**Annex 11. Item 5.1. – Chapter 3.4.7. Bovine viral diarrhoea**

2 MEETING OF THE WOAH BIOLOGICAL STANDARDS COMMISSION

3 **Paris, 4–8 September 2023**

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5 **CHAP T E R 3 . 4 . 7 .**

# 6 B OV I N E VI RA L D I A R R H OE A

7 **SUMMARY**

1. *Cattle of all ages are susceptible to infection with bovine viral diarrhoea viruses* (*BVDV*), *including BVDV*
2. *type 1* (Pestivirus bovis), *type 2* (Pestivirus tauri)*, and Hobi-like pestiviruses* (*type 3* [Pestivirus brazilense])*.*
3. *Distribution is world-wide although some countries have recently eradicated the virus. BVDV infection results*
4. *in a wide variety of clinical manifestations, including enteric and respiratory disease in any class of cattle, or*
5. *reproductive and fetal disease following infection of a susceptible breeding female. Infection may be*
6. *subclinical or extend to severe fatal disease. Animals that survive* in-utero *infection in the first trimester of*
7. *gestation are almost always persistently infected* (*PI*)*. PI animals provide the main reservoir of the virus in a*
8. *population and excrete large amounts of virus in urine, faeces, discharges, milk and semen. Identification of*
9. *such PI cattle is a key element in controlling the infection. It is important to avoid the trade of such animals.*
10. *They may appear clinically healthy, or weak and unthrifty. Many PI animals die before reaching maturity.*
11. *They may infrequently develop mucosal disease with anorexia, gastrointestinal erosions, and profuse*
12. *diarrhoea, invariably leading to death. Mucosal disease can arise only in PI animals. Latent infections*
13. *generally do not occur following recovery from acute infection. However bulls may rarely have a persistent*
14. *testicular infection and excrete virus in semen for prolonged periods.*
15. ***Detection of the agent:*** *BVDV is a pestivirus in the family* Flaviviridae *and is closely related to classical*
16. *swine fever virus* (Pestivirus suis) *and ovine border disease virus~~es~~* (Pestivirus ovis). *BVD viruses are*
17. *classified into the distinct species* Pestivirus bovis (*commonly known as BVDV type 1*)*,* Pestivirus tauri
18. (*BVDV type 2*) *and* Pestivirus brazilense (*BVDV type 3 or Hobi-like pestivirus*)*. ~~The two genotypes~~* ~~(~~*~~types 1~~*
19. *~~and 2~~*~~)~~ *~~are classified as separate species in the genus~~* ~~Pestivirus~~*~~. A third putative genotype, BVDV type 3,~~*
20. *~~has also recently been proposed.~~ Although both cytopathic and non-cytopathic biotypes of BVDV type 1 and*
21. *type 2 exist, non-cytopathic strains are usually encountered in field infections and are the main focus of*
22. *diagnostic virus isolation in cell cultures. PI animals can be readily identified by a variety of methods aimed*
23. *to detect viral antigens or viral RNA directly in blood and tissues. Virus can also be isolated by inoculation*
24. *of specimens onto susceptible cell cultures followed by immune-labelling methods to detect the replication*
25. *of the virus in the cultures. Persistence of virus infection should be confirmed by resampling after an interval*
26. *of at least 3 weeks, when virus will again be detected. PI animals are usually seronegative. Viraemia in acute*
27. *cases is transient and usually difficult to detect. Virus isolation in semen from bulls that are acutely or*
28. *persistently infected requires special attention to specimen transport and testing. RNA detection assays are*
29. *particularly useful because they are rapid, have very high sensitivity and do not depend on the use of cell*
30. *cultures.*
31. ***Serological tests:*** *Acute infection with BVDV is best confirmed by demonstrating seroconversion using*
32. *sequential paired samples, ideally from several animals in the group. The testing of paired* (*acute and*
33. *convalescent samples*) *should be done a minimum of 21 days apart and samples should be tested*
34. *concurrently in the same assay. Enzyme-linked immunosorbent assays and the virus neutralisation test are*
35. *the most widely used.*
36. ***Requirements for vaccines:*** *There is no standard vaccine for BVD, but a number of commercial*
37. *preparations are available. An ideal vaccine should be able to prevent transplacental infection in pregnant*
38. *cows. Modified live virus vaccine should not be administered to pregnant cattle* (*or to their sucking calves*)
39. *due to the risk of transplacental infection. Live vaccines that contain cytopathic strains of BVDV present a*
40. *risk of inducing mucosal disease in PI animals. Inactivated viral vaccines are safe and can be given to any*
41. *class of animal but generally require booster vaccinations. BVDV is a particularly important hazard to the*
42. *manufacture of vaccines and biological products for other diseases due to the high frequency of*
43. *contamination of batches of fetal calf serum used as a culture medium supplement.*

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#### 1. Impact of the disease

## A. INTRODUCTION

1. Cattle of all ages are susceptible to infection with bovine viral diarrhoea viruses (BVDV). Distribution of the virus is world-
2. wide although some countries have recently eradicated the virus. BVDV infection results in a wide variety of clinical
3. manifestations, including enteric and respiratory disease in any class of cattle or reproductive and fetal disease following
4. infection of a susceptible breeding female. Infection may be subclinical or extend to severe fatal disease. Clinical
5. presentations and severity of disease may vary with different strains of virus. BVDV viruses also cause immune
6. suppression, which can render infected animals more susceptible to infection with other viruses and bacteria. The clinical
7. impact may be more apparent in intensively managed livestock. Animals that survive *in-utero* infection in the first trimester
8. of gestation are almost always persistently infected (PI). PI animals provide the main reservoir of the virus in a population
9. and excrete large amounts of virus in urine, faeces, discharges, milk and semen. The virus spreads mainly by close contact
10. between PI animals and other cattle. Virus shedding by acutely infected animals is usually less important. This virus may
11. also persist in the environment for short periods or be transmitted ~~with~~ via contaminated reproductive materials. Vertical
12. transmission plays an important role in its epidemiology and pathogenesis.
13. Infections of the breeding female may result in conception failure or embryonic and fetal infection which results in abortions,
14. stillbirths, teratogenic abnormalities or the birth of PI calves. Persistently viraemic animals may be born as weak, unthrifty
15. calves or may appear as normal healthy calves and be unrecognised clinically for a long time. However, PI animals have
16. a markedly reduced life expectancy, with a high proportion dying before reaching maturity. Infrequently, some of these
17. animals may later develop mucosal disease with anorexia, gastrointestinal erosions, and profuse diarrhoea, invariably
18. leading to death. Mucosal disease can arise only in PI animals. It is important to avoid the trade of viraemic animals. It is
19. generally considered that serologically positive, non-viraemic cattle are ‘safe’, providing that they are not pregnant.
20. However, a small proportion of persistently viraemic animals may produce antibodies to some of the viral proteins if they
21. are exposed to another strain of BVDV that is antigenically different to the persisting virus. Consequently, seropositivity
22. cannot be completely equated with ‘safety’. Detection of PI animals must be specifically directed at detection of the virus
23. or its components (RNA or antigens). Latent infections generally do not occur following recovery from acute infection.
24. However, semen collected from bulls during an acute infection is likely to contain virus during the viraemic period and often
25. for a short time afterwards. Although extremely rare, some recovered bulls may have a persistent testicular infection and
26. excrete virus in semen, perhaps indefinitely.
27. While BVDV strains are predominantly pathogens of cattle, interspecies transmission can occur following close contact
28. with sheep, goats or pigs. Infection of pregnant small ruminants or pigs with BVDV can result in reproductive loss and the
29. birth of PI animals. BVDV infections have been reported in both New World and Old World camelids. Additionally, strains
30. of border disease virus (BDV) have infected cattle, resulting in clinical presentations indistinguishable from BVDV infection.
31. The birth of cattle PI with BDV and the subsequent development of mucosal disease have also been described. Whilst
32. BVDV and BDV have been reported as natural infections in pigs, the related virus of classical swine fever does not naturally
33. infect ruminants.
34. Although ubiquitous, control of BVDV can be achieved at the herd level, and even at the national level, as evidenced by
35. the progress towards eradication made in many European countries (Moennig *et al*., 2005; Schweizer *et al.,* 2021).

#### 2. The causal agent

1. Bovine viral diarrhoea virus (BVDV) is a single linear positive-stranded RNA virus in the genus *Pestivirus* of the family
2. *Flaviviridae.* The genus contains a number of species including ~~the two genotypes of bovine viral diarrhoea virus (~~BVDV)
3. (types 1 [*Pestivirus bovis*], ~~and~~ 2 [*Pestivirus tauri*] and 3 [*Pestivirus brazilense*]) and the ~~closely~~ related classical swine
4. fever (*Pestivirus suis*) and ovine border disease viruses (*Pestivirus ovis*). Viruses in these ~~genotypes~~ pestivirus species
5. show considerable antigenic difference from each other and, within the ~~type 1 and type 2~~ species *Pestivirus bovis* and
6. *tauri*, BVDV isolates exhibit considerable biological and antigenic diversity. Within the ~~two BVDV genotypes~~ species
7. *Pestivirus bovis* and *tauri*, further subdivisions are discernible by genetic analysis (Vilcek *et al*., 2001). The two ~~genotypes~~
8. species may be differentiated from each other, and from other pestiviruses, by monoclonal antibodies (MAbs) directed
9. against the major glycoproteins E2 and ERNS or by genetic analysis. Reverse-transcription polymerase chain reaction (RT-
10. PCR) assays enable virus typing direct from blood samples (Letellier & Kerkhofs, 2003; McGoldrick *et al*., 1999). Type 1
11. viruses are generally more common although the prevalence of type 2 strains can be high in North America. BVDV of both
12. ~~genotypes~~ species (*Pestivirus bovis* and *tauri*) may occur in non-cytopathic and cytopathic forms (biotypes), classified
13. according to whether or not microscopically apparent cytopathology is induced during infection of cell cultures. Usually, it
14. is the non-cytopathic biotype that circulates freely in cattle populations. Non-cytopathic strains are most frequently
15. responsible for disease in cattle and are associated with enteric and respiratory disease in any class of cattle or
16. reproductive and fetal disease following infection of a susceptible breeding female. Infection may be subclinical or extend
17. to severe fatal disease (Brownlie, 1985). Cytopathic viruses are encountered in cases of mucosal disease, a clinical
18. syndrome that is relatively uncommon and involves the ‘super-infection’ of an animal that is PI with a non-cytopathic virus
19. by a closely related cytopathic strain. The two virus biotypes found in a mucosal disease case are usually antigenically
20. closely related if not identical. Type 2 viruses are usually non-cytopathic and have been associated with outbreaks of
21. severe acute infection and a haemorrhagic syndrome. However some type 2 viruses have also been associated with a
22. disease indistinguishable from that seen with the more frequently isolated type 1 viruses. Further, some type 1 isolates
23. have been associated with particularly severe and fatal disease outbreaks in adult cattle. Clinically mild and inapparent
24. infections are common following infection of non-pregnant animals with either ~~genotype~~ virus species.
25. ~~There is an increasing awareness of an “atypical” or “HoBi-like” pestivirus – a putative BVDV type 3~~ *Pestivirus H* strains
26. are also associated with clinical disease in cattle, but they appear mainly restricted to South American and Asian cattle
27. populations~~, in cattle, also associated with clinical disease~~ (Bauermann *et al*., 2013; Chen *et al.,* 2021)~~, but its distribution~~
28. is presently unclear. These viruses are readily detected by proven pan-reactive assays such as real-time RT-PCR. Some
29. commercial antigen ELISAs (enzyme-linked immunosorbent assays) have been shown to detect these strains (Bauermann
30. *et al*., 2012); generally virus isolation, etc., follows the same principles as for BVDV 1 (*Pestivirus bovis*) and 2 (*Pestivirus*
31. *tauri*). It should be noted however, that antibody ELISAs vary in their ability to detect antibody to BVDV type 3 (*Pestivirus*
32. *brazilense*) and vaccines designed to protect against BVDV 1 and 2 may not confer full protection against infection with
33. these ~~novel~~ pestiviruses (Bauermann *et al.,* 2012*;* 2013).

