or signs of cytotoxicity.

v) Culture may then either be frozen and thawed for passage to fresh cultures, or the cover-slip may be removed, fixed in acetone and stained with direct immunofluorescent conjugate to BVDV. In this case, examine under a fluorescent microscope for diffuse, cytoplasmic fluorescence characteristic of pestiviruses.

Alternatively, cultures may be freeze/thaw harvested and passaged on to microtitre plates for culture and staining by the immunoperoxidase method (see section on microplate immunoperoxidase method for mass screening of serum samples above) or by the immunofluorescent method described here.

b) Enzyme-linked immunosorbent assay for antigen detection
Several methods for the enzyme-linked immunosorbent assay (ELISA) for antigen detection have been published (e.g. ref. 29) and a number of commercial kits are available. Most are based on the sandwich ELISA principle, with a capture antibody bound to the solid phase, and a detector antibody conjugated to a signal system, such as peroxidase. Both monoclonal- and polyclonal-based systems are described. The test is suitable for detection of persistently infected animals, and usually measures BVD antigen (NS2-3 or ERNS) in lysates of peripheral blood leukocytes; the new generation of antigen-capture ELISAs (ERNS capture ELISAs) is able to detect BVD antigen in blood as well as in plasma or serum samples. The best of the methods gives a sensitivity similar to virus isolation, and may be preferred in those rare cases where persistent infection is combined with seropositivity. Due to transient viraemia, the antigen ELISA appears to be less useful for virus detection in acute BVD infections.

The NS2-3 ELISA may be less effective in young calves that have had colostrum due to the presence of BVDV maternal antibodies. The reverse transcription PCR (RT-PCR) is probably the most sensitive detection method for this circumstance, but the ERNS ELISA has also been shown to be a sensitive and reliable test, particularly when used with ear-notch samples (18).

c) Immunohistochemistry

Enzyme-labelled methods are useful to detect BVDV antigen in tissue sections (77), particularly where suitable MAb's are available. It is important that the reagents and procedures used be fully validated, and that nonspecific reactivity be eliminated. For persistently infected cattle almost any tissue can be used, but particularly good success has been found with lymph nodes, thyroid gland, skin, brain, abomasum and placenta. Skin biopsies, such as ear-notch samples, have shown to be useful for in-vivo diagnosis of persistent BDV infection (17).

d) Nucleic acid detection

The RT-PCR method can be adapted to the detection of BVD viral RNA for diagnostic purposes (10, 36, 44, 46). This may have a special value where low-level virus contamination is suspected, for example in screening batches of FCS, or biological products such as vaccines (38). Caution is needed in the interpretation of results, as the detection of viral RNA does not imply per se that infective virus is present. A multiplex PCR can be used to amplify and type virus from cell culture, or direct from blood samples, by producing different sized PCR products (33). Newer methodologies incorporate the use of DNA fluorescently labelled probes, which confirm the identity of the PCR product, provide automated reading and can also differentiate pestiviruses in real time (53). Testing for virus after inoculation of cell cultures using PCR should be avoided as it may give false positive results if commercial bovine fetal serum contaminated with ruminant sera is used (23). Molecular tests can be prone to contamination in unskilled hands. Stringent precautions should therefore be taken to avoid DNA contamination in the test system, and rigorous controls must be mounted (see Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases).

The RT-PCR technique is also sensitive enough to enable the detection of persistently infected lactating cows in a herd of up to 100 animals or more, by testing the somatic cells within bulk milk (25, 66). A positive result indicates that at least one such animal is present in the milking herd. Follow-up virus isolation or antigen detection tests are required to identify the individual(s).

Viral nucleic acid in tissues can be detected by in situ hybridisation with enzyme-linked riboprobes (22). This is a sensitive technique that can be applied to formalin-fixed paraffin-embedded tissue, thereby allowing a retrospective analysis. Extraction of nucleic acid and RT-PCR from such samples has been described in this context, also allowing phylogenetic analysis (2).

