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Review

# Bovine viral diarrhoea: Pathogenesis and diagnosis

Sasha R. Lanyon [a,*], Fraser I. Hill [b], Michael P. Reichel [a], Joe Brownlie [c]

[a] University of Adelaide, School of Animal and Veterinary Sciences, Roseworthy Campus, Roseworthy, South Australia 5371, Australia
[b] Gribbles Veterinary, 840 Tremaine Ave, Palmerston North 4440, New Zealand
[c] Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire AL97TA, United Kingdom

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ABSTRACT

Bovine viral diarrhoea virus (BVDV) is the most prevalent infectious disease of cattle. It causes financial losses from a variety of clinical manifestations and is the subject of a number of mitigation and eradication schemes around the world. The pathogenesis of BVDV infection is complex, with infection pre- and post-gestation leading to different outcomes. Infection of the dam during gestation results in fetal infection, which may lead to embryonic death, teratogenic effects or the birth of persistently infected (PI) calves. PI animals shed BVDV in their excretions and secretions throughout life and are the primary route of transmission of the virus. These animals can usually be readily detected by virus or viral antigen detection assays (RT-PCR, ELISA), except in the immediate post-natal period where colostral antibodies may mask virus presence. PI calves in utero (the 'Trojan cow' scenario) currently defy detection with available diagnostic tests, although dams carrying PI calves have been shown to have higher antibody levels than seropositive cows carrying non-PI calves.

Acute infection with BVDV results in transient viraemia prior to seroconversion and can lead to reproductive dysfunction and immunosuppression leading to an increased incidence of secondary disease. Antibody assays readily detect virus exposure at the individual level and can also be used in pooled samples (serum and milk) to determine herd exposure or immunity. Diagnostic tests can be used to diagnose clinical cases, establish disease prevalence in groups and detect apparently normal but persistently infected animals. This review outlines the pathogenesis and pathology of BVD viral infection and uses this knowledge to select the best diagnostic tests for clinical diagnosis, monitoring, control and eradication efforts. Test methods, types of samples and problems areas of BVDV diagnosis are discussed.



## Introduction

Bovine viral diarrhoea virus (BVDV), a Pestivirus of the family Flaviviridae (Becher and Thiel, 2011), is capable of causing serious clinical disease in cattle. The virus is divided into two genotypes (BVDV-1 and BVDV-2) on the basis of antigenic and genetic differences (Vilcek et al., 2005). Infection with BVDV is known to have a significant financial impact (Houe, 1999), stemming primarily from the reproductive and immunosuppressive effects of acute infection. As such, control and eradication programs are becoming increasingly common across much of the cattle-producing world (Lindberg and Alenius, 1999; Houe et al., 2006), including legislated, regional schemes and voluntary, herd-based schemes.

While healthy, immunocompetent cattle (or late-term, immunocompetent fetuses) may suffer from acute BVDV infection resulting in seroconversion, the disease is primarily spread and maintained in cattle populations by persistently infected (PI) individuals. Persistency of infection arises from fetal infection in early gestation (Grooms, 2004), following acute infection in the dam. The majority of control programs aim to eliminate PI cattle (Lindberg and Alenius, 1999), and hence the source of continuing infection. This requires the efficient and effective use of accurate diagnostic tests, and a wide range is readily available for the detection of BVDV virus, antigen (Ag) or specific antibodies (Ab). These tests will return varying results depending on the current or historical BVDV infection status. Animals that have never been exposed to the virus will test negative for Ab, Ag, and virus. Animals or late-term, immunocompetent fetuses that have experienced an acute infection will test Ab positive and, generally, Ag or virus negative, while PI individuals will return a positive Ag or virus test and negative Ab result. However, as the range of tests available continues to grow, the challenge to BVDV diagnosis and control is the selection of the appropriate test for varying situations and goals.

## Pathogenesis

BVDV infection has a wide range of clinical presentations and unique diagnostic challenges. In order to select an appropriate

* Corresponding author. Tel.: +61 408 602 774.
*E-mail address:* sasha.lanyon@adelaide.edu.au (S.R. Lanyon).

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diagnostic test, it is important first to understand the pathogenesis of the disease.

BVDV is divided into non-cytopathogenic (ncp) and cytopathogenic (cp) biotypes based on effects on cultured cells rather than in the infected host. Cp biotypes induce apoptosis in cultured cells (Gamlen et al., 2010), while ncp biotypes do not. Non-cytopathogenic BVDV, however, appears to be the cause of acute infections and can be transmitted in a wide range of body fluids, including nasal discharge, urine, milk, semen, saliva, tears and fetal fluids (Meyling et al., 1990). Faeces are a poor source of virus (Brownlie et al., 1987). Cp BVDV has been shown to be capable of inducing acute infection under experimental conditions (Lambot et al., 1997). The most important source of ncp BVDV infection is PI cattle (Brownlie, 1990).