#### 3. Pathogenesis

##### 3.1. Acute infections

1. Acute infections with BVDV are encountered more frequently in young animals, and may be clinically inapparent or
2. associated with fever, diarrhoea (Baker 1995), respiratory disease and sometimes sudden death. The severity of disease
3. may vary with virus strain and the involvement of other pathogens (Brownlie, 1990). In particular, outbreaks of a severe
4. form of acute disease with haemorrhagic lesions, thrombocytopenia and high mortality have been reported sporadically
5. from some countries (Baker, 1995; Bolin & Ridpath, 1992). Infection with type 2 viruses (*Pestivirus tauri*) in particular
6. has been demonstrated to cause altered platelet function. During acute infections there is a brief viraemia for 7–10 days
7. and shedding of virus can be detected in nasal and ocular discharges. There may also be a transient leukopenia,
8. thrombocytopenia or temperature response, but these can vary greatly among animals. Affected animals may be
9. predisposed to secondary infections with other viruses and bacteria. Although BVDV may cause a primary respiratory
10. disease on its own, the immunosuppressive effects of the virus exacerbate the impact of this virus. BVDV is one of the
11. major pathogens of the bovine respiratory disease complex in feedlot cattle and in other intensive management systems
12. such as calf raising units.
13. Infection of breeding females immediately prior to ovulation and in the first few days after insemination can result in
14. conception failure and early embryonic loss (McGowan & Kirkland, 1995). Cows may also suffer from infertility,
15. associated with changes in ovarian function and secretions of gonadotropin and progesterone (Fray *et al*., 2002).
16. Bulls may excrete virus in semen for a short period during and immediately after infection and may suffer a temporary
17. reduction of fertility. Although the virus level in this semen is generally low it can result in reduced conception rates
18. and be a potential source of introduction of virus into a naive herd (McGowan & Kirkland, 1995).
19. **3.2. *In-utero* infections**
20. Infection of a breeding female can result in a range of different outcomes, depending on the stage of gestation at
21. which infection occurred. Before about 25 days of gestation, infection of the developing conceptus will usually result
22. in embryo-fetal death, although abortion may be delayed for a considerable time (McGowan & Kirkland, 1995).
23. Surviving fetuses are normal and uninfected. However, infection of the female between about 30–90 days will
24. invariably result in fetal infection, with all surviving progeny PI and seronegative. Infection at later stages and up to
25. about day 150 can result in a range of congenital defects including hydranencephaly, cerebellar hypoplasia, optic
26. defects, skeletal defects such as arthrogryposis and hypotrichosis. Growth retardation may also occur, perhaps as a
27. result of pituitary dysfunction. Fetal infection can result in abortion, stillbirth or the delivery of weak calves that may
28. die soon after birth (Baker, 1995; Brownlie, 1990; Duffell & Harkness, 1985; Moennig & Liess, 1995). Some PI calves
29. may appear to be normal at birth but fail to ~~grow normally~~ thrive. They remain PI for life and are usually seronegative,
30. exceptions may be young calves that ingested colostrum containing antibodies. The onset of the fetal immune
31. response and production of antibodies occurs between approximately day 90–120, with an increasing proportion of
32. infected calves having detectable antibodies while the proportion in which virus may be detected declines rapidly.
33. Infection of the bovine fetus after day 180 usually results in the birth of a normal seropositive calf.

##### 3.3. Persistent infections

1. Persistently viraemic animals are a continual source of infective virus to other cattle and are the main reservoir of
2. BVDV in a population. In a population without a rigorous BVDV control programme, approximately 1–2% of cattle are
3. PI. During outbreaks in a naive herd or breeding group, if exposure has occurred in the first trimester of pregnancy,
4. a very high proportion of surviving calves will be PI. If a PI animal dies, there are no pathognomonic lesions due to
5. BVDV and the pathology is often complicated by secondary infections with other agents. Some PI animals will survive
6. to sexual maturity and may breed successfully but the~~ir~~ progeny of female PI animals will also always be PI. Animals
7. being traded or used for artificial breeding should first be screened to ensure that they are not PI.

##### 3.4. Mucosal disease

1. Persistently viraemic animals may later succumb to mucosal disease (Brownlie, 1985). However, cases are rare. This
2. syndrome has been shown to be the outcome of the infection of a PI animal with an antigenically similar cytopathic
3. virus, which can arise either through superinfection, recombination between non-cytopathic biotypes, or mutation of
4. the persistent biotype (Brownlie, 1990). There is usually little need to specifically confirm that a PI animal has
5. succumbed to mucosal disease as this is largely a scientific curiosity and of little practical significance, other than
6. that the animal is PI with BVDV. However, cases of mucosal disease may be the first indication in a herd that BVDV
7. infection is present, and should lead to more in depth investigation and intervention.

##### 3.5. Semen and embryos

1. Bulls that are PI usually have poor quality, highly infective semen and reduced fertility (McGowan & Kirkland, 1995).
2. All bulls used for natural or artificial insemination should be screened for both acute and persistent BVDV infection.
3. A rare event, possibly brought about by acute infection during pubescence, can result in persistent infection of the
4. testes and thus strongly seropositive bulls that intermittently excrete virus in semen (Voges *et al*., 1998). This
5. phenomenon has also been observed following vaccination with an attenuated virus (Givens *et al*., 2007). Embryo
6. donor cows that are PI with BVDV also ~~re~~present a potential source of infection, particularly as there are extremely
7. high concentrations of BVDV in uterine and vaginal fluids. While oocysts without an intact zona pellucida have been
8. shown to be susceptible to infection *in vitro*, the majority of oocysts remain uninfected with BVDV. Normal uninfected
9. progeny have also been ‘rescued’ from PI animals by the use of extensive washing of embryos and *in-vitro* fertilisation.
10. Female cattle used as embryo recipients should always be screened to confirm that they are not PI, and ideally, are
11. seropositive or were vaccinated at least 4 weeks before first use.
12. Biological materials used for *in-vitro* fertilisation techniques (bovine serum, bovine cell cultures) have a high risk of
13. contamination and should be screened for BVDV. Incidents of apparent introduction of virus via such techniques have
14. highlighted this risk. It is considered essential that serum supplements used in media should be free of contaminants
15. as detailed in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for*
16. *veterinary use,* using techniques described in Section B.~~3~~ 1.1 of this chapter.

#### 4. Approaches to diagnosis and sample collection

1. The diagnosis of BVDV infection can sometimes be complex because of the delay between infection and clinical
2. expression. While detection of PI animals should be readily accomplished using current diagnostic methods, the recognition
3. of acute infections and detection of BVDV in reproductive materials can be more difficult.

##### 4.1. Acute infections

1. Unlike PI animals, acutely infected animals excrete relatively low levels of virus and for a short period of time (usually
2. about 7–10 days) but the clinical signs may occur during the later stages of viraemia, reducing the time to detect the
3. virus even further. In cases of respiratory or enteric disease, samples should be collected from a number of affected
4. animals, preferentially selecting the most recently affected. Swabs should be collected from the nares and conjunctiva
5. of animals with respiratory disease or from rectum and faeces if there are enteric signs. Lung and spleen are preferred
6. from dead animals. Viral RNA may be detected by real-time RT-PCR assays and have the advantages of high
7. sensitivity and being able to detect genome from non-infectious virus. As the virus levels are very low, it is not usually
8. practical to undertake virus isolation unless there is a need to characterise the strain of BVDV involved. Serology
9. undertaken on paired acute and convalescent sera (collected at least 21 days after the acute sample and from 8–10
10. animals) is worthwhile and gives a high probability of incriminating or excluding BVDV infection.
11. Confirmation that an abortion, stillbirth or perinatal death is caused by BVDV is often difficult to establish because
12. there can be a long delay between initial infection and death or expulsion of the fetus. Sampling should take into
13. consideration the need to detect either viral components or antibodies. Spleen and lung are preferred samples for
14. virus detection while pericardial or pleural fluids are ideal samples for serology. The stomach of newborn calves
15. should be checked to confirm that sucking has not occurred. While virus may be isolated from fetal tissue in some
16. cases, emphasis should be placed on the detection of viral antigen by ELISA or RNA by real-time RT-PCR. For
17. serology, both ELISAs and virus neutralisation test (VNT) are suitable though sample quality and bacterial
18. contamination may compromise the ability to detect antibodies by VNT. Maternal serology, especially on a group of
19. animals, can be of value, with the aim of determining whether there has been recent infection in the group. A high
20. antibody titre (>1/1000) to BVDV in maternal serum is suggestive of fetal infection and is probably due to the fetus
21. providing the dam with an extended exposure to virus.