2. Serological tests

Antibody to BVDV can be detected in cattle sera by a standard virus neutralisation (VN) test or by ELISA, using one of several published methods (27, 40, 43, 63). Control positive and negative standard sera must be included in every test. These should give results within predetermined limits for the test to be considered valid. ELISA for antibody in bulk milk samples can give a useful indication of the BVD status of a herd (58). A high ELISA value of 1.0 or more absorbance units indicates a high probability of the herd having been exposed to BVDV in the recent past, most likely through one or more persistently viraemic animals being present. In contrast, a very low or negative value (≤0.2) indicates that it is unlikely that persistently viraemic animals are present. Further categorisation has been suggested for intermediate values, but this is dependent on the husbandry system in use. ELISA values have been shown to be an unreliable indicator of the presence of persistently infected animals on farms, due to differing husbandry (78), and also due to the presence of viral antigen in bulk milk, which may interfere with the antibody assay itself (60). Determination of the antibody status of a small number of young stock (9–18 months) has also been suggested as an indicator of recent exposure to BVDV (39), but these are likewise
dependent on the degree of contact between different groups of animals in the herd. Rapid ‘spot tests’ can be used for initial screening as part of BVD control and eradication schemes (49).

a) Virus neutralisation test

Because it makes the test easier to read, most laboratories use highly cytopathogenic, laboratory-adapted strains of BVDV for VN tests, although immune-labelling techniques are now available that allow simple detection of the growth or neutralisation of noncytopathogenic strains where this is considered desirable. No single strain is likely to be ideal for all circumstances, but in practice one should be selected that detects the highest proportion of serological reactions in the local cattle population. Two widely used cytopathogenic strains are ‘Oregon C24V’ and ‘NADL’. Low levels of antibody to BVD type 2 virus may not be detectable by a neutralisation test that uses type 1 strain of the virus, and vice versa (32). It is important that BVD type 1 and BVD type 2 be used in the test and not just the one that the diagnostician thinks is present, as this can lead to under reporting.

An outline protocol for a microtitre VN test is given below (27):

i) The test sera are heat-inactivated for 30 minutes at 56°C.

ii) From a starting dilution of 1/5, 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, two or four wells are used at each dilution depending on the degree of precision required. Control positive and negative sera should also be tested.

iii) An equal volume (e.g. 50 µl) of a stock of cytopathogenic strain of BVDV 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 30–421 TCID₅₀).

iv) The plate is incubated for 1 hour at 37°C.

v) A flask of suitable cells (e.g. bovine turbinate, bovine testis) is trypsinised and the cell concentration is adjusted to 3 × 10⁵/ml. 50 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 for CPE. 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 method. A seronegative animal will show no neutralisation at the lowest dilution (1/5), equivalent to a final dilution of 1/10.

b) Enzyme-linked immunosorbent assay

Both indirect and blocking types of test can be used (40, 43, 63). A number of commercial kits are available. The chief difficulty in setting up the test lies in the preparation of a viral antigen of sufficient potency. The virus must be grown under optimal culture conditions using a highly permissive cell type. Any serum used in the medium must not inhibit growth of BVDV. The optimal time for harvest should be determined experimentally for the individual culture system. The virus can be concentrated and purified by density gradient centrifugation. Alternatively, a potent antigen can be prepared by treatment of infected cell cultures with detergents, such as Nonidet P40, N-decanoyl-N-methylglucamine (Mega 10), Triton X-100 or 1-octyl-beta-D-glucopyranoside (OGP). Some workers have used fixed, infected whole cells as antigen. In future, increasing use may be made of artificial antigens manufactured by expressing specific viral genes in bacterial or eukaryotic systems (72). Such systems should be validated by testing sera specific to a wide range of different virus strains. In the future, this technology should enable the production of serological tests complementary to subunit or marker vaccines, thus enabling differentiation between vaccinated and naturally infected cattle.

An example outline protocol for an indirect ELISA is given below (27).

i) Roller cultures of secondary calf testis cells with a high multiplicity of infection (about one), are inoculated with BVDV strain Oregon C24V, overlaid with serum-free medium and incubated for 24 hours at 37°C.

ii) The cells are scraped off and pelleted. The supernatant medium is discarded. The pellet is treated with two volumes of 2% OGP in PBS for 15 minutes at 4°C, and centrifuged to remove the cell debris. The supernatant antigen is stored in small aliquots at −70°C, or freeze-dried. Non-infected cells are processed in parallel to make a control antigen.

