Investigations into the epidemiological and clinical effects of bovine viral diarrhoea virus infection in sheep and other non-bovine species in Australia

by

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Abstract

Bovine viral diarrhoea is a disease of cattle known to cause severe reproductive dysfunction and immunosuppression in infected animals. It has also been reported that the BVD virus (BVDV) is able to cross species barriers infecting non-bovine species such as sheep, goats, deer and alpacas. The ability of BVDV to infect species other than cattle is of great concern when developing control or eradication programs for BVDV, particularly in areas where cattle regularly come into contact with other susceptible species. As such, the main objective of this thesis was to investigate the epidemiological and clinical effects associated with BVDV infections in sheep and other commonly farmed Australian livestock species.

Firstly, the clinical and reproductive outcomes following acute infections of pregnant ewes was studied. Twenty two ewes were experimentally infected with BVDV-1c between 59 to 69 days of gestation and the clinical and reproductive outcomes of these ewes were compared to those of a control group of 11 BVDV naïve and uninfected ewes. In the naïve ewe flock a lambing rate of 172.7% was recorded, lambs were born clinically normal and had a high survival rate. In comparison, a lambing rate of just 31.8% was reported for the infected ewe flock, as well as poor lamb survival and lambs presenting with a wide range of pathological lesions; including anasarca, hydranencephaly and skeletal deformities. A viable, neonatal, BVDV-1c persistently infected (PI) lamb was also born during this study.

Three experimental trials then investigated the ability of a neonatal BVDV-1c PI lamb to infect susceptible sheep and cattle. Results indicated that there was a low rate of transmission to susceptible ewes and lambs (n=9) co-paddocked with the PI lamb. The remaining two trials showed no transmission to susceptible steers; housed either adjacent to (n=5) or exposed directly to (n=5) the PI lamb. Furthermore, the transmissibility of BVDV-1c from sheep undergoing acute BVDV-1c infections was studied by co-paddocking five experimentally

infected wethers with five susceptible wethers. Results confirmed acute infection in the experimentally infected wethers however the naïve sheep did not seroconvert.

In addition, the clinical and haematological effects associated with acute BVDV-1c infections in alpacas were investigated. Results indicated that transmission of BVDV can occur naturally from PI cattle to alpacas housed together. However, there were no apparent signs of infection in any of the alpacas. Lastly, the presence *Pestivirus*-specific antibodies was determined for 875 South Australian sheep and 245 Northern Territory water buffalo at a prevalence of 0.34% and 4.5%, respectively.

This body of work has indicated that acute BVDV-1c infections of pregnant ewes can lead to severe reproductive losses and the birth of persistently BVDV-1c infected lambs. However, results also indicate that sheep are unlikely to play a pivotal role in the spread and persistence of the virus in Australia. Further work needs to be undertaken to fully understand the role other non-bovine species have in the spread and persistence of the virus in Australia.

Declaration of Originality

I certify that this work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or tertiary institution without the prior approval of the University of Adelaide and, where applicable, any partner institution responsible for the joint award of this degree.

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Caitlin. A. Evans

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Chapter 1: An introduction to ruminant pestiviruses

Bovine viral diarrhoea virus (BVDV) and Border disease virus (BDV) are economically important and highly prevalent viruses of domestic ruminants which, along with Classical swine fever virus (CSFV) in pigs, belong to the genus *Pestivirus.* While *Pestiviruses* have previously been considered to be host specific, both BVDV and BDV have been shown to cross species barriers, infecting species other than those from which they were originally isolated from (Nettleton and Entrican 1995). As a result BVDV and BDV are often referred to collectively as "ruminant pestiviruses" (McFadden *et al.* 2012).

1.1 Pestivirus diversity

Pestiviruses are enveloped, 50nm spherical particles containing single stranded RNA (Nettleton and Entrican 1995) of positive polarity and belong to the *Flaviviridae* family. Completing the *Flaviviridae* family are three other genera; *Hepacivirus, Flavivirus* and *Pegivirus* (International Committee on Taxonomy of Viruses 2014) which all up total over 50 species including well known viruses Hepatitis C (HCV), Yellow fever virus, Dengue virus, tick-borne encephalitis virus and West Nile virus. Although there are many similarities within the genetic makeup of the flaviviruses, it has been reported that pestiviruses are more closely related to the *Hepacivirus* genus than to the other Flaviviridae genera (Schweizer and Peterhans 2014).

The genus *Pestivirus* includes four well-known species; Bovine viral diarrhoea virus-1 (BVDV-1) and BVDV-2 in cattle, Border disease virus (BDV) in sheep and Classical swine fever virus (CSFV) in pigs (Broaddus *et al.* 2009). In addition there have been a growing number of new and emerging *Pestivirus* species identified (Becher *et al.* 2014) and include; Bungowannah virus - isolated from pigs in an Australian piggery (Kirkland *et al.* 2007), Atypical (HoBi-like) pestivirus - identified in Europe, Asia and South America (Stahl *et al.* 2010), Pronghorn antelope virus (Vilcek *et al.* 2005) and a giraffe species from Kenya (Harasawa *et al.* 2000).

Bovine viral diarrhoea virus

Bovine viral diarrhoea virus, previously identified as "Mucosal disease virus", was first identified in the United States of America in 1946 amongst cattle herds showing signs of severe diarrhoea and ulcerations of the alimentary tract mucosa (Olafson *et al.* 1946). Since this time it has been determined that there are two distinct BVDV species, BVDV-1 and BVDV-2 (Kahrs 2001; Ridpath *et al.* 2010). Originally this division was based primarily on phylogenetic analysis of the virus however, since this time, antigenic differences between the two species have been identified (Ridpath *et al.* 1994; Ridpath *et al.* 2000).

The main difference observed between BVDV-1 and BVDV-2 was the failure of vaccines and diagnostic tests, based on BVDV-1 strains, to control and detect BVDV-2 strains (Ridpath *et al.* 2010). The split into two species is further supported by the fact that infection with BVDV-2 often leads to more severe and sometimes fatal infections (Kahrs 2001; Houe 2003). Since the split of BVDV-1 from BVDV-2 further phylogenetic analysis has been completed and has identified 12 BVDV-1 strains (a-l) and two BVDV-2 strains (a and b) with each strain appearing to originate from different geographical areas (Ridpath *et al.* 2010).

Border Disease virus

The 'hairy' coated, trembling lambs associated with BDV were first observed in sheep flocks from the border counties of England and Wales during the 1940s, however the disease was not named until 1959 (Hughes and Kershaw 1959). Since its discovery it was questioned whether BDV was a strain of BVDV or a separate group within the *Pestivirus* genus. In 1994 a study of two ovine *Pestivirus* strains identified that they were clearly different to both BVDV and CSFV (Becher *et al.* 1994) and since this time BDV species have been phylogenetically classified in to seven numbered genotypes (Oguzoglu 2012).

Similar to BVDV, the seven BDV genotypes are classified and named according to where they were originally isolated from. The seven genotypes are; the 'classical' BDVs from the USA, UK, Australia and New Zealand forming BDV-1a and -1b (Becher *et al.* 2003; Strong *et al.* 2010), the ovine isolates from Germany forming BDV-2 (Becher *et al.* 2003), the ovine and Gifhorn isolates from Switzerland forming BDV-3 (Becher *et al.* 2003; Stalder *et al.* 2005), isolates from Pyrenean chamois and Spanish sheep forming BDV-4 (Arnal *et al.* 2004; Valdazo-Gonzalez *et al.* 2006), ovine isolates from France forming BDV-5 and BDV-6 (Dubois *et al.* 2008) and isolates from Turkey and Italy forming BDV-7 (Thabti *et al.* 2005; Giammarioli *et al.* 2011).

The birth of 'hairy' coated, trembling lambs in Australia and New Zealand resulted in the colloquial term for BDV, "Hairy shaker disease" (Manktelow *et al.* 1969).

Biotypes

In addition to the multiple species/strains of BVDV and BDV identified, there are two biotypes of each species; cytopathic and non-cytopathic (Nettleton *et al.* 1992; Schweizer and Peterhans 2014). The non-cytopathic biotype, for both BVDV and BDV, can be maintained within a population (Schweizer and Peterhans 2014) and is associated with acute infections as well as the reproductive and immunosuppressive effects commonly observed with *Pestivirus* infections (Schweizer and Peterhans 2014). It is also the non-cytopathic biotype which is responsible for the development of persistently infected individuals. In comparison, the cytopathic biotype is less common and is not maintained within a population. Instead, the cytopathic biotype, for both BVDV and BDV, only occurs in persistently infected animals through the mutation of the non-cytopathic biotype (Nettleton and Entrican 1995; Schweizer and Peterhans 2014). This mutation leads to dual infection in these animals with homologous cytopathic and non-

cytopathic biotypes, resulting in the onset of mucosal disease and high mortality rates in infected sheep and cattle (Loken 1995; Schweizer and Peterhans 2014).