### *Acute infections*

In non-pregnant non-immune cattle, acute infections with ncp BVDV result in transient viraemia (Howard, 1990), beginning on day 3 post-infection (Pedrera et al., 2011) until immunity develops, usually about 2 weeks later (Meyling et al., 1990). Nose-to-nose or sexual contact with persistently infected cattle is the most common means of spread of infection between animals, though acutely infected animals, flies, aerosolised virus and contaminated veterinary equipment or pens have also been implicated (Gunn, 1993; Niskanen and Lindberg, 2003).

CD46 has been shown to be the receptor on macrophage and lymphocyte host cell membranes where BVDV gains entry (Maurer et al., 2004). Infection of a BVDV naïve animal results in a transient viraemia of 10–14 days' duration (Howard, 1990). This can be associated with short-term leukopenia (Muller-Doblies et al., 2004), lymphopenia (Ridpath et al., 2007) and/or thrombocytopenia (Marshall et al., 1996; Blanchard et al., 2010), apoptosis in the thymus (Raya et al., 2012), immunosuppression (Wilhelmsen et al., 1990), pyrexia (Baker, 1995) and diarrhoea (Brownlie et al., 1987). The resultant immunosuppression, in turn, can allow other infectious agents to become established, or allow the recrudescence of existing infections (Potgieter, 1995). Respiratory disease is exacerbated by BVDV infection (Fulton et al., 2000) and abortion has been associated with BVDV and *Neospora caninum* co-infection (Björkman et al., 2000; Quinn et al., 2004). Immunosuppression is associated with direct effects of BVDV on circulating T and B lymphocytes (Bolin et al., 1985; Chase, 2013) and apoptosis of lymphocytes in gut associated lymphoid tissue (Pedrera et al., 2012).

Experimental nasal infection of healthy calves with ncp BVDV resulted in localisation of virus in enterocytes, Peyer's patches, thymus, spleen lymph nodes (Liebler-Tenorio et al., 1997), tonsils and liver (Pedrera et al., 2011), in decreasing order of concentration. Liebler-Tenorio et al. (2004) reported that as virus began to be cleared about 6 days post-infection there was marked lymphocyte apoptosis, and that T cell mediated destruction of lymphocytes infected with virus resulted in clearance of virus by 9–13 days post-infection. Apoptosis of lymphocytes (Wilhelmsen et al., 1990) and suppression of phagocytic function of macrophages (Marshall et al., 1996) via inactivation of caspase-9 (Pedrera et al., 2012) in lymph nodes, bronchiole-associated and gut-associated lymphoid tissue decreases the capacity of the immune system to respond to other infectious agents.

BVDV infection of the myenteric and submucosal ganglia of the gastrointestinal tract and interruption of normal intestinal neural function may be the cause of diarrhoea observed in some acutely infected cattle (Wilhelmsen et al., 1990). Acutely affected cattle may recover completely in 3 weeks (Muller-Doblies et al., 2004) if other infections are not superimposed. BVDV infected, recovered and immune cattle can, however, continue to carry virus in peripheral blood mononuclear cells for 98 days or more and transfer of infection seems possible experimentally (Collins et al., 2009) although natural transfer of infection seems unlikely.

### *Effects on fertility*

Reproductive effects of acute BVDV infection include reduced conception rates (McGowan et al., 1993), early embryonic death (McGowan et al., 1993), abortions and congenital defects (Sprecher et al., 1991). Acute infection of sexually active bulls results in reduction in sperm density and motility, plus an increase in sperm abnormalities (Paton et al., 1989). In vitro studies confirmed that ncp BVDV incubated with sperm and oocytes significantly lowered fertilisation rates (Garoussi and Mehrzad, 2011). Research has also shown that BVDV can persist in semen for 2.75 years after acute infection although transmission to susceptible animals did not ensue (Givens et al., 2009). Persistent infection of a bull was associated with testicular hypoplasia (Borel et al., 2007).

Experimental acute infection of non-pregnant heifers via intramuscular inoculation resulted in a lymphoplasmacytic oophoritis persisting at least 61 days post-infection (Ssentongo et al., 1980). Later studies demonstrated that the pre-ovulatory luteinising hormone surge observed in uninfected cattle was partially or completely absent in experimentally infected, superovulated cattle (McGowan et al., 2003). Inflammation and necrosis of granulosa cells and oocytes in follicles was associated with BVDV detected by immunolabelling (McGowan et al., 2003). Infection of bovine embryos before placenta formation has been demonstrated in cows experimentally infected with BVDV at day 26 of pregnancy (Tsuboi et al., 2011). BVDV has been found to be localised in the oocytes of PI females (Fray et al., 1998, 2000a), a possible explanation of why calves born to PI cows are always PI themselves (Meyling et al., 1990).