##### 4.2. Persistent infections

1. In the past, identification of PI animals relied heavily on the use of virus isolation in cell cultures. However, antigen
2. detection ELISAs and real-time RT-PCR assays, each with relatively high sensitivity, are widely used for the detection
3. of viral antigens or RNA in both live and dead animals. Virus isolation aimed at the detection of non-cytopathic BVDV
4. in blood is also used, while in some countries, the virus has been identified by immunohistochemistry (IHC). Skin
5. samples have been collected from live animals while a wide range of tissues from dead animals are suitable. Both
6. virus isolation and IHC are labour intensive and costly and can be technically demanding. Virus isolation from blood
7. can be confounded by the presence of maternal antibody to BVDV in calves less than 4–5 months of age (diagnostic
8. gap). Also for antigen detection ELISAs and flow cytometry from blood or blood leukocytes, there are restrictions that
9. limit when animals that ingested colostrum that contains antibodies against BVDV can be reliably tested. In older
10. animals with persistent viraemia infection, low levels of antibody may be present due to their ability to seroconvert to
11. strains of BVDV (including vaccines) antigenically different to the persisting virus (Brownlie, 1990). Bulk (tank) or
12. individual milk samples have been used to monitor dairy herds for the presence of a PI animal. Antigen ELISA, real-
13. time PCR and virus isolation have all been used. To confirm a diagnosis of persistent infection, animals should be
14. retested after an interval of at least 3 weeks by testing of blood samples for the presence of the virus and for ~~evidence~~
15. absence of seroconversion. Care should be taken with retesting of skin samples as it has been shown that, in some
16. acute cases, viral antigen may persist for many weeks in skin (Cornish *et al*., 2005).

##### 4.3. Mucosal disease

1. Although not undertaken for routine diagnostic purposes, for laboratory confirmation of a diagnosis of mucosal
2. disease it is necessary to isolate the cytopathic virus. This biotype may sometimes be isolated from blood, but it can
3. be recovered more consistently from a variety of other tissues, in particular spleen, intestine and Peyer’s patch tissue.
4. Virus isolation is readily accomplished from spleen which is easy to collect and is seldom toxic for cell culture.

##### 4.4. Reproductive materials

1. Semen donor bulls should be sampled for testing for freedom from BVDV infection prior to collection of semen, in
2. accordance with the *Terrestrial Animal Health Code.* It is necessary to confirm that these bulls are not PI, are not
3. undergoing an acute infection and to establish their serological status. This initial testing should be carried out on
4. whole blood or serum samples. To establish that a seropositive bull does not have a persistent testicular infection
5. (PTI), samples of semen should be collected on at least three separate occasions at intervals of not less than 7 days
6. due to the possibility of intermittent low level virus excretion, especially during the early stages of infection. There is
7. also a need to submit a number of straws from each collection, or an appropriate volume of raw semen. Particular
8. care should be taken to ensure that sample transport recommendations are adhered to and that the laboratory
9. documents the condition of the samples on arrival at the laboratory. Further details of collection, transport and test
10. requirements are provided in sections that follow.

## 249 B. DIAGNOSTIC TECHNIQUES

250 ***Table 1.*** *Test methods available for diagnosis of bovine viral diarrhoea and their purpose*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Method** | **Purpose** | | | | | |
| [Population](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex11_3.04.07_Table_1a_population_freedom_from_infection.pdf) [freedom](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex11_3.04.07_Table_1a_population_freedom_from_infection.pdf) [from](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex11_3.04.07_Table_1a_population_freedom_from_infection.pdf) [infection](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex11_3.04.07_Table_1a_population_freedom_from_infection.pdf) | [Individual animal](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex11_3.04.07_Table_1b_individual_freedom_to_move.pdf) [freedom from](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex11_3.04.07_Table_1b_individual_freedom_to_move.pdf) [infection prior to](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex11_3.04.07_Table_1b_individual_freedom_to_move.pdf) [movement](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex11_3.04.07_Table_1b_individual_freedom_to_move.pdf) | [Contribute 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[vaccination)](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex11_3.04.07_Table_1f_immune_status_individuals.pdf) |
| **Detection of the agent(a)** | | | | | | |
| **Virus isolation** | + | ++ ~~+~~ | ++ | ++ ~~+~~ | – | – |
| **Antigen detection by ELISA** | +++ | +++ | +++ | +++ | +++ | – |
| **Antigen detection by IHC** | – | – | – | ++ | – | – |
| **NA detection by real-time RT-PCR** | +++ | +++ | +++ | +++ | +++ | – |
| **Detection of immune response** | | | | | | |
| **ELISA** | +++ | ++ | +++ | ~~–~~ +(a) | +++ | +++ |
| **VN** | + | ++ ~~+~~ | ++ | – | + | +++ |

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

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

1. ELISA = enzyme-linked immunosorbent assay; IHC = immunohistochemistry method; NA = nucleic acid; RT-PCR = reverse-
2. transcription polymerase chain reaction; ~~ISH =~~ *~~in-situ~~* ~~hybridisation;~~ VN = virus neutralisation.
3. (a)A combination of agent detection methods applied on the same clinical sample is recommended.

#### 1. Detection of the agent

1. To prevent the shipment of either animals or animal derivatives (especially semen and embryos) that are infected with
2. BVDV, it is necessary to test for the presence of the infectious virus (virus isolation), viral antigens (antigen detection
3. ELISA) or RNA (real-time RT-PCR) in the blood of the animal being shipped, or the donor of the germplasm (semen or
4. embryos). The exception is for seropositive bulls where semen must be tested rather than the donor bull. Serology only
5. plays a role for establishing that seronegative animals are not undergoing an acute infection or, to establish the serological
6. status of donor bulls. Due to their variable sensitivity without prior virus amplification, procedures such as IHC or *in-situ*
7. hybridisation (ISH) directly on tissues are not considered to be suitable for certification for freedom from BVDV for
8. international trade purposes. In contrast, immune-staining is an essential component of virus isolation in cell culture to
9. detect the presence of non-cytopathic strains of BVDV which predominate in field infections.
10. All test methods must be extensively validated by testing on known uninfected and infected populations of cattle, including
11. animals with low- and high-titre viraemias. Methods based on polyclonal or MAb-binding assays (ELISA or IHC), immune
12. labelling (VI) or on nucleic acid recognition (PCR) must be shown to detect the full range of antigenic and genetic diversity
13. found among BVD viruses. There are ~~three~~ designated WOAH Reference Laboratories for BVDV that can assist with
14. relevant information; the reference laboratories for classical swine fever could also be approached to offer some advice.

##### 1.1. Virus isolation

1. When performed to a high standard, BVDV isolation is very reliable. However, it does have very exacting
2. requirements to ensure that the cell cultures and medium components give a system that is very sensitive and are
3. not compromised by the presence of either low levels of BVDV specific antibody or virus. Virus isolation only has the
4. capacity to detect infectious virus which imposes certain limits on sample quality. Further, to detect low levels of virus
5. that may be present in some samples, particularly semen, it may be necessary to examine larger volumes of specimen
6. than is usual. Some of these limitations can be overcome by the use of antigen detection ELISAs with proven high
7. analytical sensitivity, or the use of real-time RT-PCR.
8. The virus may be isolated in a number of bovine monolayer cell cultures (e.g. kidney, lung, testis or turbinate). In
9. some instances, ovine cells are also suitable. Primary or secondary cultures can be frozen as cell suspensions in
10. liquid nitrogen. These can then be tested over a series of passages, or seeded to other susceptible cells and checked
11. for freedom from contaminants and to evaluate their sensitivity compared to an approved batch of cells before routine
12. use. Such problems may be reduced by the use of continuous cell lines, which can be obtained BVD-free, however,
13. their BVDV-free status and susceptibility must be monitored regularly. Continuous cells should be used under a ‘seed
14. lot’ system where they are only used over a limited passage range, within which they have been shown to have
15. acceptable sensitivity to BVDV infection. Although particular continuous cell lines are considered to be appropriate
16. for use for BVDV isolation, there can be significant variation in batches of cells from different sources due to differing
17. passage histories so their suitability must still be confirmed before routine use.
18. Non-cytopathic BVDV is a common contaminant of bovine tissues and cell cultures must be checked for freedom
19. from adventitious virus by regular testing. Cells must be grown in proven cell culture medium components and a large
20. area of cells must be examined. It is not appropriate to screen a few wells of a 96 well plate – examining all wells of
21. a 96 well plate will be more convincing evidence of freedom. The fetal bovine serum that is selected for use in cell
22. culture must also be free not only from virus, but also and of equal or perhaps even greater importance, from BVDV
23. neutralising antibody. Heat treatment (56°C for 30–45 minutes) is inadequate for the destruction of BVDV in
24. contaminated serum; irradiation with a dose of at least 25 kiloGrays (2.5 Mrad) is more certain. Commercial batches
25. of fetal bovine serum mostly test positive by real-time RT-PCR even after the virus has been inactivated by irradiation.
26. Further, most commercially collected batches of fetal bovine serum contain antibodies to pestiviruses, sometimes at
27. levels that are barely detectable but sufficient to inhibit virus isolation. To overcome this, serum can be obtained from
28. BVD virus and antibody free donor animals and used with confidence. Testing of donors for both virus and antibody
29. occurs on an individual animal basis. Although horse serum has been substituted for bovine fetal serum, it is often
30. found to have poorer cell-growth-promoting characteristics. Further there has sometimes been cross contamination
31. with fetal bovine serum during processing, negating the objective of obtaining a BVDV-free product.
32. Buffy coat cells, whole blood, washed leukocytes or serum are suitable for isolation of the virus from live animals.
33. Maternal antibody may interfere with isolation from serum in young calves. Tissue suspensions from post-mortem
34. cases should be prepared by standard methods. Confirmation that a bull is not PI with BVDV is most readily achieved
35. by testing of a blood sample. However, persistent testicular infections (PTI) have been detected in some bulls that
36. have recovered from acute infection, are no longer viraemic and are now seropositive (Voges *et al*., 1998). Virus may
37. be detected in most but not all collections of semen from these bulls. Although still considered to be uncommon, to
38. exclude the potential for a PTI it is essential to screen semen from all seropositive bulls. To be confident that a bull
39. does not have a PTI, batches of semen collected over several weeks should be screened. Once a series of collections
40. have been screened, further testing of semen from a seropositive bull is not warranted. Raw semen, and occasionally
41. extended semen, is cytotoxic and must be diluted in culture medium. For these reasons, it is important to monitor the
42. health of the cells by microscopic examination at intervals during the incubation. These problems are largely
43. overcome by the use of real-time RT-PCR which has several advantages over virus isolation, including higher
44. sensitivity and the potential to be completed within a few hours rather than weeks for virus isolation.
45. There are many variations of procedure in use for virus isolation. All should be optimised to give maximum sensitivity
46. of detection of a standard virus preparation. All biological components used for cell culture should be screened and
47. shown to be free of both BVDV and antibodies to BVDV. Cell cultures (whether primary or continuous lines) should
48. be regularly checked to confirm that they maintain maximum susceptibility to virus infection. Depending on the
49. specimen type and purpose for testing, virus isolation is likely to require one or more passages in cell cultures. While
50. PI animals can be readily identified by screening blood or serum with one passage, semen should be routinely
51. cultured for three passages and biological products such as fetal bovine serum up to five times (original inoculation
52. plus four passages). Conventional methods for virus isolation are used, with the addition of a final immune-staining
53. step (immunofluorescence or, more frequently, peroxidase staining) to detect growth of non-cytopathic virus. Thus
54. tube cultures should include flying cover-slips, while microplate cultures can be fixed and labelled directly in the plate.
55. Examples are given below. Alternatively, culture supernatant from the final passage can be screened by real-time
56. RT-PCR (see below).
57. 1.1.1. Microplate immunoperoxidase method for mass screening for virus detection in serum
58. **samples (Meyling, 1984)**
59. i) 10–25 μl of the serum sample is placed into each of four wells of a 96-well tissue-culture grade
60. microplate. This is repeated for each sample. Known positive and negative controls are
61. included.
62. ii) 100 μl of a cell suspension at the appropriate concentration (usually about 150,000 cells/ml) in
63. medium without fetal calf serum (FCS) is added to all wells. *Note:* the sample itself acts as the
64. cell-growth supplement. If testing samples other than serum, use medium with 10% FCS that is
65. free of antibodies to ruminant pestiviruses.
66. iii) The plate is incubated at 37°C for 4 days, either in a 5% CO2 atmosphere or with the plate
67. sealed.
68. iv) Each well is examined microscopically for evidence of cytopathology (cytopathic effect or CPE),
69. or signs of cytotoxicity.
70. v) The cultures are frozen briefly at approximately –80°C and 50 µl of the culture supernatant is
71. passaged to new cell cultures, repeating steps ~~3~~1.1.1.i to iv above.
72. vi) The cells are then fixed and stained by one of two methods:

|  |  |  |
| --- | --- | --- |
| 344 | **●** | **Paraformaldehyde** |
| 345 | a) | Add 200 µl of a 1/10 dilution of formaldehyde solution (approximately 3% concentration) to |
| 346 |  | the plate and leave at room temperature for 10 minutes. |
| 347 | b) | The contents of the plate are then discarded and the plate is washed. |
| 348 | c) | Wash plates 5 times with 0.05% Tween 20 in water (an automatic microplate washer can be |
| 349 |  | used with a low pressure and speed setting). |
| 350 | d) | To each well add 50 µl of an antiviral antibody at the appropriate dilution (prepared in |
| 351 |  | phosphate buffered saline/ PBS containing 1% gelatin) and incubate for 60–90 minutes at |
| 352 |  | 37°C in a humidified chamber. |
| 353 | e) | Wash plates five times as in step c). |
| 354 | f) | Dilute the appropriate peroxidase conjugated antiserum to the optimum dilution in 1% gelatin/PBS |
| 355 |  | (e.g. peroxidase conjugated rabbit anti-mouse immunoglobulin when the antiviral antibody is a |
| 356 |  | mouse monoclonal). The optimum concentration should be determined for each batch of |
| 357 |  | conjugate by “checkerboard” titration against reference positive and negative controls. |
| 358 | g) | To each well of the microplate add 50 µl of the diluted peroxidase conjugate and incubate for 90 |
| 359 |  | minutes at 37°C in a humidified chamber. |
| 360 | h) | Wash plates five times as in step c). |
| 361 | i) | “Develop” the plate by adding 3-amino-9-ethyl carbazole (AEC) substrate (100 µl/well) and |
| 362 |  | allowing to react for 30 minutes at room temperature. |
| 363 | j) | Add 100 µl of PBS to each well and add a lid to each plate. |
| 364 | k) | Examine the wells by light microscopy, starting with the negative and positive control wells. There |
| 365 |  | should be no or minimal staining apparent in the cells that were uninfected (negative control). The |
| 366 |  | infected (positive control) cells should show a reddish- brown colour in the cytoplasm. |
| 367 | **●** | **Acetone** |
| 368 | a) | The plate is emptied by gentle inversion and rinsed in PBS. |
| 369 | b) | The cells are fixed as follows: the plate is dipped into a bath of 20% acetone in PBS, emptied |
| 370 |  | immediately and then transferred to a fresh bath of 20% acetone in PBS for 10 minutes. The |
| 371 |  | plate is drained thoroughly and as much fluid as possible is removed by tapping and blotting. |
| 372 |  | The plate is dried thoroughly for at least 3 hours at a temperature of 25–30°C (e.g. using |
| 373 |  | radiant heat from a bench lamp). *Note:* the drying is part of the fixation process. |
| 374 | c) | The fixed cells are rinsed by adding PBS to all wells. |
| 375 | d) | The wells are drained and the BVD antibody (50 μl) is added to all wells at a predetermined |
| 376 |  | dilution in PBS containing 1% Tween 80 (PBST) and 5% horse serum or 1% gelatin. (Horse |
| 377 |  | serum or gelatin may be added to reduce nonspecific staining.) |
| 378 | e) | Incubate at 37°C for 15 minutes. |
| 379 | f) | Empty the plate and wash three times in PBST. |
| 380 | g) | Drain and add the appropriate anti-species serum conjugated to peroxidase at a |
| 381 |  | predetermined dilution in PBST (50 μl per well) for 15 minutes at 37°C. |
| 382 | h) | Empty the plate and wash three times in PBST. |
| 383 | i) | Rinse the plate in distilled water. Ensure all fluid is tapped out from the plate. |
| 384 | j) | Add freshly prepared hydrogen peroxide substrate with a suitable chromogen, e.g. 3-amino- |
| 385 |  | 9-ethyl carbazole (AEC). |

1. An alternative substrate can be made, consisting of 9 mg diaminobenzidine tetrahydrochloride
2. and 6 mg sodium perborate tetrahydrate dissolved in 15 ml of PBS. Though the staining is not
3. quite so intense, these chemicals have the advantage that they can be shipped by air.
4. k) The plate is examined microscopically. Virus-positive cells show red-brown cytoplasmic
5. staining.
6. Alternative methods for fixation of the cells may be used and include the use of heat (see Chapter
7. 3.8.3 *Classical swine fever*, Section B.2.2.1.viii). These should be first evaluated to ensure that the
8. capacity to detect viral antigen is not compromised.
9. 1.1.2. Tube method for tissue or buffy coat suspensions
10. *Note:* this method can also be conveniently adapted to 24-well plastic dishes. *Note:* a minimum of 2 and
11. preferably 3 passages (including primary inoculation) is required.
12. i) Tissue samples are ground up and a 10% suspension in culture medium is made. This is then
13. centrifuged to remove the debris.
14. ii) Test tube cultures with newly confluent or subconfluent monolayers of susceptible bovine cells are
15. inoculated with 0.1 ml of the sample. The culture is left to adsorb for 1 hour at 37°C.
16. iii) The culture is washed with 1 ml of medium; this is then discarded and 1 ml of culture maintenance
17. medium is added.
18. iv) The culture is incubated for 4–5 days at 37°C, and examined microscopically for evidence of CPE
19. or signs of cytotoxicity.
20. v) The culture should then be frozen and thawed for passage to fresh cultures for one or preferably
21. two more passages (including the culture inoculated for the final immunostaining). At the final
22. passage, after freeze–thaw the tissue culture fluid is harvested and passaged on to microtitre plates
23. for culture and staining by the immunoperoxidase method (see section B.~~3~~1.1.1 above) or by the
24. immunofluorescent method. For immunofluorescence, cover-slips are included in the tubes and
25. used to support cultured cells. At the end of the culture period, the cover slips are removed, fixed
26. in 100% acetone and stained with an immunofluorescent conjugate to BVDV. Examine the cover
27. slips under a fluorescent microscope for diffuse, cytoplasmic fluorescence characteristic of
28. pestiviruses. Alternatively, culture supernatant from the final passage can be screened by real-time
29. RT-PCR (see below).
30. 1.1.3. Virus isolation from semen
31. The samples used for the test are, typically, extended bovine semen or occasionally raw semen.
32. Semen samples should be transported to the laboratory in liquid nitrogen, or on dry ice. The samples
33. should be stored in liquid nitrogen or at lower than –70°C (for long-term storage) or 4°C (for short-
34. term storage of not more than 1–2 days). The receiving laboratory should document the condition
35. under which samples are received. Raw semen is generally cytotoxic and should be prediluted (e.g.
36. 1/10 in BVDV free bovine serum) before being added to cell cultures. At least 0.1 ml of raw semen
37. should be tested with three passages in cell culture. Toxicity may also be encountered with extended
38. semen. For extended semen, an approximation should be made to ensure that the equivalent of a
39. minimum of 0.1 ml raw semen is examined (e.g. a minimum of 1.0 ml extended semen). If toxicity is
40. encountered, multiple diluted samples may need to be tested to reach a volume equivalent to 0.1 ml
41. raw semen (e.g. 5 × 1 ml of a sample of extended semen that has been diluted 1/5 to reduce toxicity).
42. A suggested method is as follows:
43. i) Dilute 200 μl fresh semen in 1.8 ml bovine serum containing antibiotics. This can be the same
44. serum as is being used for supplementing the cell cultures, and must be shown to be free from
45. antibodies against BVDV.
46. ii) Mix vigorously and leave for 30 minutes at room temperature.
47. iii) Inoculate 1 ml of the semen/serum mixture into a monolayer of susceptible cells (see virus
48. isolation from tissue above) in cell culture tubes or a six-well tissue culture plate.
49. iv) Incubate the cultures for 1 hour at 37°C.
50. v) Remove the mixture, wash the monolayer several times with maintenance medium and then
51. add new maintenance medium to the cultures.
52. vi) Include BVDV negative and positive controls in the test. Special caution must be taken to avoid
53. accidental contamination of test wells by the positive control, for example always handling the
54. positive control last.
55. vii) Observe plates microscopically to ensure freedom from contamination and cytotoxicity. No
56. cytopathology is expected as a result of BVDV infection but other viruses such as BHV-1 could
57. be inadvertently isolated.
58. viii) After 5–7 days, the cultures are frozen at or below approximately –70°C and thawed, clarified
59. by centrifugation, and the supernatant used to inoculate fresh monolayers.
60. ix) At the end of the second passage, the supernatant from the freeze-thaw preparation should be
61. passaged onto cultures in a suitable system for immunoperoxidase staining or other antigen
62. detection or by real-time RT-PCR after 5 days of culture. This is most readily achieved in 96
63. well microplates. The sample is considered to be negative, if there is no evidence of viral antigen
64. or BVDV RNA detected.