1.2 Outcomes of infection

Infections with ruminant pestiviruses can either result in acute or persistent infections. Acute infections with non-cytopathic biotypes of BVDV or BDV are generally clinically inapparent, but can result in immunosuppression and the onset of other diseases including mastitis and respiratory disease (Fulton *et al.* 2002; Wellenberg *et al.* 2002). The more severe clinical signs associated with *Pestivirus* infections arise from the ability of these viruses to cross the placenta and establish an infection within the developing foetus (Nettleton and Entrican 1995; Niskanen and Lindberg 2003). When infection develops in pregnant females, poor pregnancy rates, early embryonic death, abortions, stillbirths, physical malformations and the birth of PI animals are common (Grooms 2004; Lindberg *et al.* 2006; Broaddus *et al.* 2009).

The severity of the clinical signs observed as a result of *Pestivirus* infections in ruminants is dependent upon the pregnancy status of the animal, the type of infection that animal is experiencing (acute or persistent) and the infecting species or strain of *Pestivirus*. While the severity of infection has not been reported to differ clinically between the 7 BDV genotypes, the outcomes of infection associated with BVDV appear to be dependent on the infecting strain. Acute infections with any of the BVDV-1 strains generally result in clinically mild signs compared to the BVDV-2 strains which have been shown to lead to severe clinical disease, including pyrexia and high mortality rates (Schweizer and Peterhans 2014).

Acute Infections

When susceptible, non-pregnant cattle develop an acute infection with one of the many BVDV-1 strains, clinical signs are often mild or inapparent. The clinical signs associated with acute

infections with non-cytopathic biotypes can include, but are not limited to, moderate fever, oculo-nasal discharge, inappetance, decreased milk production and mild diarrhoea (Nettleton and Entrican 1995). These acute BVDV-1 infections have also been shown to cause immunosuppression and result in an increased susceptibility to other diseases such as mastitis and bovine respiratory disease (Fulton *et al.* 2002; Wellenberg *et al.* 2002) (Lindberg *et al.* 2006; Laureyns *et al.* 2010). Often these secondary infections are recognised and treated while the underlying BVDV infection goes undetected (Lindberg *et al.* 2006).

Acute infection with cytopathic biotypes are less common, however they have been associated with interstitial oophortis (Grooms 2004). In comparison, acute infections with BVDV-2 strains in susceptible, non-pregnant cattle have been shown to result in thrombocytopenia, pyrexia and high mortality rates (Schweizer and Peterhans 2014)

In sheep, acute infections with BDV are short lived with generally inapparent or mild clinical signs observed (Nettleton 1990). The clinical sings often associated with acute BDV infections in non-pregnant sheep have included pyrexia, conjunctivitis, nasal discharge and diarrhoea (Nettleton *et al.* 1998).

Foetal Infection

Acute infections with either BVDV or BDV in susceptible, pregnant females have been reported to result in severe reproductive losses. These reproductive losses can occur throughout pregnancy, however what clinical outcomes are observed is dependent upon the stage of gestation at which infection develops (Nettleton *et al.* 1998; Grooms 2004).

The foetus is first able to respond to an antigenic stimuli (immunocompetence) during mid gestation (100-125 days in cattle (Grooms 2004); 60-80 days in sheep (Nettleton *et al.* 1998)). As such, the outcomes following foetal infection with ruminant *Pestiviruses* are dependent on

whether infection developed before, during or after this stage of foetal development (Nettleton 1990; Nettleton and Entrican 1995; Broaddus *et al.* 2009).

When acute infection develops prior to, or soon after, conception (-9-45 days in cattle (Grooms 2004); 0-60 days in sheep (Nettleton *et al.* 1998)) poor conception rates, poor pregnancy rates and early embryonic death are commonly reported (Grooms 2004). Similarly, infection of a pregnant female during early gestation (0-100 days in cattle (Grooms 2004); 0-60 days in sheep (Nettleton and Entrican 1995)) can often result in foetal death.

It is thought that due to the high levels of virus present in both the dam and the foetus, virus replication is so fast and uncontrolled that the death of the foetus is most likely (Nettleton 1990). Foetal death can occur soon after maternal infection has developed, resulting in the foetus being absorbed/aborted or foetal death may not develop until weeks after initial infection, when an abortion or stillbirth is observed (Nettleton *et al.* 1998). As foetal death can occur at any time throughout gestation, irrespective of when the maternal infection with BVDV or BDV occurred, pinpointing the time of infection in the dam is often difficult.

A critical developmental period for the foetus occurs at organogenesis (100-150 days in cattle (Grooms 2004); 60-80 days in sheep (Nettleton *et al.* 1998)). If foetal infection with either BVDV or BDV occurs during this period then neurological and/or physical malformations may occur in newborns. Developmental malformations associated with *Pestivirus* infections have included cataracts, cerebellar hypoplasia, porencephaly and hydrocephaly, skeletal deformities, arthrogryposis and anasarca (Baker 1990; Kahrs 2001). In addition, lambs infected with BDV may present with a small and weak stature, an inability to stand, have violent trembling/contractions of the muscles in the hind legs and most commonly have what is termed 'fuzzy', hair-like wool (Nettleton and Entrican 1995; Nettleton *et al.* 1998). Colloquially these lambs have been known as 'hairy shakers'. Infection of the foetus after

organogenesis/immunocompetence (150 days in cattle; 80 days in sheep) generally results in foetuses born normal and antibody positive to BVDV or BDV.

Persistent Infection

Prior to the development of foetal immunocompetence (0-120 days in cattle (Grooms 2004); 0-80 days in sheep (Nettleton *et al.* 1998)), the foetus is immunotolerant and unable to identify between self-antigens and antigens of the infecting virus. If maternal infection with a non-cytopathic biotype of BVDV or BDV was to develop during this period then the foetus can be born persistently infected (PI) (Nettleton *et al.* 1998; Grooms 2004).

Persistent infection results due to viral proteins being recognised, by the developing foetus, as self-antigens and thus are not cleared from the body (Grooms 2004). These animals generally have poor growth rates, remain stunted, are more susceptible to other diseases (Houe 1993) and continuously shed the virus in their excretions and secretions for the duration of their lives (Brownlie *et al.* 1987; Lindberg and Houe 2005). As such PI animals are epidemiologically the most significant reservoir of transmissible infection of ruminant *Pestiviruses* and their presence in a population allows for the continual spread of the virus on farms (Laureyns *et al.* 2010). Although it is not uncommon for PI animals to die at a young age, many have been reported to survive up until, and past, breeding age (Nettleton 1990; Brock *et al.* 1997).

Persistent infections do not result from maternal infection with the cytopathic biotype. Rather, mutation of the non-cytopathic biotype in PI animals can occur, resulting in dual infection with homologous cytopathic and non-cytopathic biotypes. This dual infection can lead to the onset of Mucosal Disease and high mortality rates in PI animals (Loken 1995; Schweizer and Peterhans 2014).

1.3 Economic Cost

Infections with BVDV or BDV have been reported to have a significant financial impact on producers. The major financial losses associated with ruminant *Pestivirus* infections are attributed to reproductive disease and resultant calving/lambing losses, as well as the immunosuppressive effects which can result in poor growth, decreased production and increased susceptibility to other diseases (Houe 2003). It has previously been mentioned that infection with ruminant pestiviruses can impair the immunity of infected animals, which can increase the susceptibility to and severity of other diseases such as mastitis, diarrhoea and respiratory disease (Fulton *et al.* 2002; Wellenberg *et al.* 2002; Laureyns *et al.* 2010). While these diseases are identified and treated, the underlying BVDV or BDV infection often goes unidentified and untreated (Lindberg *et al.* 2006; Laureyns *et al.* 2010) and the economic losses associated with ruminant *Pestivirus* infections are higher as a result (Laureyns *et al.* 2010).

Bovine viral diarrhoea virus

In regards to BVDV infections in cattle, many estimates have been calculated. Where acute infections are clinically inapparent and the majority of losses are related to reproductive disease and the production of PI animals, financial losses to producers have been estimated at between $AUD 31 and $AUD 199 per cow (in the infected herd) (Houe 2003; Gunn *et al.* 2004; Lindberg *et al.* 2006; Reichel *et al.* 2008). However, where a BVDV outbreak occurs simultaneously with other diseases, particularly mastitis (Niskanen *et al.* 1995; Waage 2000) financial losses have been estimated up to $AUD 500 per cow (in the infected herd) (Houe 2003; Lindberg *et al.* 2006). This increase in losses is thought to be primarily due to the increased incidence, ongoing treatment and resultant reduction in milk premiums (for dairy herds) due to infection with these secondary diseases (Houe 2003). Similarly, a BVDV outbreak in a South Australian dairy herd resulted in financial losses to that producer of an estimated $AUD 144,700 over two years due

primarily to production losses (calves and milk) as well as losses relating to secondary infections (Lanyon.S.R. *et al.* 2012).

Border disease virus

Unlike BVDV, estimates regarding the financial losses associated with BDV infection in sheep are limited. One report from 1984 estimated that an earlier BDV outbreak was thought to cost a producer more than 20% of the expected years earnings (Sharp and Rawson 1986). This loss was associated primarily with a decrease in lambing rates and poor growth rates of surviving lambs (Sharp and Rawson 1986). Since this time other estimates relating to the economic effect of BDV infection on producers have not been reported.