### *Fetal infection*

The effects of fetal infections are complex and depend on the age of the fetus when BVDV infection first occurred. During the first 18 days of pregnancy, while the embryo is unattached, no infection of the embryo occurs as BVDV does not penetrate the zona pellucida (Moennig and Liess, 1995). Once cotyledons develop, viraemia of the dam from days 29 to 41 post-conception can result in embryonic infection leading directly to embryonic death (Carlsson et al., 1989; McGowan et al., 1993), and reduced pregnancy rates (Grahn et al., 1984).

Infection of the dam after day 30 of gestation and during the first trimester can result in the birth of PI calves (Brownlie et al., 1998). Infection between 80 and 150 days of gestation can lead to teratogenic effects in the fetus. These include cerebellar atrophy (Brown et al., 1974), ocular degeneration (Brown et al., 1975), brachygnathism (Blanchard et al., 2010), pseudocyst formation in the brain (Montgomery et al., 2008), and thymus, bone (Webb et al., 2012) and lung growth retardation (Done et al., 1980). Viral infection at this stage can also lead to fetal death and abortion without any effect on the cow (Done et al., 1980). Vasculitis found in the cerebellum (Brown et al., 1974) leads to white matter oedema, cerebellar swelling and external germinal layer necrosis, resulting in cerebellar atrophy. Cerebellar hypoplasia then ensues, resulting in ataxic calves at birth (Trautwein et al., 1986). White matter cavitation (hydrocephalus) and hypomyelination (Otter et al., 2009) may also be a consequence.

### *Persistent infection*

Infection of the dam in the first trimester can result in the birth of a PI calf (Brownlie et al., 1998). The reported window for the creation of PI calves varies but is generally accepted to be between

approximately 25 and 90 days of gestation, with occasional occurrences as early as day 18 and as late as day 125 (Grooms, 2004). This window may vary from fetus to fetus, as illustrated by reports of a PI calf with a seropositive twin (Schoder et al., 2004).

The ability of ncp BVDV to inhibit the induction of type I interferon in the fetus (Charleston et al., 2001; Peterhans and Schweizer, 2013) enables the virus to survive in the host and establish PI animals. These PI animals do not mount an Ab response or clear the virus, and will shed large amounts of virus in all excretions and secretions including milk, semen, saliva, nasal secretions, urine, blood and aerosols (Brownlie et al., 1987; Nettleton and Entrican, 1995). BVDV is widely distributed in the lymph nodes, gastrointestinal tract epithelial and lymphoid cells, lungs, skin, thymus and the brain (Liebler-Tenorio et al., 2004) of PI animals. The distribution of virus in the central nervous system is within neurones, astrocytes, oligodendroglia and blood vessel-associated cells but not the endothelium (Montgomery, 2007).

PI animals can be clinically healthy, but some may appear small, weak and ill-thrifty (Baker, 1995). Some PI cattle show decreased weight gain, stunted growth and chronic ill thrift (Voges et al., 2006). Temperature, respiration rate and heart rate of PI calves have been reported within normal ranges (Constable et al., 1993), however, their thyroid hormone concentrations have been shown to be significantly lower than healthy calves (Larsson et al., 1995).

PI animals are regularly reported to be susceptible to secondary infections (Voges et al., 2006), suggesting poor immune function. This, combined with susceptibility to mucosal disease, leads to poor survivability of most PI animals (Houe, 1993; Voges et al., 2006), although recent data suggests that as many as 28% of PIs in a population may be over 2 years of age (Booth and Brownlie, 2012).

### *Mucosal disease*

Mucosal disease only develops in PI cattle and is inevitably fatal. Disease is associated with the appearance of a cp BVDV biotype arising from mutation of ncp BVDV already circulating in the PI animal (Brownlie et al., 1984). Mucosal disease can be experimentally induced via superinfection with a cp strain that is antigenically homologous to the persisting ncp strain (Brownlie et al., 1984), or naturally transmitted between PI animals that are PI with the same homologous ncp BVDV isolate. Mutations underlying the change in biotype include insertion of cellular sequences, gene duplications, deletion (Tautz et al., 1994) and single nucleotide changes (Kummerer et al., 2000). All cp biotypes produce the non-structural (NS) protein NS3, whereas in ncp biotypes only the uncleaved form NS2/3 can be detected (Peterhans et al., 2003).

Cytopathogenic BVDV localises in the germinal centres of lymph nodes (Fray et al., 2000b), tonsils, and gut associated lymphoid tissue of Peyer's patches before spreading to gastrointestinal epithelium (Liebler-Tenorio et al., 1997, 2000). Cytopathogenic BVDV promotes monocyte activation and differentiation, while at the same time inhibiting Ag presentation to T cells. This leads to uncontrolled inflammation and enhanced viraemia, while impairing antiviral defences (Lee et al., 2009). Young cattle (<7 months of age) tended to develop respiratory disease in one study (Bachofen et al., 2010) while older cattle (>7 months of age) developed enteric mucosal disease.