iii) The antigen is diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6. Alternate rows of an ELISA-grade microtitre plate are coated with virus and control antigens overnight at 4°C. The plates are then washed in PBS with 0.05% Tween 20 or Tween 80 (PBST) before use in the test.
iv) Test sera are diluted 1/50 in serum diluent (0.5 M NaCl; 0.01 M phosphate buffer; 0.05% Tween 20; 0.001 M ethylene diamine tetra-acetic acid; 1% polyvinyl pyrrolidone, pH 7.2) and added to virus- and control-coated wells for 1 hour at 37°C. The plates are then washed five times in PBST.

v) Rabbit anti-bovine IgG peroxidase conjugate is added at a predetermined dilution (in serum diluent) for 1 hour at 37°C, then the plates are again washed five times in PBST.

vi) A suitable enzyme substrate is added, such as hydrogen peroxide/tetramethyl benzidine. After colour development, the reaction is stopped with sulphuric acid and the absorbance is read on an ELISA plate reader. The value obtained with control antigen is subtracted from the test reaction to give a net absorbance value for each serum.

vii) It is recommended to convert net absorbance values to sample:positive ratio (or percentage positivity) by dividing net absorbance by the net absorbance on that test of a standard positive serum that has a net absorbance of about 1.0. This normalisation procedure leads to more consistent and reproducible results.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Infection via the oropharynx and respiratory tract is probably the most important route of transmission of BVDV on farms. Protection against spread in this way would have a beneficial effect on controlling disease due to the virus, particularly in the young animal. The formulation of a vaccine that will provide protection to the fetus is also required in order to prevent the wide range of syndromes that result from in utero infection (13).

A standard vaccine for protection against infection has not yet been developed, but a number of commercial preparations are available in, for example, Europe and North America. Traditionally, BVD vaccines have been based on a cytopathogenic strain of the virus and fall into two classes: modified live virus or inactivated vaccines. Although live virus vaccines are available in some countries, they should be used under careful veterinary control because a cytopathogenic strain may precipitate mucosal disease by superinfection of persistently viraemic animals, while in pregnant cattle, a noncytopathogenic component of the vaccine may cross the placenta and infect the fetus as described in Section B.b. Live virus vaccine may also be immunosuppressive and precipitate other infections. On the other hand, modified live virus vaccines may only require a single dose. Properly constituted vaccines containing killed virus are safe to use but, to obtain satisfactory levels of immunity, they usually require booster vaccinations, which may be inconvenient. A combined vaccination protocol using inactivated followed by live vaccine may reduce the risk of adverse reaction to the live strain (31).

Experimental inactivated vaccines based on baculovirus-expressed BVD viral glycoprotein E2 have been described. They offer a future prospect of ‘marker vaccines’ when used in connection with a complementary serological test (15). However, it should be noted that such vaccines for the closely related classical swine fever virus have not proven so effective, probably because of their inability to induce a strong cell-mediated immune response.

BVDV is particularly important as a hazard in the manufacture of biological products for other diseases because of the high frequency of contamination of batches of FCS used as a culture medium supplement (38). Particular attention should be paid to sera designed for administration to animals, or used as a growth supplement in embryo transfer or in-vitro fertilisation procedures. Serum used for such purposes should be treated so as to assure sterility. It is recommended that post-treatment tests, such as are detailed in Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials, be used to ensure that serum is free of BVDV.

Guidelines for the production of veterinary vaccines are 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.

1. Seed management

a) Characteristics of the seed

An ideal vaccine should contain a strain (or strains) of virus that has been shown to give protection against the wide diversity of antigenicity that has been demonstrated by BVDV. A good appreciation of the antigenic characteristics of individual strains can be obtained by screening with panels of MAbs (64). The identity of the seed virus should be confirmed by sequencing (68).

The emergence of genotype 2 BVD has raised questions regarding the degree of protection conferred by type 1 vaccines against genotype 2. An in-vitro study of the neutralising ability of sera induced by one
vaccine revealed broad reactivity with diverse strains from Europe and the USA, including type 2 strains (37). Other work has shown that vaccine derived from one genotype can afford a degree of protection from the other (19, 21, 52). However, the efficacy of vaccination of whatever genotype, particularly with a killed vaccine, in preventing transplacental transmission is less predictable, as viraemia is rarely completely prevented.