1.4 Diagnosis

Effective diagnostic differentiation between individuals that are naïve, acutely infected or PI is critical for diseases such as BVDV and BDV. In order for this to be done, effective and accurate diagnostic tests first need to be established. The diagnostic accuracy (utility) of a test is based upon the ability of that test to differentiate between individuals which are diseased and non-diseased. Diagnostic sensitivity relates to the percentage of diseased individuals which are correctly identified as positive by the test, while diagnostic specificity relates to the percentage of non-diseased individuals which are correctly identified as negative by the test (Saah and Hoover 1997). Current diagnostic tests for ruminant Pestiviruses include those for the detection of BVDV or BDV-specific antibodies (Ab), BVDV or BDV-specific antigen (Ag) or the virus itself (Saliki and Dubovi 2004).

Antibody testing

Acute infections with BVDV or BDV result in a short period of viral excretion between 3 and 10 days post infection which is then followed by the development of antibodies generally

between 7 and 14 days (Collins *et al.* 2009). These antibodies can be detected, retrospectively, through the use of antibody detection methods including virus neutralisation test (VNT), antibody enzyme linked immunosorbent assays (ELISA) and agarose gel immunodiffusion (AGID).

The most commonly used diagnostic tests for detecting BVDV/BDV-specific antibodies are the VNT and AGID (Saliki and Dubovi 2004). While the VNT is a highly specific test, it has limitations including cross-reactivity with other *Pestivirus* strains (Kirkland and Mackintosh 2006), requires tissue culture facilities, is time consuming and is expensive to run (Cho *et al.* 1991; Houe *et al.* 2006). Testing by VNT is also limited to serum samples. Similarly, testing by AGID is highly sensitive and is used for serum samples. While AGID can reliably detect antibodies to *Pestiviruses,* the test is also cross-reactive across all *Pestivirus* infections (Kirkland and Mackintosh 2006). Antibodies induced by vaccination have not been shown to effect AGID testing (Kirkland and Mackintosh 2006).

In comparison, the ELISA is quick, requires less infrastructure, is suited to processing large numbers of samples and is relatively inexpensive and simple to perform (Saliki and Dubovi 2004). Sensitivities and specificities for antibody ELISA's have been reported, relative to VNT, to range from 95% to 99% and 97% to 100%, respectively (Cho *et al.* 1991; Horner and Orr 1993; Kramps *et al.* 1999; Lanyon *et al.* 2013) making them an accurate and rapid alternative to VNT and AGID. Antibody ELISAs can also be used to test a variety of samples including serum, bulk milk, colostrum and pooled serum (Nettleton and Entrican 1995; Lanyon *et al.* 2013; Jenvey *et al.* 2015). There has been some concern regarding the use of antibody ELISAs in areas where vaccination is common as it is believed that the antigenic composition of BVDV vaccines inhibits the accuracy of the ELISA (Saliki and Dubovi 2004).

While a positive result on any one of these Ab tests indicates that an animal has previously undergone an acute infection to BVDV or BDV and is generally not a PI, a negative result cannot conclude that the animal is naïve to these viruses. An animal persistently infected with BVDV or BDV will also, generally, return a negative result for antibodies to *Pestivirus* infections. Persistent infection with BVDV or BDV implies that these animals were infected prior to immune-competence developing and have not undergone an acute infection, thus antibodies are not present in these animals. As such further antigen or virus-specific testing needs to be undertaken to identify if these animals are naïve or PI.

Similarly, in countries where there are multiple circulating strains of BVDV, PI animals can become acutely infected with a BVDV strain other than that of the persisting strain (Fulton *et al.* 2003). Likewise, young PI animals will often test positive for antibodies to BVDV due to the presence of circulating colostral antibodies (Fux and Wolf 2012). As such, a positive result for antibodies to BVDV cannot definitively rule out an animal as being persistently infected. If an animal is suspected of being persistently infected with BVDV then virus or virus-specific antigen testing should also be undertaken.

Virus or virus-specific antigen testing

The detection of BVD or BD viruses or virus-specific antigen is generally undertaken to identify those animals which are persistently infected. Commonly used antigen testing includes immunohistochemistry (IHC) and antigen ELISA while virus isolation and reverse transcriptase polymerase chain reaction (RT-PCR) is used for virus detection.

Immunohistochemistry is considered as a highly reliable diagnostic method as it is suitable for large scale testing and has been shown to detect PI animals with 100% sensitivity and 98.8% specificity when used on ear notch tissue samples (Cornish *et al.* 2005). A limitation of IHC is

that it is restricted to tissue samples, is labour intensive, is prone to technical error and as such requires experienced personnel to subjectively score each sample (Driskell and Ridpath 2006).

In comparison, the antigen ELISA is a simple, rapid and cost effective method for identifying samples positive for BVDV or BDV-specific antigen (Shannon *et al.* 1991; Horner *et al.* 1995). Antigen ELISAs can be used on a variety of samples including serum, milk and ear notch supernatants and have been considered a worthy alternative to other viral detection methods (Shannon *et al.* 1991; Horner *et al.* 1995). The sensitivity and specificity of antigen ELISAs, when compared to virus isolation, have been reported to range from 67% to 100% and 90% to 100%, respectively (Mignon *et al.* 1992; Sandvik and Krogsrud 1995; Brinkhof *et al.* 1996). However, testing of serum samples has been reported as being less sensitive compared to testing of ear notch supernatants (Saliki and Dubovi 2004; Cleveland *et al.* 2006; Lanyon *et al.* 2014b). This reduction in sensitivity for serum samples is due to the ability of colostral antibodies to inhibit the accurate detection of PI individuals for up to several weeks after birth (Fux and Wolf 2012). This phenomenon has previously been reported in calves and is often referred to as the 'colostral diagnostic gap' (Fux and Wolf 2012). As a result it is often considered best practice to test serum samples from young animals after 3 months of age (Zimmer *et al.* 2004) or combine antigen testing with RT-PCR to ensure accurate identification of animals persistently infected with BVDV or BDV.

While the antigen ELISA is considered an accurate and rapid testing method, in recent years the concept of the antigen ELISA has been refined into an even quicker and more convenient point-of-care test. The point-of-care test allows animals to quickly be tested before, or as, they come onto a farm and takes just 20 minutes to perform. This quick protocol thus allows the point-of-care tests to be run in the field. Similar to the standard antigen ELISA, point-of-care tests can be used on serum, milk and ear notch tissue samples (IDEXX 2010). However, unlike

the standard antigen ELISA, point-of-care tests are not considered suitable for large scale testing.

Virus-specific antigen testing is used to identify individual animals which are persistently infected however, it is unable to differentiate between different virus species or strains. In order to determine what the infecting *Pestivirus* species is within a population virus, testing should be undertaken in addition to antigen and/or antibody testing. It was thought for many years that virus isolation was the gold standard for BVDV and BDV diagnosis (Saliki and Dubovi 2004). Although virus isolation is still used today, RT-PCR is becoming more widely used and accepted as the gold standard for P*estivirus* diagnosis (Lanyon *et al.* 2014a). In comparison to virus isolation, RT-PCR is quicker, cheaper, is not restricted to labs with cell culture facilities and is highly sensitive (Kim and Dubovi 2003; Houe *et al.* 2006). RT-PCR can also be used to test a variety of samples including blood, milk and tissue samples (Bhudevi and Weinstock 2003; Kim and Dubovi 2003), is reliable even when colostral antibodies are present (Zimmer *et al.* 2004) and is able to differentiate between *Pestivirus* species including BVDV-1 and BVDV-2 (Letellier *et al.* 1999).

Although both virus and virus-specific antigen testing is predominantly used to identify animals persistently infected with BVDV or BDV, these diagnostic methods have also been shown to detect acutely infected individuals. It has been reported that animals suffering from an acute *Pestivirus* infection undergo a short period of viral excretion between 3 and 10 days post infection (Brownlie *et al.* 1987; Nettleton and Entrican 1995; Collins *et al.* 2009). As such the testing of acutely infected animals soon after infection may return a positive result for BVD or BD virus or virus-specific antigen despite not being persistently infected. Consecutive testing of individuals for virus at least 19 days apart, will allow for the correct identification of individuals which are acutely or persistently infected (Meyling *et al.* 1990).

1.5 Control and eradication

While control and eradication programs have been initiated in many countries, particularly throughout Europe, they are predominantly in relation to BVDV infection in cattle. So far, there are currently no peer-reviewed documents relating to the control or eradication of BDV in sheep. As such this section relates solely to BVDV control in cattle, however due to the epidemiological similarities between BVDV and BDV, equivalent methods could be used when controlling BDV in sheep.

In the past, many BVDV control programs focussed on vaccination use as the primary means to reduce/prevent the development of clinical disease within a herd (Stahl and Alenius 2012). While vaccination is thought to be efficient in preventing against clinical disease and protecting animals from viraemia and/or foetal infection, PI calves have still been shown to develop in vaccinated herds (Van Campen *et al.* 2000). As such many of the current BVDV control and eradication programs do not solely rely on vaccination/protecting animals from acute infection (Lindberg and Houe 2005; Laureyns *et al.* 2010). Current control programmes embody two primary elements: 1. preventing infection of susceptible animals and 2. identifying and eliminating animals which are persistently infected. These elements can then be used in conjunction with, or without, vaccination use.