An NS3 protease expressed by the cp BVDV results in the induction of apoptosis (Adler et al., 1997; Gamlen et al., 2010). Double stranded RNA is produced by the virus in infected cells triggering apoptosis by intrinsic and extrinsic pathways (Yamane et al., 2006; Pedrera et al., 2012). Intrinsic pathways are regulated by the release of cytochrome C from mitochondria inducing activation of the death regulator, apoptotic protease-activating factor. External pathways include up-regulation of tumour necrosis factor alpha (TNF-α), a key cytokine participating in apoptosis execution (Yamane et al., 2005). These changes occur primarily in the Peyer's patches leading to lymphoid depletion and atrophy. Microvilli disappear from the lamina propria over the Peyer's patches. Cell debris and mucous accumulate in dilated intestinal gland crypts giving the appearance of necrosis.

Necrosis of keratinocytes in the stratum spinosum leads to disruption of intercellular junctions in the keratinised epithelium of the skin, muzzle, oral cavity, oesophagus, rumen, reticulum and omasum (Bielefeldt-Ohmann, 1995). Normal wear and tear at the epithelial surface leads to erosion and ulceration of the weakened surface exposing underlying connective tissues. Leakage of fluid from the denuded surface of the gastrointestinal tract leads to diarrhoea and dehydration, while bacterial infection and inflammation at the exposed sites results in secondary septicaemia. Diarrhoea, erosions and inflammation induce noticeable disease in affected animals bringing them to the attention of the farmer and veterinarian. Death may occur within a few days or be protracted and take a few weeks (Bolin, 1995).

### *PIs in utero: The 'Trojan cow'*

A non-PI cow that is carrying a PI fetus is known colloquially as a 'Trojan cow'. In such cases the dam appears immune to BVDV and healthy and thus a benign risk whilst, in fact, harbouring a potent source of infectious virus within the fetal unborn calf. However, these animals do present an epidemiological risk: once the calf is born, it will shed copious amounts of BVDV and represent a very high infectious pressure. Previous observations have shown that Trojan cows have Ab titres during mid-late pregnancy significantly higher than that of seropositive cows carrying normal calves (Brownlie et al., 1998; Lindberg and Alenius, 1999). This high Ab titre is most likely a result of continual antigenic challenge of the cow.

## Diagnosis

### *Available diagnostic tools*

Diagnostic testing is available for the detection of the virus, BVDV specific Ag, and BVDV specific Abs. These tests are generally reported as being very reliable (Saliki and Dubovi, 2004; Dubovi, 2013).

### *Virus or virus-specific antigen detection*

Whether investigating individual disease cases, endeavouring to eradicate BVDV from a herd or region, or identifying infected animals posing an epidemiological threat, it is vital to accurately detect BVD virus or specific Ag. The use of virus isolation, Ag detection (including Ag enzyme linked immunosorbent assay (ELISA) and immunohistochemistry [IHC]), nucleic acid probe hybridisation and reverse transcriptase polymerase chain reaction (RT-PCR) for the diagnosis of BVDV infection was reviewed by Saliki and Dubovi in 2004. Since this time, there has been significant progress.

Saliki and Dubovi (2004) refer to virus isolation as the 'gold standard' for BVDV diagnosis. While this is still the case today, the use of PCR has become increasingly common, with RT-PCR (Hertig et al., 1991) now being widely accepted as the standard for BVDV diagnosis. RT-PCR is often preferable to virus isolation as it is less time consuming, less expensive, not restricted to laboratories with cell culture facilities and is also highly sensitive (Kim and Dubovi, 2003; Houe et al., 2006). The limitations associated with virus isolation are discussed by Dubovi (2013). A variety of samples, including blood, milk, follicular fluid, saliva and tissue

samples, can be tested successfully by RT-PCR (Bhudevi and Weinstock, 2003; Kim and Dubovi, 2003; Kliucinskas et al., 2008), with prolonged storage having minimal effect (Vilcek et al., 2001; Bhudevi and Weinstock, 2003). Application of primers specific to the 5′ untranslated region has shown that it is possible to successfully identify type I and type II BVDV using RT-PCR (Letellier et al., 1999). Both acute and persistent infections can be detected by RT-PCR (Bhudevi and Weinstock, 2003). As such, repeat testing at a minimum 4-week interval is advised, with successive positive results indicating PI.