##### 1.2. Nucleic acid detection

1. Conventional gel based RT-PCR has in the past been used for the detection of BVD viral RNA for diagnostic
2. purposes. A multiplex RT-PCR has been used for the simultaneous amplification and typing of virus from cell culture,
3. or direct from blood samples. However, gel based RT-PCR has the disadvantage that it is relatively labour intensive,
4. expensive and prone to cross contamination. These problems had been markedly reduced following the introduction
5. of probe-based real-time or quantitative RT-PCR methods. Nevertheless, stringent precautions should still be taken
6. to avoid nucleic acid contamination in the test system and general laboratory areas where samples are handled and
7. prepared (see Chapter 1.1.6 *Principles and methods of validation of diagnostic assays for infectious diseases* and
8. Chapter 2.2.3 *Development and optimisation of nucleic acid assays*). These assays have even higher sensitivity than
9. gel based RT-PCR and can be completed in a few hours. They are in widespread use for the diagnosis of infectious
10. diseases, allowing the direct detection of viral RNA from a wide range of specimens including serum, whole blood,
11. tissues, milk and semen. The high analytical sensitivity allows the adoption of strategies to screen pools of individual
12. samples or testing of bulk tank milk. By using this approach the presence of one or more PI animals can be identified
13. in herds containing several hundred cows. However, it is not appropriate to pool blood samples taken from calves
14. between day 7 and 40 of life, when colostrum that contains antibodies against BVDV was ingested. During this time
15. the sensitivity of PCR can be reduced and infected animals escape detection. In contrast, the detection of viral RNA
16. in skin biopsy samples remains unaffected (Fux & Wolf*,* 2012). Although slightly more expensive than immunostaining
17. methods, real-time RT-PCR is a quick and reliable method that can also be used to screen culture supernatant from
18. the final passage of cell cultures. While real-time RT-PCR has very high sensitivity and can be applied to the screening
19. of biological materials used for vaccine manufacture, caution is needed in the interpretation of results, as the detection
20. of viral RNA does not imply *per se* that infective virus is present. Real-time RT-PCR assays based on fluorescent-
21. labelled DNA probes can also be used to differentiate pestiviruses (e.g. McGoldrick *et al*., 1999).
22. Primers for the assay should be selected in highly conserved regions of the genome, ideally the 5’-noncoding region,
23. or the NS3 (p80 gene). There are published assays that are broadly reactive across the pestivirus genus, detecting
24. all BVDV types (*Pestivirus bovis, tauri* and *brazilense*), CSFV (*Pestivirus suis*), some strains of BDV (*Pestivirus ovis*)
25. and ~~most of the~~ several ‘atypical’ pestiviruses (e.g. Hoffman *et al*., 2006). A sensitive broadly reactive assay is
26. recommended for diagnostic applications because interspecies transfer of different pestiviruses is occasionally
27. encountered. When further identification of the specific virus is required, pestivirus species-specific assays can be
28. applied to further type the virus. It is important to thoroughly optimise all aspects of the real-time RT-PCR assay,
29. including the nucleic acid extraction and purification. Optimal concentrations of Mg2+, primers, probe and polymerase,
30. and the cycling parameters need to be determined. However, fully formulated and optimised ‘ready to use’
31. ‘mastermixes’ are now available commercially and only require addition of optimised concentrations of primers and
32. probe. Optimised cycling conditions are often recommended for a particular mastermix.
33. A variety of commercially available nucleic acid purification systems are available in kit form and several can be semi-
34. automated. Systems based on the capture and purification of RNA using magnetic beads are in widespread use and
35. allow rapid processing of large numbers of samples. Specific products should be evaluated to determine the optimal
36. kit for a particular sample type and whether any preliminary sample processing is required. For whole blood samples,
37. the type of anticoagulant and volume of blood in a specimen tube is important. More problems with inhibitors of the
38. PCR reaction are encountered with samples collected into heparin treated blood than EDTA. These differences are
39. also exacerbated if the tube does not contain the recommended volume of blood, thereby increasing the concentration
40. of anticoagulant in the sample. To identify possible false-negative results, it is recommended to spike an exogenous
41. (‘internal control’) RNA template into the specimen prior to RNA extraction (e.g. Hoffman *et al*., 2006). By the inclusion
42. of PCR primers and probe specific to the exogenous sequence, the efficiency of both the RNA extraction and also
43. the presence of any PCR inhibitors can be monitored. While valuable for all sample types, the inclusion of an internal
44. control is particularly desirable when testing semen and whole blood. When using an internal control, extensive testing
45. is necessary to ensure that PCR amplification of the internal control does not compete with the diagnostic PCR and
46. thus lower the analytical sensitivity (see also chapter 1.1.6).
47. When it is suspected that a sample may contain substances that are adversely affecting either the efficiency of RNA
48. extraction or the real-time RT-PCR assay, modest dilution of the sample in saline, cell culture medium or a buffer
49. solution (e.g. phosphate buffered gelatin saline [PBGS]) will usually overcome the problem. Dilution of a semen
50. sample by 1/4 and whole unclotted blood at 1/10 is usually adequate. As the real-time RT-PCR has extremely high
51. analytical sensitivity, dilution of the sample rarely has a significant impact on the capacity of the assay to detect viral
52. RNA when present.
53. 1.2.1. Real-time polymerase chain reaction for BVDV detection in semen
54. Real-time RT-PCR has been shown to be extremely useful to screen semen samples to demonstrate
55. freedom from BVDV and, apart from speed, often gives superior results to virus isolation in cell
56. culture, especially when low virus levels are present, such as may be found in bulls with a PTI. The
57. real-time RT-PCR described here uses a pair of sequence-specific primers for amplification of target
58. ~~D~~ RNA and a 5’-nuclease oligoprobe for the detection of amplified products. The oligoprobe is a
59. single, sequence-specific oligonucleotide, labelled with two different fluorophores. The primers and
60. probe are available commercially and several different fluorophores options are available. This pan-
61. pestivirus real-time RT-PCR assay is designed to detect viral ~~D~~ RNA of all strains of BVDV types 1
62. (*Pestivirus bovis*) ~~and BVDV~~, 2 (*Pestivirus tauri*) and 3 (*Pestivirus brazilense*) as well as ~~BDV,~~ CSFV
63. (*Pestivirus suis*), some strains of BDV (*Pestivirus ovis*) and most atypical pestiviruses. The assay
64. selectively amplifies a 208 base pair sequence of the 5’ non-translated region (5’ NTR) of the
65. pestivirus genome. Details of the primers and probes are given in the protocol outlined below.
66. i) Sample preparation, equipment and reagents
67. a) The samples used for the test are, typically, extended bovine semen or occasionally raw
68. semen. If the samples are only being tested by real-time RT-PCR, it is acceptable for them
69. to be submitted chilled but they must still be cold when they reach the laboratory.
70. Otherwise, if a cold chain cannot be assured or if virus isolation is being undertaken, the
71. semen samples should be transported to the laboratory in liquid nitrogen or on dry ice. At
72. the laboratory, the samples should be stored in liquid nitrogen or at lower than –70°C (for
73. long-term storage) or 4°C (for short-term storage of up to 7 days). *Note:* samples for virus
74. isolation should not be stored at 4°C for more than 1–2 days.
75. b) Due to the very high analytical sensitivity of real-time RT-PCR, much smaller volumes of
76. semen may be used. However, at least three straws (minimum 250 µl each) from each
77. collection batch of semen should be processed. The semen in the three straws should be
78. pooled and mixed thoroughly before taking a sample for nucleic acid extraction.
79. c) A real-time PCR detection system, and the associated data analysis software, is required
80. to perform the assay. A number of real-time PCR detection systems are available from
81. various manufacturers. ~~Other equipment required for the test includes a micro-centrifuge,~~
82. ~~a chilling block, a micro-vortex, and micropipettes.~~ As real-time RT-PCR assays are able
83. to detect very small amounts of target nucleic acid molecules, appropriate measures are
84. required to avoid contamination. ~~, including dedicated and physically separated ‘clean’~~
85. ~~areas for reagent preparation (where no samples or materials used for PCR are handled),~~
86. ~~a dedicated sample processing area and an isolated area for the PCR thermocycler and~~
87. ~~associated equipment. Each area should have dedicated reagents and equipment.~~
88. Furthermore, a minimum of one negative sample should be processed in parallel to
89. monitor the possibility of low level contamination. Sources of contamination may include
90. product carry-over from positive samples or, more commonly, from cross contamination
91. by PCR products from earlier work.
92. d) The real-time RT-PCR assay involves two separate procedures.
93. 1) Firstly, BVDV RNA is extracted from semen using an appropriate validated nucleic
94. acid extraction method. Systems using magnetic beads for the capture and
95. purification of the nucleic acid are recommended. It is also preferable that the beads
96. are handled by a semi-automated magnetic particle handling system.
97. 2) The second procedure is the RT-PCR analysis of the extracted RNA template in a
98. real-time RT-PCR system.
99. ii) Extraction of RNA
100. RNA or total nucleic acid is extracted from the pooled (three straws collected at the same time
101. from the same animal) semen sample. Use of a commercially available magnetic bead based
102. extraction kit is recommended. However, the preferred kit should be one that has been
103. evaluated to ensure optimal extraction of difficult samples (semen and whole blood). Some
104. systems and kit protocols are sufficiently refined that it is not necessary to remove cells from
105. the semen sample. Prior to extraction dilute the pooled semen sample 1/4 in ~~phosphate buffered~~
106. ~~gelatin saline (~~PBGS) or a similar buffered solution. Complete the RNA extraction by taking 50
107. µl of the diluted, pooled sample and add it to the sample lysis buffer. Some commercial
108. extraction kits may require the use of a larger volume. It has also been found that satisfactory
109. results are obtained by adding 25 µl of undiluted pooled sample to sample lysis buffer. Complete
110. the extraction by following the kit manufacturer’s instructions.
111. iii) Real-time RT-PCR assay procedure
112. a) Reaction mixture: There are a number of commercial real-time PCR amplification kits
113. available from various sources and the particular kits selected need to be compatible with
114. the real-time PCR platform selected. The required primers and probes can be synthesised
115. by various commercial companies. The WOAH Reference Laboratories for BVDV can
116. provide information on suitable suppliers.
117. b) Supply and storage of reagents: The real-time PCR reaction mixture is normally provided
118. as a 2 × concentration ready for use. The manufacturer’s instructions should be followed
119. for application and storage. Working stock solutions for primers and probe are made with
120. nuclease-free water at the concentration of 20 μM and 3 μM, respectively. The stock
121. solutions are stored at –20°C and the probe solution should be kept in the dark. Single-
122. use or limited use aliquots can be prepared to limit freeze–thawing of primers and probes
123. and extend their shelf life.
124. c) Primers and probe sequences
125. Selection of the primers and probe are outlined in Hoffmann *et al*. (2006) and summarised
126. below.

|  |  |  |
| --- | --- | --- |
| 577 | *Forward:* | BVD 190-F 5’-GRA-GTC-GTC-ART-GGT-TCG-AC |
| 578 | *Reverse:* | V326 5’-TCA-ACT-CCA-TGT-GCC-ATG-TAC |
| 579 | *Probe:* | TQ-pesti 5’-FAM-TGC-YAY-GTG-GAC-GAG-GGC-ATG-C-TAMRA-3’ |