Preventing infection of susceptible animals

In order to effectively reduce both the immunological and reproductive effects of BVDV infection on a population, preventing susceptible animals, particularly those that are pregnant, from infection is essential. The primary method by which this is undertaken is by increasing on-farm biosecurity measures. These can include using only farm-bred replacement heifers, minimizing the movement of cattle between properties, reducing the co-mingling of different management herds, serological testing of new cattle for BVD virus and quarantining new

animals before joining the herd (Nettleton and Entrican 1995; Houe *et al.* 2006). Increasing these biosecurity measures not only assists in the self-clearance of BVDV from a population and subsequent reduction in BVDV prevalence (Stahl and Alenius 2012) but may also help reduce reinfection of cleared herds. While improved biosecurity is considered by some to be the central element of BVDV control (Stahl and Alenius 2012), many programs have used these measures in conjunction with the identification and elimination of PI individuals to reduce virus circulation (Houe *et al.* 2006).

Identifying and eliminating persistently infected animals

Direct contact between PI and susceptible animals is the most common and effective route of transmission for BVDV (Lindberg and Houe 2005). As such the identification and removal of these PI animals can be beneficial to the control the disease within infected populations (Brock 2004; Presi *et al.* 2011). The identification and subsequent removal of these PI animals is undertaken through the detection of individual PI animals using antigen/viral and antibody testing or differentiating between PI and non-infected herds through antibody testing (Presi *et al.* 2011).

The first of the large-scale BVDV eradication and control programs was initiated during the early 1990s, in Denmark, Finland, Norway and Sweden (Lindberg *et al.* 2006; Presi *et al.* 2011). These programs relied upon differentiating between persistently BVDV infected and non-infected herds using antibody screening (Presi *et al.* 2011). Herds identified as being persistently infected were subsequently tested for individual PI animals. Regardless of the initial prevalence of disease, it took approximately 10 years for each of these countries to reach the final phase of eradication (Lindberg *et al.* 2006; Presi *et al.* 2011). In comparison, the Swiss eradication program used diagnostic testing to identify individual, PI animals by antigen testing every animal and removing those which returned positive results (Presi *et al.* 2011; Stahl and Alenius 2012). Although a logistically challenging method this program eliminated 97% of PI

animals in the first phase of the program and after 2 years 17,000 PI animals had been identified (Presi *et al.* 2011; Stahl and Alenius 2012).

Regardless of which identification method is used, the final element for effective BVDV control is through continuous monitoring of negative herds to ensure that these herds remain free from the virus. This is generally achieved by antigen testing newborn calves (e.g. ear-notch samples) and newly purchased animals (e.g. blood/serum samples).

Risks to Pestivirus control

While eliminating PI animals has been shown to efficiently reduce the prevalence of BVDV within a herd, there have been studies which have reported the persistence of the virus even in the absence of these PI animals (Barber and Nettleton 1993; Moerman *et al.* 1993). For herds considered cleared of BVDV and thus susceptible to reinfection it is important that other potential sources of infection are understood and considered in management/control programs. While there are numerous potential sources of infection, such as on farm equipment, flies, bedding and workers clothing (Lindberg and Alenius 1999; Lindberg and Houe 2005), only those which involve direct transmission from infected animals will be discussed here.

It has been reported that despite the absence of, or contact with, a PI animal, BVDV was able to persist in a herd for 2 and a half years. This study suggested that the persistence of BVDV was as a result of animals undergoing an acute infection and the short period of viral excretion these animals undergo prior to the development of antibodies (Brownlie *et al.* 1987; Collins *et al.* 2009). Although acutely infected animals have been shown to undergo a short period of viral excretion, the amount of virus excreted by these animals is substantially less than the amounts excreted by animals which are persistently infected with BVDV (Young *et al.* 2006; Kosinova *et al.* 2007). Nevertheless there is a risk that acutely BVDV infected animals aid in the persistence of BVDV in a population

Virus has also been identified in semen from acutely BVDV infected bulls, a phenomenon called "prolonged testicular infection" whereby a localised persistent BVDV infection occurs in the testes (Kirkland *et al.* 1997; Givens *et al.* 2009). Despite testing antibody positive in serum, virus can be consistently detected in the semen of these animals for up to 33 months following an acute infection (Givens *et al.* 2003; Givens *et al.* 2009). While the potential for viral transmission from bulls with prolonged testicular infection appears to be low, transmission of BVDV from bulls with prolonged testicular infection to susceptible females has been shown to occur following insemination (Kirkland *et al.* 1997; Niskanen *et al.* 2002).

Cross-species transmission of BVDV has been shown to occur in species such as sheep, goats and deer as BVDV-specific antibodies and, in some species, the birth of PI animals have been reported (Snowdon *et al.* 1975; Scherer *et al.* 2001). In many countries the potential for viral transmission from these non-bovine species is low to non-existent, as cattle rarely come in to contact with these species. However, for many countries the co-pasturing of cattle and non-bovine species is common, and as such cross species transmission could pose a risk to the effective control of BVDV.

Understandably there is not a "one-size-fits-all" approach to the control and eradication of *Pestiviruses* (Ridpath 2010). The design of each program varies and is dependent on factors including; the prevalence of ruminant pestiviruses within the country, the density of animal populations, animal movement protocols, the accurate identification of infected animals/herds and the ability for ruminant pestiviruses to cross species barriers and succeed within other potential hosts.

1.6 Cross species transmission

Historically, *Pestiviruses* were named after the species from which they were originally isolated; bovine pestivirus (BVDV) from cattle and ovine pestivirus (BDV) from sheep (Uttenthal *et al.* 2005). However, numerous studies have indicated that *Pestiviruses*, particularly BVDV and BDV, are capable of infecting a wide range of species, not just those from which they were originally isolated (Edwards *et al.* 1995; Vilcek and Belak 1996). Both BVDV and BDV have been shown to elicit an antibody response in multiple species, (Nettleton 1990) primarily from the order *Artiodactyla*, an order made up of 10 families and 240 species (Grubb 2005).

Bovine viral diarrhoea virus

Since its discovery in 1946, antibodies to BVDV have been identified in cattle, sheep (Scherer *et al.* 2001), goats (Bachofen *et al.* 2013), deer (Nettleton 1990), camels (Gao *et al.* 2013), alpaca (Goyal *et al.* 2002) and a wide variety of ruminant wildlife (Nettleton 1990). While cross-species transmission of BVDV has been recorded in many species, it is of particular importance to understand its effects on sheep due to farming methods often resulting in the interaction of cattle and sheep.

Infections with BVDV in sheep have been shown to develop either by the natural transmission of the virus between cattle and sheep or through experimental, inoculation trials. The clinical effects on sheep, following BVDV infections have been reported as being similar to those seen in BVDV affected cattle or BDV affected sheep (Scherer *et al.* 2001). Table 1 shows a subset of these occurrences and the outcomes associated with BVDV infection in sheep and other domestic ruminant species.

Table 1: Outcomes of infection with BVDV in domestic ruminant species other, than cattle

Species	Strain	Cause of infection	Outcome	Reference
Sheep	BVDV-1	Unknown natural infection	Antibody and virus detected, lambs with low birth weight, poor growth, sporadic abortions	(Pratelli *et al.* 2001)
	BVDV-2	Unknown natural infection	Antibody and virus detected, lambs with low birth weight, poor growth, sporadic abortions	(Pratelli *et al.* 2001)
	MDV	Experimental infection via intravenous inoculation	Antibody response in ewes, poor lambing rates, PI lamb born	(Snowdon *et al.* 1975)
	BVDV	Experimental infection via intravenous inoculation	Antibody response in ewes, high rate of abortions and foetal deaths, low birth weights of lambs	(Parsonson *et al.* 1979)
	BVDV	Experimental infection via inoculation	Arthrogryposis, brachygnathia, anasarca, porencephaly, hydranencephaly, cerebellar hypoplasia, leukoenceophalomalacic lesions,	(Hewicker-Trautwein *et al.* 1995)
	BVDV-2	Experimental infection via intravenous and intranasal inoculation	Antibody response and severe ulcerative placentitis in ewes, poor lambing rates, birth of PI lamb	(Scherer *et al.* 2001)
	BVDV-1c	Experimental infection by subcutaneous inoculation	Antibody response in ewes, poor lambing rates, birth of PI lamb	(Evans *et al.* 2015)
Goat	BVDV-1	Unknown natural infection	Antibody and virus detected, kids with low birth weight, poor growth, sporadic abortions	(Pratelli *et al.* 2001)
	BVDV-1	Natural transmission, possibly from cattle	PI goat detected	(Krametter-Froetscher *et al.* 2010a)
	BVDV	Experimental infection via intramuscular inoculation	Antibody response in does, high rate of reproductive failure	(Loken *et al.* 1991a)
	BVDV	Infected vaccine	Antibody response in does, severe reproductive failure	(Loken *et al.* 1991b)
	BVDV-1	Natural infection via experimental exposure to PI calf	Antibody response in does, high rate of abortion, two PI kids	(Bachofen *et al.* 2013)
	BVDV-1	Natural infection via exposure to PI goat	Antibody response in does, development of PI kids	(Bachofen *et al.* 2013)

Table 1 continued.