Quantitative RT-PCR has been applied to BVDV detection with excellent analytical sensitivity and specificity (Bhudevi and Weinstock, 2001). Limits of detection as low as 1000 and 100 copies of BVDV-1 and -2, respectively, high repeatability and 100% agreement with conventional PCR were demonstrated by Letellier and Kerkhofs (2003). A linear relationship between the CT value and the quantity of viral RNA present (Bhudevi and Weinstock, 2001) suggests that qRT-PCR can be used to differentiate between acute and PIs, with lower levels of virus expected to be present during an acute infection; however, the use of qRT-PCR for this purpose has never been practically demonstrated.

RT-PCR can be used on bulk tank milk (BTM) samples to detect PI cows contributing to the tank. The maximum theorised herd size in which a single PI cow can be detected has been estimated to be as high as 5000 milking cows (Radwan et al., 1995), while practically, detection of PIs in herds has been reported to range from one PI animal in a herd of 132 to two PI cows in a herd of 800 (Drew et al., 1999; Renshaw et al., 2000; Hill et al., 2010). A positive BTM RT-PCR result may also indicate one or several acute infections in the milking herd, as opposed to the presence of PI cows. While a positive BTM RT-PCR result indicates BVDV infection (i.e. specific), a negative BTM RT-PCR result does not necessarily indicate that the herd is not infected – just that the infected individual was not contributing to the BTM at that time point. Animals that are not contributing to the BTM tank should be tested separately by blood or ear notch sampling.

The same principle can be applied to pooled serum samples (Munoz-Zanzi et al., 2000). Application of RT-PCR may detect any infected individuals contributing to the pool in pools of up to 50 (Smith et al., 2008; Yan et al., 2011), with pools returning a positive test result prompting testing of individual samples. Smaller pools may be more economical when searching for a PI; for example, Munoz-Zanzi et al. (2000) concluded the least-cost pooling strategy to be initial pools of size 20, with a re-pooling step of pools of 5 prior to individual testing.

The Ag ELISA presents a simple, rapid method for detection of PI animals that is ideal for high throughput applications, such as herd screening (Mignon et al., 1991; Shannon et al., 1991; Horner et al., 1995). Sensitivities and specificities of Ag ELISAs have been reported, ranging from 67% to 100% and 98.8% to 100%, respectively, when compared with virus isolation (Shannon et al., 1991; Mignon et al., 1992; Sandvik and Krogsrud, 1995; Brinkhof et al., 1996; Saliki et al., 1997, 2000).

Since last reviewed in 2004 (Saliki and Dubovi), further progress has been made, with multiple BVDV Ag ELISAs now available commercially, for use with various samples, such as serum, milk and ear notches. The Ag ELISA is a very robust, simple, cost-efficient diagnostic method; the test requires no cell-culture facilities and results are minimally affected by prolonged storage (Shannon et al., 1991; Saliki and Dubovi, 2004). This concept has been further refined, leading to the recent commercial release of a SNAP test for rapid, cow-side detection of PI animals.[1] Unlike Ab ELISAs, Ag ELISAs cannot return useful results on pooled serum samples, with reported sensitivity of <15% in pools of just two serum samples (Cleveland et al., 2006). In addition, colostral antibodies may affect the sensitivity of the Ag assay on samples from suckling calves (Fux and Wolf, 2013). Cross-reactivity with border disease virus has been observed in a commercially available BVDV Ag ELISA (McFadden et al., 2012).

Immunohistochemistry is one of the most popular methods of BVDV Ag detection in the USA (Driskell and Ridpath, 2006), and has been shown to detect PI animals with 100% sensitivity when used on ear notch tissue samples (Cornish et al., 2005). The same study also showed that IHC returned a positive result on ear notches from some acutely infected calves. Indeed, three of the eight calves identified as acutely infected, whilst negative for BVDV by RT-PCR and virus isolation on buffy coat samples on all testing occasions, were positive by IHC on two separate occasions, 90 days apart, showing that IHC can detect BVDV Ag in tissue samples long after the period of acute viraemia.

While IHC is perceived as robust and suitable for large numbers of samples, it faces disadvantages in that it is restricted to tissue samples, is labour intensive, is prone to technical error, relies on a subjective scoring system, requiring experienced personnel to ensure accuracy (Cornish et al., 2005; Driskell and Ridpath, 2006) and is unreliable for use on samples stored in formalin for >15 days (Khan et al., 2011).

### *BVDV specific antibody detection*

Detection of Abs in cattle is a valuable way of determining an individual animal's immune status and any previous exposure to BVDV. A positive antibody test in an unvaccinated individual will not only indicate that an animal has been previously exposed to BVDV, but that it is not PI. A positive result in a pregnant female will indicate the possibility that she is carrying a PI fetus. However, a negative antibody result in an individual does not confirm the animal as BVDV naive; further virus or Ag testing is required to confirm the animal is not PI.