580 d) Preparation of reaction mixtures

581 The PCR reaction mixtures are prepared in a separate room that is isolated from other

582 PCR activities and sample handling. For each PCR test, appropriate controls should be

583 included. As a minimum, a no template control (NTC), appropriate negative control (NC)

584 and two positive controls (PC1, PC2) should be included. The positive and negative

585 controls are included in all steps of the assay from extraction onwards while the NTC is

586 added after completion of the extraction. The PCR amplifications are carried out in a

587 volume of 25 μl. The protocol described is based on use of a 96 well microplate based

588 system but other options using microtubes are also suitable. Each well of the PCR plate

589 should contain 20 µl of reaction mix and 5 µl of sample as follows:

|  |  |  |
| --- | --- | --- |
| 590 | 12.5 µl | 2× RT buffer – from a commercial kit. |
| 591 | 1 µl | BVD 190-F Forward primer (20 µM) |
| 592 | 1 µl | V326 Reverse primer (20 µM) |
| 593 | 1 µl | TQ-pesti Probe (3 µM) |
| 594 | 2 µl | tRNA (40 ng/µl) |
| 595 | 1.5 µl | nuclease free water |
| 596 | 1 µl | 25× enzyme mix |
| 597 | 5 µl | sample (or controls – NTC, NC, PC1, PC2) |

1. e) Selection of controls
2. NTC: usually consists of nuclease free water or tRNA in nuclease free water that is added
3. in place of a sample when the PCR reaction is set up.
4. NC: In practice, many laboratories use PBGS or a similar buffer. Ideally the controls for
5. testing of semen samples should be negative semen, from seronegative bulls. However,
6. as a minimum, the assay in use should have been extensively validated with negative and
7. positive samples to confirm that it gives reliable extraction and amplification with semen.
8. PCs: There are two positive controls (PC1=moderate – [Ct 29-32] and PC2=weak [Ct 32–
9. 35] positive). Positive semen from naturally infected bulls is preferable as a positive
10. control. However, this is likely to be difficult to obtain. Further, semen from a PI bull is not
11. considered suitable because the virus loads are usually very high and would not give a
12. reliable indication of any moderate reduction in extraction or assay performance. Negative
13. semen spiked with defined quantities of BVDV virus could be used as an alternative. If
14. other samples are used as a routine PC, as a minimum the entire extraction process and
15. PCR assay in use must have been extensively validated using known positive semen from
16. bulls with a PTI or from bulls undergoing an acute infection. If these samples are not
17. available and spiked samples are used for validation purposes, a number of samples
18. spiked with very low levels of virus should be included. On a day to day basis, the inclusion
19. of an exogenous control with each test sample will largely compensate for not using
20. positive semen as a control and will give additional benefits by monitoring the efficiency of
21. the assay on each individual sample. Positive control samples should be prepared
22. carefully to avoid cross contamination from high titred virus stocks and should be prepared
23. in advance and frozen at a ‘ready to use’ concentration and ideally ‘single use’ volume.
24. f) Extracted samples are added to the PCR mix in a separate room. The controls should be
25. added last, in a consistent sequence in the following order: NTC, negative and then the
26. two positive controls.
27. g) Real-time polymerase chain reaction
28. The PCR plate or tubes are placed in the real-time PCR detection system in a separate,
29. designated PCR room. Some mastermixes have uniform reaction conditions that are
30. suitable for many different assays. As an example, the PCR detection system is
31. programmed for the test as follows:
32. 1× 48°C 10 minutes
33. 1× 95°C 10 minutes
34. 45 × (95°C 15 seconds, 60°C 1 minute)
35. h) Analysis of real-time PCR data
36. The software program is usually set to automatically adjust results by compensating for any
37. background signal and the threshold level is usually set according to the manufacturer’s
38. instructions for the selected analysis software used. In this instance, a threshold is set at 0.05.
39. i) Interpretation of results
40. a) Test controls – all controls should give the expected results with positive controls (PC1
41. and PC2) falling within the designated range and both the negative control (NC) and no
42. template control (NTC) should have no Ct values.
43. b) Test samples
44. 1) Positive result: Any sample that has a cycle threshold (Ct) value less than 40 is
45. regarded as positive.
46. 2) Negative result: Any sample that shows no Ct value is regarded as negative. However,
47. before reporting a negative result for a sample, the performance of the exogenous
48. internal control should be checked and shown to give a result within the accepted range
49. for that control (for example, a Ct value no more than 2–3 Ct units higher than the
50. NTC).

##### 1.3. Enzyme-linked immunosorbent assay for antigen detection

1. Antigen detection by ELISA has become a widely adopted method for the detection of individual PI animals. These
2. assays are not intended for the detection of acutely infected animals (though from to time this may be achieved).
3. Importantly, these assays are not designed for screening of semen or biological materials used in assays or vaccine
4. manufacture. Several methods for the ELISA for antigen detection have been published and a number of commercial
5. kits are available. Most are based on the sandwich ELISA principle, with a capture antibody bound to the solid phase,
6. and a detector antibody conjugated to a signal system, such as peroxidase. Amplification steps such as the use of
7. biotin and streptavidin in the detection system are sometimes used to increase assay sensitivity. Both monoclonal-
8. and polyclonal-based systems are described. The test measures BVD antigen (NS2-3 or ERNS) in lysates of peripheral
9. blood leukocytes; the new generation of antigen-capture ELISAs (ERNS capture ELISAs) are able to detect BVD
10. antigen in blood as well as in plasma or serum samples. The best of the methods gives a sensitivity similar to virus
11. isolation, and may be preferred in those rare cases where persistent infection is combined with seropositivity. Due to
12. transient viraemia, the antigen ELISA is less useful for virus detection in acute BVD infections.
13. The ~~NS2-3~~ antigen detection ELISAs may be less effective in young calves that have had colostrum due to the
14. presence of BVDV maternal antibodies, especially when blood samples or blood leucocytes are tested (Fux & Wolf,
15. 2012). Blood or blood leucocytes should not be tested in the first month (ERNS capture ELISA) or the first 3 months
16. (NS2-3 ELISA) of life due to the inhibitory effect of maternal antibodies. The real-time RT-PCR is probably the most
17. sensitive detection method for this circumstance, but the ERNS ELISA has also been shown to be a sensitive and
18. reliable test, ~~particularly~~ when used with skin biopsy (ear-notch) samples (Cornish *et al*., 2005).

##### 1.4. Immunohistochemistry

1. Enzyme-labelled methods are useful to detect BVDV antigen in tissue sections, particularly where suitable MAbs are
2. available. However, these assays are not appropriate to certify animals for international trade and use should be
3. limited to diagnostic investigations. It is important that the reagents and procedures used be fully validated, and that
4. nonspecific reactivity be eliminated. For PI cattle almost any tissue can be used, but particularly good success has
5. been found with lymph nodes, thyroid gland, skin, brain, abomasum and placenta. Skin biopsies, such as ear-notch
6. samples, have shown to be useful for *in-vivo* diagnosis of persistent BVDV infection.

#### 2. Serological tests

1. Antibody to BVDV can be detected in cattle sera by a standard VNT or by ELISA, using one of several published methods
2. or with commercial kits (e.g. Edwards, 1990). Serology is used to identify levels of herd immunity, for the detection of the
3. presence of PI animals in a herd, to assist with investigation of reproductive disease and possible involvement of BVDV
4. and to establish the serological status of bulls being used for semen collection and to identify whether there has been a
5. recent infection. ELISA for antibody in bulk milk samples can give a useful indication of the BVD status of a herd (Niskanen,
6. 1993). High ELISA values ~~(0.8 or more absorbance units)~~ in an unvaccinated herd indicates a high probability of the herd
7. having been exposed to BVDV in the recent past, most likely through one or more persistently viraemic animals being
8. present. In contrast, ~~a~~ very low or negative values ~~(≤0.2)~~ indicate~~s~~ that it is unlikely that persistently viraemic animals are
9. present. However, ELISA values are not always a reliable indicator of the presence of PI animals on farms, due to differing
10. husbandry (Zimmer *et al*., 2002), recent administration of vaccine and also due to the presence of viral antigen in bulk milk,
11. which may interfere with the antibody assay itself. Determination of the antibody status of a small number of young stock
12. (9–18 months) has also been utilised as an indicator of recent transmission of BVDV in the herd (Houe *et al*., 1995), but
13. this approach is also dependent on the degree of contact between different groups of animals in the herd and the potential
14. for exposure from neighbouring herds. VN tests are more frequently used for regulatory purposes (e.g. testing of semen
15. donors) while ELISAs (usually in the form of commercially prepared kits) are commonly used for diagnostic applications.
16. Whether ELISA or VNT, control positive and negative standard sera must be included in every test. These should give
17. results within predetermined limits for the test to be considered valid. In the VNT, a ‘serum control’ to monitor sample
18. toxicity should also be included for each test sample.

##### 2.1. Virus neutralisation test

1. Selection of the virus strain to include in a VNT is very important. No single strain is likely to be ideal for all
2. circumstances, but in practice one should be selected that detects the highest proportion of serological reactions in
3. the local cattle population. Low levels of antibody to BVD type 2 virus (*Pestivirus tauri*) may not be detectable by a
4. neutralisation test that uses type 1 strain of the virus, and vice versa (Fulton *et al*., 1997). It is important that BVD
5. type 1 and BVD type 2 (*Pestivirus bovis* and *tauri*) be used in the test and not just the one that the diagnostician
6. thinks is present, as this can lead to under reporting. Because it makes the test easier to read, most laboratories use
7. highly cytopathic, laboratory-adapted strains of BVDV for VN tests. Two widely used cytopathic strains are ‘Oregon
8. C24V’ and ‘NADL’. However immune-labelling techniques are now available that allow simple detection of the growth
9. or neutralisation of non-cytopathic strains where this is considered desirable, especially to support the inclusion of a
10. locally relevant virus strain. An outline protocol for a microtitre VN test is given below (Edwards, 1990):
11. 2.1.1. Test procedure
12. i) The test sera are heat-inactivated for 30 minutes at 56°C.
13. ii) From a starting dilution of 1/4, serial twofold dilutions of the test sera are made in a cell-culture
14. grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each
15. sample, three or four wells are used at each dilution depending on the degree of precision
16. required. At each dilution of serum, for each sample one well is left without virus to monitor for
17. evidence of sample toxicity that could mimic viral cytopathology or interfere with virus
18. replication. Control positive and negative sera should also be included in each batch of tests.
19. iii) An equal volume (e.g. 50 μl) of a stock of cytopathic strain of BVDV containing 100 TCID50 (50%
20. tissue culture infective dose) is added to each well. A back titration of virus stock is also done
21. in some spare wells to check the potency of the virus (acceptance limits 30–300 TCID50).
22. iv) The plate is incubated for 1 hour at 37°C.
23. v) A flask of suitable cells (e.g. bovine turbinate, bovine testis) is trypsinised and the cell
24. concentration is adjusted to 1.5 × 105/ml. 100 μl of the cell suspension is added to each well of
25. the microtitre plate.
26. vi) The plate is incubated at 37°C for 4–5 days, either in a 5% CO2 atmosphere or with the plate
27. sealed.
28. vii) The wells are examined microscopically for CPE or fixed and stained by immunoperoxidase
29. staining using an appropriate monoclonal antibody. The VN titre for each serum is the dilution
30. at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman–
31. Kärber or Reed Muench methods. A seronegative animal will show no neutralisation at the
32. lowest dilution (1/4), equivalent to a final dilution of 1/8. For accurate comparison of antibody
33. titres, and particularly to demonstrate significant (more than fourfold) changes in titre, samples
34. should be tested in parallel in the same test.