Species	**Strain**	**Cause of infection**	**Outcome**	**Reference**
Goat	BVDV-1b	Natural infection via experimental exposure to PI calf	Antibody response	(Broaddus *et al.* 2007)
	BVDV-2a	Natural infection via experimental exposure to PI calf	Antibody response	(Broaddus *et al.* 2007)
Alpaca	BVDV	Natural infection	Antibodies to BVDV detected	(Goyal *et al.* 2002)
	BVDV-1b	Natural infection	Anorexia and lethargy in acutely infected animals, abortion, birth of PI cria	(Carman *et al.* 2005)
	BVDV-1	Natural infection	Persistent infection of cria	(Mattson *et al.* 2006)
	BVDV-1	Natural infection	Antibodies to BVDV detected, stillbirths, congenital disease and stunted growth of cria	(Barnett *et al.* 2008)
	BVDV	Natural infection	Antibodies to BVDV detected	(Cockcroft *et al.* 2015)
	BVDV-1b	Natural infection via experimental exposure to PI alpaca	Antibodies to BVDV detected, mild clinical signs including nasal discharge and elevated body temp	(Byers *et al.* 2011)
Llama	BVDV-1	Experimental infection	Antibodies to BVDV detected, no signs of disease, abortion	(Wentz *et al.* 2003b)

Irrespective of the route of transmission, acute BVDV infections in sheep have been shown to result in the development of BVDV-specific antibodies between 14 and 30 days after exposure (Scherer *et al.* 2001; Evans *et al.* 2015) although there are often no observable clinical signs of infection in these animals (Evans *et al.* 2015). While acute BVDV infections in non-pregnant sheep are clinically inapparent, acute BVDV infections in pregnant ewes have led to severe reproductive losses, similar to those observed in BVDV infected cattle. Previous studies have reported lamb/foetal losses of anywhere from 52% to 100% following infection of pregnant ewes with BVDV (Snowdon *et al.* 1975; Parsonson *et al.* 1979; Scherer *et al.* 2001; Evans *et al.* 2015). Studies have also reported severe neurological and physical abnormalities in lambs born to mothers infected during mid gestation with abnormalities including; arthrogryposis, brachygnathia, hydranencephaly, porencephaly and anasarca (Hewicker-Trautwein *et al.* 1994;

Hewicker-Trautwein *et al.* 1995). The birth of immunotolerant and persistently BVDV infected lambs have also been reported following maternal infection between 38 and 78 days of gestation (Snowdon *et al.* 1975; Parsonson *et al.* 1979; Scherer *et al.* 2001; Evans *et al.* 2015). While many BVDV PI lambs have survived only to a few weeks of age (Evans *et al.* 2015), some BVDV-2 PI sheep have been reported to survive past 6 months of age (Scherer *et al.* 2001). Either way the presence on farm of a PI lamb poses a potential risk to the spread and persistence of BVDV not only within sheep populations but also within cattle herds.

It has also been reported that alpacas and llamas (South American Camelids) can be acutely infected with BVDV and develop BVDV-specific antibodies following exposure. Similar to acute infections in cattle and sheep the clinical signs of acute BVDV infections in alpacas are often mild or non-existent but can include lethargy, anorexia and a break in the fleece(Carman *et al.* 2005). However, unlike in cattle and sheep, diarrhoea does not appear to be a consistent feature of acute BVDV infections in alpaca (Carman *et al.* 2005).

As acute BVDV infections in camelids tend to go undetected, it is more often the chronic or persistent form of infection that is reported. Common signs of chronic or persistent BVDV infections in camelids include chronic ill-thrift, poor weight gain or being underweight, severe nasal discharge and pneumonia (Carman *et al.* 2005; Mattson *et al.* 2006). Similar to cattle and sheep, when acute BVDV infections develop in pregnant camelids reproductive losses such as early embryonic death, abortion, stillbirth, premature birth (Wentz *et al.* 2003a; Carman *et al.* 2005) and the birth of persistently BVDV infected cria (Mattson *et al.* 2006; Byers *et al.* 2009) are possible.

Border disease virus

While there have been fewer reports relating to the cross-species transmission of BDV, compared to BVDV, a number of non-ovine species have shown to develop acute and/or

persistent BDV infections. These species include cattle (McFadden *et al.* 2012), goats (Nettleton *et al.* 1998) and pigs (Vilcek and Belak 1996). Table 2 shows a subset of these occurrences and the outcomes associated with BDV infection in some of these domestic species.

Table 1: Outcomes of infection with BDV in domestic ruminant species, other than sheep

Species	Strain	Cause of infection	Outcome	Reference
Cattle	BDV-1a	Mating with bull PI with BDV	Antibody response and low pregnancy rate in heifers	(McFadden *et al.* 2012)
	BDV	Mixed grazing with sheep	Antibody response in cattle	(Braun *et al.* 2013)
	BDV	Experimental infection by oral inoculation	Antibody response in one calf	(Braun *et al.* 2014)
	BDV	Co-mingling with PI sheep	Antibody response in calves	(Braun *et al.* 2014)
	BDV-3	Natural transmission from PI sheep	Antibody response in heifers, >50% abortion rate and the birth of a PI calf	(Krametter-Froetscher *et al.* 2010b)
	BDV	Natural transmission from PI calf	Antibody response in heifers, persistent foetal infection	(Braun *et al.* 2015)
Goats	BDV	Unknown natural infection	Abortions, poor kid survival, nervous signs in kids	(Toplu *et al.* 2011)
	BDV	Experimental infection via intramuscular inoculation	Antibody response in dams, high rate of reproductive failure	(Loken *et al.* 1991a)
	BDV-3	Unknown natural infection	Antibodies detected, severe diarrhoea, rough hair coat and slow growth in kids	(Li *et al.* 2013)
Deer	BDV-4	Unknown natural infection	Antibody response detected in one red deer	(Marco *et al.* 2011)

Acute infections with BDV in cattle have been shown to result from direct contact with sheep naturally and persistently infected with BDV or by experimental infection. It has been reported that these acute infections can result in seroconversion, abortion in heifers (Krametter-Froetscher *et al.* 2008; Krametter-Froetscher *et al.* 2010b) and the birth of persistently BDV infected calves (Braun *et al.* 2015).

It has also been reported that BDV can transmit naturally from persistently BDV infected cattle to naïve and susceptible heifers. In New Zealand in 2012 it was identified that poor reproductive performance in 62 dairy heifers and a low pregnancy rate of 23% was associated with a bull which was persistently infected with BDV (McFadden *et al.* 2012). Similarly, in 2015, the co-mingling of a BDV PI calf with six pregnant heifers resulted in seroconversion in all six heifers and persistent infection in the foetuses of three others (Braun *et al.* 2015).

Studies have also shown serological evidence that BDV infection can occur in goats in Africa, America, Australia and Europe (Nettleton 1990). Experimental infections of BDV in goats have led to severe placentitis and clinical and pathological signs similar to those seen in sheep infected with BDV (Nettleton 1990).

1.7 History of ruminant pestiviruses in Australia

Clinical and pathological symptoms of mucosal disease virus (MDV) were first reported in Australian cattle in 1957 (Blood *et al.* 1957). While diarrhoea and nasal/buccal mucosal lesions were observed in these animals antibodies to the virus were not reported and transmission of the virus to susceptible animals was unsuccessful. The next documented case of BVDV related disease was two years later, in 1959, where severe scouring was observed in a group of South Australian yearling cattle (McCormack *et al.* 1959). Despite these two earlier cases, the first serological evidence for BVDV was not reported in Australia until 1962 (French 1962). Since this time the understanding and effect of BVDV in Australian cattle has increased and it is now considered as one of the top five cattle diseases in Australia, estimated to cost the cattle industry a total of $114.4 million per annum (MLA 2016).

Unlike many other cattle producing countries there are a limited number of BVDV strains identified in Australia. A study in 2010 reported that the more clinically severe BVDV-2 species was not present in Australia and that while BVDV-1a was identified, there was a 96%

predominance of BVDV-1c in Australia (Ridpath *et al.* 2010). This limited variability in strains is thought to be as a result of Australia's geographical location and strict biosecurity measures.

In comparison, the first reported case of *Pestivirus* infection in sheep was in 1964. This study identified antibodies to the C24V virus (BVDV-1*)* in sheep from Victoria and Tasmania (French and Snowdon 1964). In 1972 a condition of lambs, presenting with hairy birth coat, poor growth, nervous abnormalities and suggestive of BDV, was reported (Acland *et al.* 1972). Despite the similarity with BDV it was noted that these signs could also be due to infection with BVDV. While it appears that infections with BDV were not common in Australian sheep, many of these earlier reports did not have the diagnostic methods which are available today. As a result the misdiagnosis of the infecting *Pestivirus* strain may have resulted. Nonetheless, there is limited published information available regarding *Pestivirus* infections in Australian sheep, particularly from the last 30 years.