At a herd or region level, high prevalence of positive antibody results is indicative of a high likelihood that the population is currently infected (containing a PI animal), while largely negative test results indicate that the population is unlikely to contain a PI individual. Furthermore, low antibody seroprevalence in a herd or region is suggestive of severe consequences should the infection be introduced and provides evidence supporting the need for careful protection of the population. Conversely, high seroprevalence suggests that little benefit will be gained from vaccination against BVDV.

Several Ab detection methods are available including: a simple, inexpensive, reliable, rapid dot-blot enzyme immunoassay (Hemmatzadeh and Amini, 2009), an agarose gel immunodiffusion (AGID) test (Lanyon et al., 2013) and a microsphere based immunoassay. That immunoassay was reported to have a sensitivity of 99.4% and specificity of 98.3% relative to ELISA (Xia et al., 2010). However, by far the most common methods for detecting specific Abs to BVDV are the serum neutralisation test (SNT) and the Ab ELISA (Dubovi, 2013).

The SNT is a highly specific test, but it is expensive and time consuming due to a need for tissue culture (Cho et al., 1991; Horner and Orr, 1993; Houe et al., 2006). The reference test across Europe, the SNT is based on inhibition of viral replication by Abs present in a serum sample (Houe et al., 2006), but can have variable results between laboratories as a result of the use of different virus strains or cell types (Dubovi, 2013).

The SNT titre in a given animal will continue to rise for at least 3 months following acute infection (Kirkland and MacKintosh, 2006). A positive relationship was observed between Ab ELISA results and SNT titres, as well as between Ab ELISA results and AGID

[1] See: www.idexx.com.

scores, showing that Ab ELISAs also return quantitative results (Lanyon et al., 2013), with optical densities (ODs) expected to rise for 10–12 weeks post-infection.

Early Ab ELISAs were unreliable, with difficulties attaching the appropriate Ag to a reaction plate or with high background readings being experienced (Cho et al., 1991). However, this has been overcome, resulting in Ab ELISAs with high specificity and sensitivity of up to 99% and 98%, respectively, relative to the SNT (Cho et al., 1991; Horner and Orr, 1993; Kramps et al., 1999; Beaudeau et al., 2001b). A variety of samples can be tested by Ab ELISA, and as they are rapid and inexpensive, Ab ELISAs are an efficient and economical alternative to SNT (Nettleton and Entrican, 1995). While the SNT will detect a rise in Abs following vaccination, Ab ELISAs may not (Raue et al., 2011). Low SNT titres, along with prolonged storage or repeated freeze–thawing can predispose a positive sample to testing negative by Ab ELISA (Horner and Orr, 1993).

Multiple ELISAs are commercially available for the detection of BVDV-specific Abs and have been validated for use in various samples, including serum, milk and bulk milk and will detect colostral antibodies in suckling calves (Fux and Wolf, 2013). Testing BTM can be a valuable, efficient and cost-effective method for determining herd immunity. Antibody concentrations in BTM are indicative of the prevalence of immune cows in the milking herd (Beaudeau et al., 2001a; Eiras et al., 2012) and, in turn, the likelihood of the herd being infected (Beaudeau et al., 2001a). This method of identifying herds that are likely to be infected will, unlike BTM PCR, return a positive result even when the PI animal maintaining the infection is not contributing to the BTM, for example, a cow being treated for mastitis, a bull, or a heifer.

There are rare occurrences in very small herds, where a PI individual may be contributing (at peak lactation) more than the other cows to the BTM and the resultant viral load is neutralising the Abs contributed to the BTM from the remainder of the herd, causing a negative BTM Ab test result (Sandvik et al., 2001). As such, the value of BTM Ab testing is increased by simultaneous RT-PCR testing of the sample (Sandvik et al., 2001). In addition, as Abs persist at least for several years, BTM Ab testing is more valuable when used for regular surveillance so changes in seroprevalence can be observed.

Testing pooled serum samples by Ab ELISA can estimate seroprevalence amongst those individuals contributing to the pool (Lanyon et al., 2010). This is particularly valuable in non-lactating stock, including young or dry dairy stock, beef cattle and bulls.

### *Diagnosis of acute infection*

As clinical signs associated with acute BVDV infection are often mild, the purpose of diagnosing acute infection in an individual is often either to determine (1) whether a pregnant female is at risk of delivering a PI calf, (2) whether an infection is secondary to BVDV-associated immunosuppression, or (3) whether reproductive loss is a result of acute BVDV infection.