Isolates of cytopathic virus are often mixed with the noncytopathic biotype. The separation and purification of the two biotypes from an initial mixed culture depends on either three cycles of a limiting dilution technique for the noncytopathogenic virus, or three cycles of plaque selection for the cytopathogenic virus. Purity of the cytopathogenic virus should be confirmed by at least one additional passage at limiting dilution. When isolates have been cloned, their identity should be confirmed by direct or indirect staining with specific antibody linked to fluorescein or enzyme.

b) Method of culture

Both biotypes will grow in a variety of cell cultures of bovine origin. Standard procedures may be used, with the expectation for harvesting noncytopathogenic virus on days 5–7 and cytopathogenic virus on days 2–4. The details for optimal yield depend on several factors, including the cell culture and isolate used and the initial seeding rate of virus (42).

c) Validation as a vaccine

All vaccines should pass standard tests for safety and efficacy. It is crucial to ensure that the cell cultures and fetal bovine serum included in culture medium be free from adventitious BVDV and antibody (described in Section B), and other microorganisms. Live vaccines must either be demonstrated to be safe in pregnant cattle (i.e. no transmission to the fetus), or should be licensed with a warning not to use them in pregnant animals. Live vaccines containing cytopathogenic strains should have an appropriate warning of the risk of inducing mucosal disease in persistently infected cattle.

Efficacy tests of BVD vaccines in non-pregnant cattle are limited by the difficulty of establishing a satisfactory challenge model. Tests should include as a minimum the demonstration of seroconversion following vaccination, a reduction in virus shedding after challenge in vaccinated cattle, and a diminution in measurable clinical parameters, such as rectal temperature response and leukopenia (4, 13, 42). Vaccines intended for use in adult breeding cattle should be evaluated for their efficiency in reducing transplacental transmission, ideally achieving complete prevention. In this case, a suitable challenge system can be established by intranasal inoculation of noncytopathogenic virus into pregnant cows at under 90 days gestation (13). Usually this system will reliably produce persistently viraemic offspring in non-immune cows.

2. Method of manufacture

There is no standard method for the manufacture of a BVD 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 or beta-propiolactone inactivation (42, 61). A variety of adjuvants may be used (42, 57).

3. In-process control

Cultures should be inspected regularly to ensure that they are free from contamination, and to monitor the health of the cells and the development or absence of CPE, as appropriate.

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

It is essential that all the infectivity be removed during preparation of an inactivated vaccine, and samples should be subjected to several passages in cell culture to ensure the absence of live BVDV. It may also be necessary to ensure the absence of various proscribed agents (prior to inactivation) before use of the vaccine is permitted.

c) Potency

Ideally, the potency of the vaccine should be determined by inoculation into seronegative and virus negative calves, followed by monitoring of the antibody response; however, this is prohibitively expensive for batch control. Antigen content can be assayed by ELISA and adjusted as required to a standard level for the
particular vaccine (4, 51). Standardised assay protocols applicable to all vaccines do not exist. Live vaccine batches may be assayed by infectivity titration.

d) Duration of immunity

There are few published data on the duration of antibody following vaccination with a commercial product. Protocols for their use usually recommend a primary course of two inoculations and boosters at yearly intervals. Only limited data are available on the antibody levels that correlate with protection against respiratory infections (7, 41) or in utero infection (13).

e) Stability

There are no accepted guidelines for the stability of BVD vaccines, but it can be assumed that attenuated virus vaccine (freeze-dried) should remain potent for at least 1 year if kept at 4°C. Inactivated virus vaccine could have a longer shelf life at 4°C. Lower temperatures could prolong shelf life for either type, but adjuvants in killed vaccine may preclude this.

f) Precautions

BVDV is not considered to be a human health hazard. Standard good microbiological practice should be adequate for handling the virus in the laboratory.

5. Tests on the final product

a) Safety tests

The safety of the final product formulation of both live and inactivated vaccines should be assessed in susceptible calves for any local reactions following administration, and in pregnant cattle for their effects on the unborn calf. Tests for individual batches are described in Section C.4.b.

b) Potency tests for antigenicity

BVD vaccines must be demonstrated to produce adequate immune responses, as outlined in Section C.4.c above, when used in their final formulation according to the manufacturer’s published instructions. In-vitro assays (Section C.4.c) may be used to monitor individual batches.

REFERENCE


**NB:** There are OIE Reference Laboratories for Bovine viral diarrhoea (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).