Prevalence

Reported historical prevalences for BVDV (or MDV) and/or BDV in Australian sheep and cattle are presented in Table 3. The first seroprevalence survey for antibodies to BVDV in Australian livestock was a 1964 study. This study reported seroprevalences in cattle, at the individual animal level, ranging from 13% in South Australia to 65% in Victoria. This study also reported seroprevalences in sheep at the individual animal level of 14% in Victoria and 24% in Tasmania (French and Snowdon 1964). Similarly, the first national seroprevalence study was undertaken in 1967 and reported that 61% of Australian cattle and 89% of Australian cattle herds were positive for BVDV antibodies (St George *et al.* 1967b). Since this time national prevalences to BVDV appear to have remained stable in cattle populations. A non-peer reviewed seroprevalence study from 2010 reported a national herd seroprevalence of 86% while state seroprevalences ranged from 83% in Queensland to 100% in South Australia (Taylor 2010).

In comparison, the most recent disease prevalence study, as far as the authors are aware, relating to *Pestivirus* infections in sheep was in 1984. While this study reported that <4% of Victorian sheep flocks showed signs of BDV infection, results were determined through a postal survey sent to stud sheep producers and not by diagnostic testing (Lim and Carnegie 1984). The most recent seroprevalence study in sheep was in 1971 and reported a national flock seroprevalence of 13% (St George 1971). Since this time it remains unknown to what extent BDV or BVDV are present in Australian sheep populations.

Table 3: Reported prevalences of *Pestivirus*-specific antibodies in Cattle and Sheep from Australia

Year	State	Species	Prevalence	Herd/Flock or Animal level	Infecting Pestivirus Species	Number Sampled	Methods	Reference
1964	VIC	Cattle	65%	Animal	BVDV-1	215	VNT	(French and Snowdon 1964)
	QLD	Cattle	51%	Animal	BVDV-1	142	VNT	(French and Snowdon 1964)
	WA	Cattle	63%	Animal	BVDV-1	157	VNT	(French and Snowdon 1964)
	TAS	Cattle	51%	Animal	BVDV-1	69	VNT	(French and Snowdon 1964)
	SA	Cattle	13%	Animal	BVDV-1	15	VNT	(French and Snowdon 1964)
	VIC	Sheep	14%	Animal	BVDV-1	208	VNT	(French and Snowdon 1964)
	TAS	Sheep	24%	Animal	BVDV-1	59	VNT	(French and Snowdon 1964)
1967	National	Cattle	61%	Animal	MDV	4504	VNT	(St George *et al.* 1967a)
	National	Cattle	89%	Herd	MDV	315	VNT	(St George *et al.* 1967a)
1971	National	Sheep	13%	Flock	MDV	416	VNT	(St George 1971)
1971	National	Sheep	8%	Animal	MDV	832	VNT	(St George 1971)

Table 3 continued

Year	State	Species	Prevalence	Herd/Flock or Animal level	Infecting Pestivirus Species	Number Sampled	Methods	Reference
1984	VIC	Sheep	<4%	Flock	BDV	481	Postal survey for symptoms of BDV	(Lim and Carnegie 1984)
1997	SA	Cattle	77%	Animal	BVDV	617	AGID	(Durham and Paine 1997)
2006	QLD	Cattle	45%	Animal	BVDV	7838	VNT	(Taylor *et al.* 2006)
2010	NSW	Cattle	97%	Herd	BVDV	91	AGID/ELISA	(Taylor 2010)
	NT	Cattle	96%	Herd	BVDV	25	AGID/ELISA	(Taylor 2010)
	QLD	Cattle	83%	Herd	BVDV	105	AGID/ELISA	(Taylor 2010)
	SA	Cattle	100%	Herd	BVDV	7	AGID/ELISA	(Taylor 2010)
	TAS	Cattle	87%	Herd	BVDV	23	AGID/ELISA	(Taylor 2010)
	WA	Cattle	100%	Herd	BVDV	146	AGID/ELISA	(Taylor 2010)
	VIC	Cattle	94%	Herd	BVDV	9	AGID/ELISA	(Taylor 2010)
	National	Cattle	86%	Herd	BVDV	406	AGID/ELISA	(Taylor 2010)

1.8 Thesis outline

In order to effectively control and/or eradicate BVDV, understanding cross-species transmission and the role of non-bovine species in the spread and persistence of the virus is critical. A large number of non-bovine species have previously been reported as susceptible to BVDV infection however, for this body of work, sheep were of particular interest due to the close affiliation between BDV and BVDV and the large number of sheep farmed in Australia.

In 2015 Meat and Livestock Australia reported that the National herd sizes for sheep and cattle (both beef and dairy) in Australia were 70.9 million and 27.4 million, respectively (MLA 2016). Of these, 74% of sheep and 39% of cattle are farmed across South Australia, Victoria and New South Wales (MLA 2016) a total area less than 30% of Australia's total land mass (Geoscience Australia 2004). While it is unknown what the prevalence of BVDV is in Australian sheep flocks, in 2010 it was reported that the majority (86%) of Australian cattle herds have been exposed to BVDV (Taylor 2010). The high prevalence of BVDV in cattle herds and the close proximity, and large numbers, of sheep and cattle in Australia highlights a need in understanding the effects of cross-species transmission of BVDV between cattle and sheep.

While previous studies have reported various effects relating to BVDV infection in sheep, these studies have used types or strains of the virus not predominant in Australia with the majority of these studies undertaken prior to 2002. As such, results may not be indicative of the effects relating to current acute and/or persistent infections with BVDV-1c in sheep. It remains unknown if current acute BVDV infections in sheep are clinically apparent, what reproductive outcomes result from infection of pregnant ewes during early/mid gestation, if viable BVDV PI lambs can develop and if they can in turn infect susceptible sheep and cattle. It is also unknown what the seroprevalence to *Pestiviruses* is in Australian sheep flocks and what other Australian livestock species are at risk of infection. Therefore the objective of this thesis was

to investigate important aspects regarding the epidemiological and clinical impacts of BVDV infection in sheep and other commonly farmed Australian livestock species.

The individual projects of this thesis aimed to:

1. validate a commercially available, diagnostic test for the accurate detection of BVDV-specific antibodies in sheep serum
2. determine what clinical signs and reproductive loses are associated with acute BVDV-1c infection in pregnant ewes
3. characterise the pathological consequences seen in lambs following acute maternal BVDV-1c infection between 59 and 69 days gestation
4. investigate the infectivity of a BVDV-1c PI lamb with regards to susceptible sheep and cattle
5. produce viable and persistently BVDV-1c infected lambs
6. investigate the infectivity of sheep undergoing an acute BVDV-1c infection
7. determine what clinical signs are associated with acute BVDV-1c infection in alpacas
8. understand the prevalence of BVDV-specific antibodies in potential, non-bovine, reservoir hosts in Australia

In Chapter 2 three serological assays for the detection of BVDV-specific antibodies were validated for use on sheep serum to enable the accurate detection of acute BVDV infections in sheep. In Chapter 3 the clinical effects associated with acute BVDV-1c infections in pregnant ewes was investigated. The first paper in this chapter reports on the clinical and reproductive outcomes observed in ewes acutely infected with BVDV-1c during early/mid gestation. The second paper reports on the pathological malformations in lambs associated with foetal infection with BVDV-1c during early/mid gestation. In Chapter 4 the ability of BVDV-1c infected sheep to infect susceptible sheep and cattle was investigated. The first paper in this chapter reports on the infectivity of a neonatal, BVDV-1c PI lamb and its ability to infect

susceptible sheep and cattle. The second paper reports on whether sheep undergoing an acute BVDV-1c infection can infect co-housed, susceptible sheep. In Chapter 5 the clinical and haematological effects associated with acute BVDV-1c infections in alpacas was investigated. Lastly, in Chapter 6, seroprevalences for *Pestivirus*-specific antibodies were determined for South Australian sheep and Northern Territory water buffalo.

Chapter 2: Diagnosis of BVDV-specific antibodies in sheep serum

To effectively control a disease such as BVDV, access to accurate and reliable diagnostic tools is required. For BVDV, the differentiation between individual animals that are naïve, acutely infected or persistently infected with the virus is important. Current diagnostic tests for BVDV detection are used to identify either BVDV-specific antibodies, BVDV-specific antigen or the virus itself (Saliki and Dubovi 2004).

In cattle, diagnostic tests for BVDV antibodies are commonly used to screen herds for sero-converted animals. Such diagnostic tests include agarose gel immunodiffusion (AGID), virus neutralisation test (VNT) and enzyme linked immunosorbent assay (ELISA) (Lanyon *et al.* 2014a). In Australia, the AGID has been the preferred antibody detection method for many years (Kirkland and Mackintosh 2006), although antibody ELISAs are growing in popularity due to being rapid to perform, relatively inexpensive and appropriate when testing large numbers of samples (Lanyon *et al.* 2014a).

Although BVDV is predominantly a disease of cattle, antibodies to the virus have been reported in a wide range of non-bovine species, including sheep (Pratelli *et al.* 2001). Despite the quantity of diagnostic tests available for the detection of BVDV-specific antibodies in cattle, the validation of many of these tests is lacking for non-bovine species. In order to accurately determine the full effect of BVDV infections on sheep and, in turn determine whether sheep populations might be acting as a reservoir source of BVDV infection, an accurate test for identifying BVDV-specific antibodies in sheep first needs to be identified. Due to the large number of samples to be tested and financial constraints associated with the following studies, an accurate, rapid and relatively inexpensive diagnostic method was required.