##### 2.2. Enzyme-linked immunosorbent assay

1. Both indirect and blocking types of test can be used. A number of commercial kits are available. As with the virus
2. neutralisation test, ELISAs configured using antigen from one ~~genotype~~ species of BVDV may not efficiently detect
3. antibody induced by another ~~genotype~~ virus species. Tests should therefore be selected for their ability to detect
4. antibody to the spectrum of types and strains circulating in the country where the test is to be performed.
5. The chief difficulty in setting up the test lies in the preparation of a viral antigen of sufficient potency. The virus must
6. be grown under optimal culture conditions using a highly permissive cell type. Any serum used in the medium must
7. not inhibit growth of BVDV. The optimal time for harvest should be determined experimentally for the individual culture
8. system. The virus can be concentrated and purified by density gradient centrifugation. Alternatively, a potent antigen
9. can be prepared by treatment of infected cell cultures with detergents, such as Nonidet P40, N-decanoyl-N-
10. methylglucamine (Mega 10), Triton X-100 or 1-octylbeta-D-glucopyranoside (OGP). Some workers have used fixed,
11. infected whole cells as antigen. ~~In the future,~~ Increasing use ~~may be~~ is made of artificial antigens manufactured by
12. expressing specific viral genes in bacterial or eukaryotic systems. Such systems should be validated by testing sera
13. specific to a wide range of different virus strains. In the future, this technology should enable the production of
14. serological tests complementary to subunit or marker vaccines, thus enabling differentiation between vaccinated and
15. naturally infected cattle. An example outline protocol for an indirect ELISA is given below (Edwards, 1990).
16. 2.2.1. Test procedure
17. i) Roller cultures of secondary calf testis cells with a high multiplicity of infection (about one), are
18. inoculated with BVDV strain Oregon C24V, overlaid with serum-free medium and incubated for
19. 24 hours at 37°C.
20. ii) The cells are scraped off and pelleted. The supernatant medium is discarded. The pellet is
21. treated with two volumes of 2% OGP in PBS for 15 minutes at 4°C, and centrifuged to remove
22. the cell debris. The supernatant antigen is stored in small aliquots at –70°C, or freeze-dried.
23. Non-infected cells are processed in parallel to make a control antigen.
24. iii) The antigen is diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6. Alternate
25. rows of an ELISA-grade microtitre plate are coated with virus and control antigens overnight at
26. 4°C. The plates are then washed in PBS with 0.05% Tween 20 or Tween 80 (PBST) before use
27. in the test.
28. iv) Test sera are diluted 1/50 in serum diluent (0.5 M NaCl; 0.01 M phosphate buffer; 0.05% Tween
29. 20; 0.001 M ethylene diamine tetra-acetic acid; 1% polyvinyl pyrrolidone, pH 7.2) and added to
30. virus- and control-coated wells for 1 hour at 37°C. The plates are then washed five times in
31. PBST.
32. v) Rabbit anti-bovine IgG peroxidase conjugate is added at a predetermined dilution (in serum
33. diluent) for 1 hour at 37°C, then the plates are again washed five times in PBST.
34. vi) A suitable enzyme substrate is added, such as hydrogen peroxide/tetramethyl benzidine. After
35. colour development, the reaction is stopped with sulphuric acid and the absorbance is read on
36. an ELISA plate reader. The value obtained with control antigen is subtracted from the test
37. reaction to give a net absorbance value for each serum.
38. vii) It is recommended to convert net absorbance values to sample:positive ratio (or percentage
39. positivity) by dividing net absorbance by the net absorbance on that test of a standard positive
40. serum that has a net absorbance of about 1.0. This normalisation procedure leads to more
41. consistent and reproducible results.

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#### 1. Background

## C. REQUIREMENTS FOR VACCINES

1. BVDV vaccines are used primarily for disease control purposes. Although they can convey production advantages
2. especially in intensively managed cattle such as in feedlots. In some countries where BVDV eradication is being
3. undertaken, PI animals are removed and remaining cattle are vaccinated to maintain a high level of ~~infection~~ antibody
4. positivity and prevent the generation of further PI animals. Vaccination to control BVDV infections can be challenging due
5. in part to the antigenic variability of the virus and the occurrence of persistent infections that arise as a result of fetal
6. infection. Ongoing maintenance of the virus in nature is predominantly sustained by PI animals that are the product of *in-*
7. *utero* infection. The goal for a vaccine should be to prevent systemic viraemia and the virus crossing the placenta. If this
8. is successfully achieved it is likely that the vaccine will prevent the wide range of other clinical manifestations, including
9. reproductive, respiratory and enteric diseases and immunosuppression with its secondary sequelae. There are many
10. different vaccines available in different countries. Traditionally, BVD vaccines fall into two classes: modified live virus or
11. inactivated vaccines. Experimental recombinant subunit vaccines based on BVD viral glycoprotein E2 expressed with
12. baculovirus, ~~or~~ transgenic plants or heterologous viruses and BVDV E2 DNA vaccines have been described but few, if
13. any, are in commercial production. They offer a future prospect of ‘marker vaccines’ when used in connection with a
14. complementary serological test.

##### 1.1. Characteristics of a target product profile

1. Traditionally, BVD vaccines fall into two classes: modified live or inactivated virus vaccines. The essential requirement
2. for both types is to ~~afford~~ provide a high level of fetal ~~infection~~ protection. Many of the live vaccines have been based
3. on a cytopathic strain of the virus which is considered to be unable to cross the placenta. However, it is important to
4. ensure that the vaccine virus does not cause fetal infection. In general, vaccination of breeding animals should be
5. completed well before insemination to ensure optimal protection and avoid any risk of fetal infection. Live virus vaccine
6. may also be immunosuppressive and precipitate other infections. On the other hand, modified live virus vaccines may
7. only require a single dose. Use of a live product containing a cytopathic strain of BVDV may precipitate mucosal
8. disease by superinfection of persistently viraemic animals. Properly formulated inactivated vaccines are very safe to
9. use but, to obtain satisfactory levels of immunity, they usually require booster vaccinations, which may be
10. inconvenient. A combined vaccination protocol using inactivated followed by live vaccine may reduce the risk of
11. adverse reaction to the live strain. Whether live or inactivated, because of the propensity for antigenic variability, the
12. vaccine should contain strains of BVDV that are closely matched to viruses found in the area in which they are used.
13. For example, in countries where strains of BVDV type 2 (*Pestivirus tauri*) are found, it is important for the vaccine to
14. contain a suitable type 2 strain. For optimal immunity against type 1 strains (*Pestivirus bovis*), antigens from the
15. dominant subtypes (e.g. 1a and 1b) should be included. Due to the need to customise vaccines for the most commonly
16. encountered strains within a country or region, it is not feasible to produce a vaccine antigen bank that can be drawn
17. upon globally.
18. Guidance for the production of veterinary vaccines is given in Chapter 1.1.8 *Principles of veterinary vaccine*
19. *production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be
20. supplemented by national and regional requirements.

#### 2. Outline of production and minimum requirements for vaccines

##### 2.1. Characteristics of the seed

1. For optimal efficacy, it is considered that there should be a close antigenic match between viruses included in a
2. vaccine and those circulating in the target population. BVDV type 2 strains (*Pestivirus tauri*) should be included as
3. appropriate. Due to the regional variations in ~~genotypes~~ species and subtypes of BVDV, many vaccines contain more
4. than one strain of BVDV to give acceptable protection. A good appreciation of the antigenic characteristics of
5. individual strains can be obtained by screening with panels of MAbs (Paton *et al*., 1995).
6. 2.1.1. Biological characteristics of the master seed
7. Isolates of cytopathic virus are often mixed with the noncytopathic biotype. The separation and
8. purification of the two biotypes from an initial mixed culture is important to maintain the expected
9. characteristics of the ~~seen~~ seed and depends on several cycles of a limiting dilution technique for the
10. noncytopathic virus, or plaque selection for the cytopathic virus. Purity of the cytopathic virus should
11. be confirmed by at least one additional passage at limiting dilution. When isolates have been cloned,
12. their identity and key antigenic characteristics should be confirmed. The identity of the seed virus
13. should be confirmed by sequencing. Where there are multiple isolates included in the vaccine, each
14. has to be prepared separately.
15. While retaining the desirable antigenic characteristics, the strains selected for the seed should not
16. show any signs of disease when susceptible animals are vaccinated. Live attenuated vaccines should
17. not be transmissible to unvaccinated ‘in-contact’ animals and should not be able to infect the fetus.
18. Ideally seeds prepared for the production of inactivated vaccines should grow to high titre to minimise
19. the need to concentrate the antigens and there should be a minimal amount of protein from the cell
20. cultures incorporated into the final product. Master stocks for either live or inactivated vaccines should
21. be prepared under a seed lot system involving master and working stocks that can be used for
22. production in such a manner that the number of passages can be limited and minimise antigenic drift.
23. While there are no absolute criteria for this purpose, as a general guide, the seed used for production
24. should not be passaged more than 20 times beyond the master seed and the master seed should be
25. of the lowest passage from the original isolate as is practical.
26. 2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)
27. It is crucial to ensure that all materials used in the preparation of the bulk antigens have been
28. extensively screened to ensure freedom from extraneous agents. This should include master and
29. working seeds, the cell cultures and all medium supplements such as bovine serum. It is particularly
30. important to ensure that any serum used that is of bovine origin is free of both adventitious BVDV of
31. all ~~geno~~types and antibodies against BVDV strains because low levels of either virus or antibody can
32. mask the presence of the other. Materials and vaccine seeds should be tested for sterility and
33. freedom from contamination with other agents, especially viruses as described in the chapter 1.1.8
34. and chapter 1.1.9.
35. 2.1.3. Validation as a vaccine strain
36. All vaccines should pass standard tests for efficacy. Tests should include as a minimum the
37. demonstration of a neutralising antibody response following vaccination, a reduction in virus shedding
38. after challenge in vaccinated cattle and ideally a prevention of viraemia. Efficacy tests of BVD
39. vaccines by assessing clinical parameters in non-pregnant cattle can be limited by the difficulty of
40. consistently establishing clinical signs but, when employed, clinical parameters such as a reduction
41. in the rectal temperature response and leukopenia should be monitored. Although it can be difficult
42. by using virus isolation in cell culture to consistently demonstrate the low levels of viraemia associated
43. with an acute infection, real-time PCR could be considered as an alternative method to establish the
44. levels of circulating virus.
45. If a vaccine passes basic tests, the efficacy of vaccination should ultimately be measured by the
46. capacity to prevent transplacental transmission. If there is a substantial reduction and ideally
47. complete prevention of fetal infection, a vaccine would be expected to be highly effective in other
48. situations (for example prevention of respiratory disease). A suitable challenge system can be
49. established by intranasal inoculation of noncytopathic virus into pregnant cows between 60 and 90
50. days of gestation (Brownlie *et al*., 1995). Usually this system will reliably produce persistently viraemic
51. offspring in non-immune cows. In countries where BVDV type 2 viruses (*Pestivirus tauri*) are
52. commonly encountered, efficacy in protecting against BVDV type 2 infections should be measured.