It is regularly reported that RT-PCR is one of the most sensitive methods for BVDV detection (Vaniddekinge et al., 1992; Horner et al., 1995), and is said to be capable even of detecting relatively low levels of virus shed during acute infections (Bhudevi and Weinstock, 2003). As mentioned above, the presence of virus has been demonstrated in peripheral blood mononuclear cells up to 98 days post-exposure (Collins et al., 2009). It is possible that, due to its high analytical sensitivity, RT-PCR may be capable of detecting this viral presence in blood. Immunohistochemistry and Ag ELISA were also demonstrated to return positive results up to 90 days post-infection when used to test tissue samples (Cornish et al., 2005). However, detection of virus on a single occasion presents an ambiguous result as it could signify either an acute or, more likely, a persistent infection. Development of qRT-PCR protocols (Bhudevi and Weinstock, 2001) for BVDV detection may offer the ability to distinguish between acute and persistent infections based on the amount of virus present. Alternatively, absence of virus on a subsequent sample collected at least 19 days later will confirm acute infection (Meyling et al., 1990).

An acutely infected animal should become seropositive for BVDV-specific Abs within 2–3 weeks post-infection. Hence, testing for antibody (e.g. Ab ELISA) several weeks after initial testing can be used to distinguish between acute and persistent infection in animals with positive RT-PCR results. Alternatively, paired Ab tests pre- and post-infection showing a rise in Ab concentration may be sufficient to confirm the occurrence of an acute infection. However, pre-infection samples or samples collected during the period of acute viraemia are often difficult to obtain. Therefore, serial post-infection Ab tests may be the most common method of detecting acute infection, with a rising Ab titre, demonstrated by SNT or Ab ELISA, suggesting that acute infection occurred in the previous 10–12 weeks (Lambot et al., 1997; Fredriksen et al., 1999).

### *Diagnosis of fetal malformation*

If the lesions induced by BVDV infection are severe, the fetus will die and be aborted (Brownlie et al., 1998). Other fetuses may survive and be born with a variety of malformations depending on the tissue affected. Demonstration of BVDV in any tissues of affected fetuses or calves by virus isolation, IHC (Ellis et al., 1995; Njaa et al., 2000), Ag ELISA testing of fetal fluid or skin, and PCR testing of fetal fluid (Hyndman et al., 1998) would confirm fetal BVDV infection. When infection is acquired after 150–180 days of gestation, the fetus is able to mount an effective immune response, clear the virus, and will be born Ab positive and virus or Ag negative (Hansen et al., 2010). These animals, therefore, will test positive by SNT or Ab ELISA prior to colostrum intake. Demonstration of either virus or Ab in aborted fetuses or pre-colostral ingestion in calves will confirm fetal infection.

### *Diagnosis of abortion or reproductive failure*

To investigate if reproductive failure was due to BVDV infection would require demonstration of seroconversion of the dam over the period of early pregnancy. Serial blood samples from the dam 4–6 weeks apart (using a serum neutralisation test or Ab ELISA to demonstrate a rising titre post-infection) would indicate acute infection, however abortion of a PI fetus leads to a decline in antibody concentration (Brownlie et al., 1984) making titres difficult to interpret. Vaccination history would also be needed to interpret the titres. While it is difficult to conclusively diagnose BVDV as the direct cause of reproductive failure, the virus should be acknowledged as a significant contributor to reproductive disease. Herd-level evaluation is warranted when BVDV is suspected in individual cases.

If abortion occurs and the fetus can be recovered, testing as detailed above, for diagnosis of fetal infection, could be undertaken on fetal skin or fluids.

### *Diagnosis of PI*

Due to the exceptionally high viral load in PI cattle, detection of adult PIs is reasonably straightforward with several diagnostic tests including virus isolation, IHC, RT-PCR and Ag ELISA, achieving excellent sensitivity and specificity when used for this purpose (Saliki et al., 2000; Kim and Dubovi, 2003). If performance were comparable, then Ag ELISA would be the preferred method as it is substantially less expensive than virus isolation or RT-PCR for testing of an individual, and is less labour intensive than IHC. However, when attempting to diagnose PI in young, colostrum-fed

calves, the efficacy of Ag ELISAs is questionable with both positive and negative outcomes reported (Shannon et al., 1991; Shannon et al., 1993; Brinkhof et al., 1996; Bock et al., 1997; Zimmer et al., 2004). Virus isolation tests are similarly reported to be inhibited by the presence of maternal Abs (Zimmer et al., 2004). Interference from maternal Abs has a much smaller effect on RT-PCR (Horner et al., 1995; Zimmer et al., 2004). Therefore, RT-PCR is the preferable diagnostic method for this purpose.