2.1 Identifying BVDV-1specific antibodies in sheep serum

The primary outcome for this manuscript was to compare three serological diagnostic assays: the AGID and two commercially available ELISAs, and validate them for the detection of BVDV-specific antibodies in sheep serum, following experimental infection with BVDV-1c. This manuscript has been published by the *Journal of Veterinary Diagnostic Investigation*, vol 29(2), pp 181-185.

Original article: Investigation of AGID and two commercial ELISAs for the detection of Bovine viral diarrhea virus-specific antibodies in sheep serum

CA Evans, SR Lanyon and MP Reichel (2017)

Investigation of AGID and two commercial ELISAs for the detection of Bovine viral diarrhea virus-specific antibodies in sheep serum

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Contribution to the Paper	Designed and implemented animal trial, managed animal trial, collected and analysed samples, undertook data analysis and interpretation, drafted and edited manuscript.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 24/7/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Sasha R Lanyon
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Signature	Date 24/6/17

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Investigation of AGID and two commercial ELISAs for the detection of *Bovine viral diarrhea virus*–specific antibodies in sheep serum

Caitlin A. Evans,[1] Sasha R. Lanyon, Michael P. Reichel

Abstract. Effective control and the eventual eradication of *Bovine viral diarrhea virus* (BVDV) from cattle populations depend on the accurate identification of infected animals. Although typically a disease agent of cattle, BVDV is known to infect a wide variety of nonbovine species, including sheep. However, validation of serologic tests in these nonbovine species, particularly sheep, is lacking. We analyzed 99 sheep sera (57 samples from *Pestivirus*-naive sheep, and 42 samples from BVDV-inoculated sheep) in order to investigate 3 serologic tests: the agarose gel immunodiffusion (AGID) and 2 commercial enzyme-linked immunosorbent assays (ELISAs) for detection of BVDV antibodies. At the manufacturer's cutoff thresholds, the AGID performed with 95.2% diagnostic sensitivity; ELISA-A performed with sensitivity of 90.5% and ELISA-B with 69.1%. All 3 tests performed with 100% diagnostic specificity. Two-graph receiver operating characteristic analysis showed that performance characteristics were optimized, such that both diagnostic sensitivity and diagnostic specificity were >95% for both ELISAs, if the thresholds were altered to 34.9% inhibition for ELISA-A and 63.5 signal-to-noise ratio for ELISA-B.

Key words: *Bovine viral diarrhea virus*; serologic tests; sheep; test validation.

Bovine viral diarrhea virus (BVDV; family *Flaviviridae*) is a virus of the *Pestivirus* genus, which also includes *Border disease virus* (BDV) in sheep and *Classical swine fever virus* in pigs.[2] BVD is a highly prevalent disease of cattle that is associated with immunosuppression[4] and reproductive losses.[7] It is widely acknowledged that BVDV has significant economic impacts on cattle industries, with estimates ranging from US$40,000 to US$100,000 per infected herd per year.[9] Several studies have shown that systematic control or eradication of BVDV is economically beneficial in endemically infected cattle populations.[8,13] Control of BVDV is largely reliant on testing for BVDV-specific antibodies, virus, viral RNA, or viral antigen. Detection of the virus (or its subunits) is critical for the identification of persistently infected (PI) individuals, which are epidemiologically important for the maintenance and spread of the virus.[3] The detection of specific antibodies indicates seroconversion in response to a previous infection in unvaccinated animals. Serologic tests for BVDV antibodies enable naive and exposed herds to be identified. Animals testing negative in a seropositive herd are consistent with PI animals and may warrant further testing for the presence of the virus.

In Australia and New Zealand, there are a number of serologic tests available for the identification of BVDV-specific antibodies in cattle, including agarose gel immunodiffusion (AGID), virus neutralization test (VNT), and enzyme-linked immunosorbent assay (ELISA; Australian and New Zealand Standard Diagnostic Procedures. Ruminant pestivirus infections, 2006, https://goo.gl/NwD5xN).[20] Although it is still thought of as the gold standard for identification of BVDV infection, VNT is relatively expensive and labor intensive when compared to ELISA and AGID, which are rapid and inexpensive, even for large-scale testing. One commercial ELISA has been validated relative to the VNT and was shown to have excellent performance on cattle sera, with diagnostic sensitivity (DSe) of 96.7% and diagnostic specificity (DSp) of 97.1%.[10] That same study showed that the performance of the AGID did not differ significantly from that of the ELISA.[10]

Although BVD is most well known as a disease of cattle, many studies have identified infection in a wide range of other ruminant and pseudoruminant species, including

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sheep,[21] goats,[1] deer,[15] and alpaca.[6] Similarly, BDV infection has been reported in cattle, with a bull in New Zealand identified as being persistently infected with BDV.[14] Infection with BVDV in sheep is of particular importance given their proximity to cattle in some commercial production systems, and the reproductive losses that have been observed following infection of pregnant ewes with BVDV.[5] For example, abortion rates of 52–100% have been observed in ewes infected with BVDV at 25–100 d gestation,[5,16,22] and the birth of BVDV PI lambs has also been reported.[5,22]

In Australia and New Zealand, there is little information available regarding the current state of infections with pestiviruses in sheep. The only known study regarding the prevalence of pestiviruses in sheep in Australia is a postal survey in 1979, which identified only 4% of flocks with *Pestivirus* infection.[11] However, the prevalence of BVDV in cattle is reported to be >86% in Australia (Taylor L. Findings of an Australia wide serological survey of beef and dairy herds for bovine viral diarrhoea virus conducted between 2007 and 2009. Australian Cattle Vets 2010;57) and 63% in New Zealand.[12] It has also been identified that only the BVDV-1 genotypes can be found in this region,[11,23] with a predominance (96%) of BVDV-1c in Australia.[18] It is clear that there is a gap in the knowledge regarding *Pestivirus* infections in sheep, but, in order to accurately undertake a prevalence survey of this region, the correct detection techniques need to be identified. Therefore, we sought to optimize 3 serologic assays: the AGID and 2 commercially available ELISAs for the detection of BVDV-specific antibodies in sheep serum.

Materials and methods

Animals

Fifty-four sheep were used in our study: 25 wethers and 29 ewes; all were Merino × Border Leicester ewes and wethers from the University of Adelaide's commercial flock at its Roseworthy Campus, South Australia. The flock is known to be *Pestivirus* naive based on previous testing. All animal experiments were approved by the University of Adelaide Animal Ethics Committee prior to this project (S-2012-240 and S-2012-248).

Sera

Blood samples were collected from all 54 sheep at the beginning of the project. The naive status was confirmed in all of the assays used in our study. Forty-two of the sheep were then given subcutaneous inoculations of 2 mL of freeze–thawed serum previously collected from a South Australian BVDV PI cow. Serum from the PI cow was typed as BVDV-1c.[5] Three sheep were not inoculated, although remained with the flock; the remaining 9 sheep were excluded from further participation in the project for practical reasons.

Blood samples were collected 6 wk postinoculation from the 42 inoculated sheep and from the 3 sheep that were not inoculated. Therefore, in total, 57 blood samples were collected from *Pestivirus*-naive sheep, and 42 samples from BVDV-inoculated sheep. All blood samples were centrifuged at 3,000 × *g* for 20 min, and serum was aliquoted into 1.2-mL microtubes before storage at −80°C until assayed.

Detection of BVDV antibodies using AGID and ELISA

The 99 serum samples were tested using an AGID[a] assay that uses the C24V reference strain (BVDV-1a)[19] as the antigen, with a score of 0 considered negative and scores of 1–3 considered positive. The AGID is recognized to be group reactive and capable of detecting antibodies to both BVDV and BDV (https://goo.gl/15GnYh).

The serum samples were also tested using 2 commercial BVDV antibody ELISAs. Samples were analyzed by the first ELISA (A)[b] as per the manufacturer's instructions where results were expressed in percentage inhibition (%Inh). The manufacturer's recommended cutoff for positive samples was >50%Inh. This ELISA has been stated by the manufacturer to detect antibodies to BVDV-1a, -1b, and -2.

Samples were also analyzed by a second ELISA (B),[c] wherein results were expressed as signal-to-noise ratio (S/N). The manufacturer's recommended cutoff for positive samples for that assay is ≤40 S/N (note: as this is a blocking ELISA, low S/N values signify a positive result). This ELISA has been stated by the manufacturer to detect antibodies to both BVDV and BDV.

Statistical analysis

The performance characteristics of the AGID and both ELISAs were calculated using EpiTools (https://goo.gl/NCMJU0). The ELISA results were dichotomized using the manufacturer's recommended cutoffs, and the true exposure status (inoculated or uninoculated) was used as the reference status. A confidence level of 95% was used.