##### 2.2. Method of manufacture

1. 2.2.1. Procedure
2. Both cytopathic and noncytopathic biotypes will grow in a variety of cell cultures of bovine origin.
3. Standard procedures may be used, with the expectation for harvesting noncytopathic virus on days
4. 4–7 and cytopathic virus on days 2–4. The optimal yield of infectious virus will depend on several
5. factors, including the cell culture, isolate used and the initial seeding rate of virus. These factors
6. should be taken into consideration and virus replication kinetics investigated to establish the optimal
7. conditions for large scale virus production. Whether a live or inactivated vaccine, the essential aim
8. will be to produce a high-titred virus stock. This bulk antigen preparation can subsequently be
9. prepared according to the type of vaccine being considered.
10. 2.2.2. Requirements for ingredients
11. Most BVDV vaccines are grown in cell cultures of bovine origin that are frequently supplemented with
12. medium components of animal origin. The material of greatest concern is bovine serum due to the
13. potential for contamination with BVD viruses and antibodies to these viruses. These adventitious
14. contaminants not only affect the efficiency of production but also may mask the presence of low levels
15. of infectious BVDV that may have undesirable characteristics. In addition to the virus seeds, all
16. materials should be tested for sterility and freedom from contamination with other agents, especially
17. viruses as described in chapters 1.1.8 and 1.1.9. Further, materials of bovine or ovine origin should
18. originate from a country with negligible risk for transmissible spongiform encephalopathies [TSEs]
19. (see chapter 1.1.9).
20. 2.2.3. In-process controls
21. In-process controls are part of the manufacturing process. Cultures should be inspected regularly to
22. ensure that they remain free from contamination, and to monitor the health of the cells and the
23. development or absence of CPE, as appropriate. While the basic requirement for efficacy is the
24. capacity to induce an acceptable neutralising antibody response, during production, target
25. concentrations of antigen required to achieve an acceptable response may be monitored indirectly
26. by assessment of the quantity of infectious virus or antigen mass that is produced. Rapid diagnostic
27. assays such as the ELISA are useful to monitor BVDV antigen production. Alternatively, the quality
28. of a batch of antigen may be determined by titration of the quantity of infectious virus present,
29. although this may underestimate the quantity of antigen. For inactivated vaccines, infectivity is
30. evaluated before inactivation. For inactivated vaccines the inactivation kinetics should be established
31. so that a suitable safety margin can be determined and incorporated into the routine production
32. processes. At the end of production, *in-vitro* cell culture assays should be undertaken to confirm that
33. inactivation has been complete. These innocuity tests should include a sufficient number of passages
34. and volume of inoculum to ensure that very low levels of infectious virus would be detected if present.
35. 2.2.4. Final product batch tests
36. i) Sterility
37. Tests for sterility and freedom from contamination of biological materials intended for veterinary use
38. may be found in Chapter 1.1.9.
39. ii) Identity
40. Identity tests should demonstrate that no other strain of BVDV is present when several strains are
41. propagated in a facility producing multivalent vaccines.
42. iii) Safety
43. Safety tests shall consist of detecting any abnormal local or systemic adverse reactions to the vaccine
44. by all vaccination route(s). Batch-to-batch safety tests are required unless safety of the product is
45. demonstrated and APPROVED in the registration dossier and production is consistent with that
46. described in chapter 1.1.8.
47. The safety test is different to the inocuity test (see above).
48. Live vaccines must either be demonstrated to be safe in pregnant cattle (i.e. no transmission to the
49. fetus), or should be licensed with a warning not to use them in pregnant animals. Live vaccines
50. containing cytopathic strains should have an appropriate warning of the risk of inducing mucosal
51. disease in PI cattle.
52. iv) Batch potency
53. BVD vaccines must be demonstrated to produce adequate immune responses, when used in their
54. final formulation according to the manufacturer’s published instructions. The minimum quantity of
55. infectious virus and/or antigen required to produce an acceptable immune response should be
56. determined. *In-vitro* assays should be used to monitor individual batches during production.

##### 2.3. Requirements for authorisation/registration/licensing

1. 2.3.1. Manufacturing process
2. For registration of a vaccine, all relevant details concerning manufacture of the vaccine and quality
3. control testing should be submitted to the relevant authorities. Unless otherwise specified by the
4. authorities, information should be provided from three consecutive vaccine batches with a volume
5. not less than 1/3 of the typical industrial batch volume.
6. There is no standard method for the manufacture of a BVD vaccine, but conventional laboratory
7. techniques with stationary, rolled or suspension (micro-carriers) cell cultures may be used.
8. Inactivated vaccines can be prepared by conventional methods, such as binary ethylenimine or beta-
9. propiolactone inactivation (Park & Bolin, 1987). A variety of adjuvants may be used.
10. 2.3.2. Safety requirements
11. *In-vivo* tests should be undertaken using a single dose, overdose (for live vaccines only) and repeat
12. doses (taking into account the maximum number of doses for primary vaccination and, if appropriate,
13. the first revaccination/booster vaccination) and contain the maximum permitted antigen load and,
14. depending on the formulation of the vaccine, the maximum number of vaccine strains.
15. i) Target and non-target animal safety
16. The safety of the final product formulation of both live and inactivated vaccines should be assessed
17. in susceptible young calves that are free of maternally derived antibodies and in pregnant cattle. They
18. should be checked for any local reactions following administration, and, in pregnant cattle, for any
19. effects on the unborn calf. Live attenuated vaccines may contribute to immunosuppression that might
20. increase mortality. It may also contribute to the development of mucosal disease in PI animals that
21. is an animal welfare concern. Therefore vaccination of PI animals with live attenuated vaccines
22. containing cytopathic BVDV should be avoided. Live attenuated vaccines must not be capable of
23. being transmitted to other unvaccinated animals that are in close contact.
24. ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations
25. Virus seeds that have been passaged at least up to and preferably beyond the passage limit specified
26. for the seed should be inoculated into young calves to confirm that there is no evidence of disease.
27. If a live attenuated vaccine has been registered for use in pregnant animals, reversion to virulence
28. tests should also include pregnant animals. Live attenuated vaccines should not be transmissible to
29. unvaccinated ‘in-contact’ animals.
30. iii) Precautions (hazards)
31. BVDV is not considered to be a human health hazard. Standard good microbiological practice should
32. be adequate for handling the virus in the laboratory. A live virus vaccine should be identified as
33. harmless for people administering the product. However adjuvants included in either live or
34. inactivated vaccines may cause injury to people. Manufacturers should provide adequate warnings
35. that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion
36. vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator
37. is aware of any danger.
38. 2.3.3. Efficacy requirements
39. The potency of the vaccine should be determined by inoculation into seronegative and virus negative
40. calves, followed by monitoring of the antibody response. Antigen content can be assayed by ELISA
41. and adjusted as required to a standard level for the particular vaccine. Standardised assay protocols
42. applicable to all vaccines do not exist. Live vaccine batches may be assayed by infectivity titration.
43. Each production batch of vaccine should undergo potency and safety testing as batch release criteria.
44. BVD vaccines must be demonstrated to produce adequate immune responses, as outlined above,
45. when used in their final formulation according to the manufacturer’s published instructions.

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| 964 | **2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)** |
| 965 | To date, there are no commercially available vaccines for BVDV that support use of a true DIVA |
| 966 | strategy. Experimental subunit vaccines based on baculovirus-expressed BVD viral glycoprotein E2 |
| 967 | have been described but are not available commercially. They offer a future prospect of ‘marker |
| 968 | vaccines’ when used in connection with a complementary serological test. Experimental BVDV E2 |
| 969 | DNA vaccines and BVDV E2 subunit vaccines expressed using transgenic plants and alphavirus |
| 970 | replicon or chimeric pestivirus vaccines have also been described. |
| 971 | **2.3.5. Duration of immunity** |
| 972 | There are few published data on the duration of antibody following vaccination with a commercial |
| 973 | product. Protocols for their use usually recommend a primary course of two inoculations and boosters |
| 974 | at yearly intervals. Only limited data are available on the antibody levels that correlate with protection |
| 975 | against respiratory infections (Bolin & Ridpath, 1995; Howard *et al*., 1989) or *in-utero* infection |
| 976 | (Brownlie *et al*., 1995). However, there are many different commercial formulations and these involve |
| 977 | a range of adjuvants that may support different periods of efficacy. Consequently, duration of |
| 978 | immunity data must be generated separately for each commercially available product by undertaking |
| 979 | challenge tests at the end of the period for which immunity has been claimed. |
| 980 | **2.3.6. Stability** |
| 981 | There are no accepted guidelines for the stability of BVD vaccines, but it can be assumed that |
| 982 | attenuated virus vaccine (freeze-dried) should remain potent for at least 1 year if kept at 4°C. |
| 983 | Inactivated virus vaccine could have a longer shelf life at 4°C. Lower temperatures could prolong |
| 984 | shelf life for either type, but adjuvants in killed vaccine may preclude this. Bulk antigens that have not |
| 985 | been formulated into finished vaccine can be reliably stored frozen at low temperatures but the |
| 986 | antigen quality should be monitored with *in-vitro* assays prior to incorporation into a batch of vaccine. |
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| 1059 | **NB:** There are WOAH Reference Laboratories for bovine viral diarrhoea (please consult the WOAH Web site: |
| 1060 | <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>) |
| 1061 | Please contact the WOAH Reference Laboratories for any further information on |
| 1062 | diagnostic tests, reagents and vaccines for bovine viral diarrhoea |
| 1063 | **NB:** First adopted in 1990. Most recent updates adopted in 2015. |