A new approach to sample collection, namely ear notching, has become increasingly common in recent years (Driskell and Ridpath, 2006), by combining sampling for BVDV testing with routine ear tagging procedures (Kuhne et al., 2005). These small skin biopsy samples (ear notches), can be tested by Ag ELISA, IHC, virus isolation or RT-PCR (Cornish et al., 2005; Kuhne et al., 2005; Kennedy, 2006). Ear notch supernatants can be pooled for testing by RT-PCR with good success (Kennedy, 2006). It is believed that ear notch sample supernatants can be tested by Ag ELISA without interference by maternal Abs in colostrum-fed PI calves (Kuhne et al., 2005), however, as recent data questions the accuracy of some of these tests in the first 90–158 days (Fux and Wolf, 2013), calves should be tested prior to colostrum intake or after the effects of colostrum have waned. Ear notching is a convenient sample collection method that can be carried out by farmers, making it an appealing option for PI detection.

*Diagnosis of mucosal disease*

In order to confirm a diagnosis of mucosal disease, one must first confirm the PI state, as described previously. In order to virologically confirm mucosal disease, both cp and ncp BVDV must be isolated from the affected animal. However, identification of PI combined with pathological lesions of mucosal disease is sufficient to confirm the diagnosis.

*Diagnosis of the 'Trojan cow'*

Current diagnostic tests will identify a Trojan cow as virus negative and Ab positive and cannot be differentiated from an immune animal carrying a normal calf. While Trojan cows are known to have very high Ab levels (Brownlie et al., 1998; Lindberg et al., 2002), using Ab levels to distinguish between Trojan cows and cows carrying normal calves has met with only moderate success with 4/13 cows designated to be carrying a normal calf found to be carrying PI calves (Brownlie et al., 1998).

Similarly, Lindberg et al. (2001) observed that the sensitivity of such a test improved significantly towards the end of pregnancy and as the selected cut off decreased. However, to achieve acceptable sensitivity, the specificity of the test was compromised (Lindberg et al., 2001). While it is crucial to detect all cows carrying a PI fetus due to the high infective risk associated with their introduction, the specificity of the test must still be acceptable for the test to achieve good acceptance by producers.

An alternative method for detecting Trojan cows is to test amniotic or allantoic fluids collected via intrauterine puncture for viral Ag (Lindberg et al., 2002; Stokstad et al., 2003). This test has proved useful, with 8/9 samples collected testing positive by RT-PCR in one study (Lindberg et al., 2002) and 15/16 in another (Stokstad et al., 2003). No complications were reported from the sampling procedure in two studies (Lindberg et al., 2002; Stokstad et al., 2003). However, the method requires sedation and local anaesthesia (Lindberg et al., 2002), and is a veterinary procedure which is likely to be expensive. In addition, there are risks associated with such a sampling procedure (Lindberg et al., 2002) (despite no complications in some experimental studies). These factors make the test impractical for widespread field application, and unlikely to experience wide uptake. At present, virus testing of calves soon after birth remains the most practical way of assessing neonate BVDV status.

## Control and eradication

The wide range of diagnostic tools available has allowed successful BVDV control and eradication schemes to become a reality. With the understanding that PI individuals are the primary transmission source, these animals naturally become the target for eradication. Test and cull schemes have successfully been applied in many countries, including all or regions of Austria, Scotland, The Netherlands, Norway, Denmark, Sweden, Switzerland, Italy, Slovenia, Germany, France, Ireland and Finland (Ferrari et al., 1999; Grom and Barlic-Maganja, 1999; Lindberg and Alenius, 1999; Synge et al., 1999; Greiser-Wilke et al., 2003; Sandvik, 2004; Hult and Lindberg, 2005; Joly et al., 2005; Mars and Van Maanen, 2005; Rossmanith et al., 2005; Anon, 2010; Presi and Heim, 2010; Van Campen, 2010; Barrett et al., 2011).

These schemes have been thoroughly reported, with common features identified and reviewed (Lindberg and Alenius, 1999; Sandvik, 2004; Houe et al., 2006). Generally, herds likely to be infected are identified during a herd level testing phase; the virus is cleared from infected herds through the removal of identified PI animals, and re-infection is prevented by ongoing application of high levels of biosecurity. Several schemes have reported substantial reductions in the prevalence of BVDV following the introduction of control measures, with Switzerland having reduced the prevalence of PI animals from 1.8% to under 0.2% in just 2 years (Presi et al., 2011), while Norway observed a steadily decreasing risk of seroconversion (from 0.12 to 0.02 in high risk herds) from 1993 to 1997, after introduction of their control scheme. Scandinavia, as a whole, along with the dairy industry in Switzerland, is now largely regarded as BVDV-free, due to the implementation of successful systematic eradication programs.

## Conclusions

An understanding of the pathology and pathogenesis of BVDV infection will guide and optimise diagnostic approaches. As the age of the animals and stage of infection varies, different tests and testing protocols need to be utilised. Highly sophisticated, but relatively cheap diagnostic tests, such as a combination of qRT-PCR and Ab monitoring on pooled material allow classification of the BVDV infection status of individuals and herds.

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