A 2-graph receiver operating characteristic (TG-ROC) analysis (https://goo.gl/LG9XZx) was performed on the data from each ELISA. Data from the blocking ELISA-B were transformed using the following formula: 100 – (S/N). From the TG-ROC analysis, optimal cutoff values for maximizing test efficiency (minimizing misclassification) or maximizing Youden J statistic were determined, and the corresponding test performance characteristics, with confidence intervals, were calculated. Cutoff values

Table 1. Performance characteristics of the agarose gel immunodiffusion (AGID) and 2 enzyme-liked immunosorbent assays (ELISAs) at the manufacturer's cutoffs.*

Test	Diagnostic sensitivity	Diagnostic specificity
AGID[a]	95.2 (83.8–99.4)	100.0 (93.7–100.0)
ELISA-A[b]	90.5 (77.4–97.3)	100.0 (93.7–100.0)
ELISA-B[c]	69.1 (52.9–82.4)†	100.0 (93.7–100.0)

* Numbers in parentheses are 95% confidence intervals. An AGID score of 0 was considered negative; scores of 1–3 were considered positive. ELISAs were interpreted according to manufacturer's cutoffs for positive samples of >50%Inh and <40 S/N, for ELISA-A and ELISA-B, respectively. Lowercase letters refer to Sources and Manufacturers section.
† Two-tailed *z*-test showed significant difference from AGID ($p = 0.0018$) and ELISA-A ($p = 0.0146$).

for ELISA-B were extracted from the TG-ROC analysis in the 100 – (S/N) manipulated form, then back-calculated and are presented here as S/N.

Pairwise 2-tailed *z*-tests (https://goo.gl/xDBbGz) were performed to examine statistically significant differences in performance characteristics between tests, and before and after optimization. A *p* value of <0.05 was considered significant.

Results

The baseline samples from the 57 uninoculated sheep all tested negative for BVDV antibodies in all 3 assays at the manufacturer's cutoffs. Conversely, of the 42 exposed sheep, 27 of these tested positive on each of the 3 assays and all but 1 (41) sheep were found to be positive in at least 1 of the assays. The 3 assays (interpreted at manufacturer's cutoffs) all performed with 100% DSp but lower DSe (Table 1), with ELISA-B showing significantly lower sensitivity than the AGID ($p = 0.0018$) and ELISA-A ($p = 0.0146$).

At the manufacturer's cutoff value for each of the 3 tests, DSp was optimal (100%) but DSe was limited, particularly for ELISA-B (Table 1). AGID had the highest sensitivity (95.2%), followed by ELISA-A (90.5%) and ELISA-B (69.1%).

The TG-ROC analysis identified 2 possible optimal cutoffs for each of the ELISAs, in order to maximize test efficiency (minimize misclassification) and maximize Youden J statistic, respectively (Figs. 1, 2). For ELISA-A, DSe improved at both optimized cutoffs relative to the manufacturer's cutoff, but the improvements were not statistically significant. No changes in DSp were observed for ELISA-A. For ELISA-B, significant improvements in DSe were observed at both the adjusted cutoff for maximal test efficiency ($p = 0.0005$) and the cutoff for maximized Youden J statistic ($p < 0.0001$), with no significant reductions in DSp. The optimal cutoffs and corresponding test characteristics are presented in Table 2. There were no significant differences in performance characteristics among the 3 assays (AGID, ELISA-A, and ELISA-B) when the optimal ELISA thresholds were used.

Discussion

In order to improve the DSe of both ELISA kits, TG-ROC analysis was undertaken. TG-ROC results for ELISA-A indicated that by lowering the manufacturer's cutoff from 50%Inh to 34.9%Inh, specificity remained optimal but sensitivity increased from 90.5% to 95.2% for both test efficiency and Youden J statistic. In comparison, TG-ROC analysis for ELISA-B returned different cutoff values for test efficiency and Youden J statistic. Results of the TG-ROC analysis indicated that in order to increase test efficiency and minimize the number of misclassified samples, increasing the manufacturer's cutoff from 40 S/N to 63.5 S/N maintained an optimal specificity while increasing sensitivity from 69.1% to 97.6%. However, in order to maximize the Youden J statistic, the manufacturer's cutoff had to be increased to 83 S/N. At this cutoff, DSe was 100%; however, specificity decreased from 100% to 98.2%. Both identified cutoff values for ELISA-B are valid options.

Which cutoff value is preferable is dependent on the purpose of the test and the costs associated with false-negatives relative to false-positives. If the accurate identification of seropositive individuals is the objective, then the 83 S/N cutoff is appropriate wherein sensitivity is maximized. By contrast, if the accurate identification of negative animals is critical—for example, if antibody screening is being used for identification of negative individuals that are potential PI animals as a precursor to virus testing—then specificity is critical and the 63.5 S/N is appropriate. The correct identification of these individuals is critical in most control and eradication programs, as the elimination of a PI animal removes the most epidemiologically important source of infection.[17] However, in areas that use vaccination, PI animals may be antibody-positive as a result of an immunologic response to a heterologous strain. Therefore, caution should be taken when interpreting results from herds that use vaccination as a control method.

The AGID and 2 commercial ELISAs assessed in our study have useful performance characteristics for detection of BVDV-specific antibodies in sheep sera. We suggest that in order to optimize performance characteristics to >95% for both DSe and DSp of both ELISAs, the thresholds be altered to 34.9%Inh for ELISA-A and 63.5 S/N for ELISA-B. It is, however, important to be aware of limitations in the ability of serologic tests to discriminate between *Pestivirus* species. To ensure that the identification of BVDV-infected or non-infected sheep is correct, further work is required on the

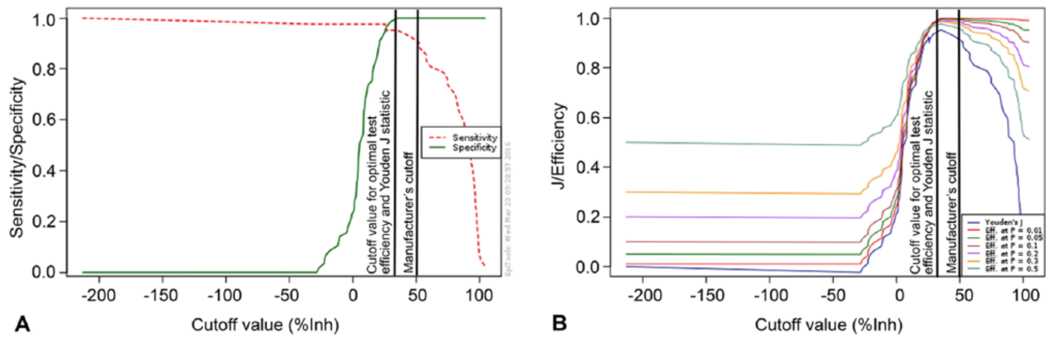

Figure 1. Output of a 2-graph receiver operating characteristics analysis (**A**) and Youden J statistic and test efficiency (**B**) of ELISA-A[b] for the detection of antibodies specific to *Bovine viral diarrhea virus*, using the true infection status of 99 sheep sera as the reference standard. ELISA results are expressed as percent inhibition (%Inh). Solid black lines indicate suggested cutoff values for Youden J statistic and test efficiency as well as the manufacturer's recommended cutoff.

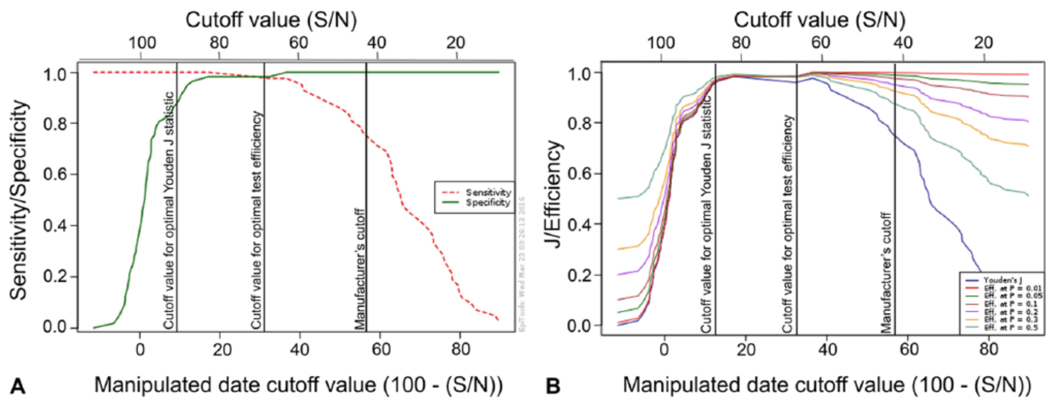

Figure 2. Output of a 2-graph receiver operating characteristics analysis (**A**) and Youden J statistic and test efficiency (**B**) of ELISA-B[c] for the detection of antibodies specific to *Bovine viral diarrhea virus*, using the true infection status of 99 sheep sera as the reference standard. ELISA results are expressed as signal-to-noise (S/N) ratio (top axis); however, data were transformed (100 – S/N; bottom axis). Solid black lines indicate suggested cutoff values for Youden J statistic and test efficiency as well as the manufacturer's recommended cutoff.

Table 2. Optimal cutoff values as determined by 2-graph receiver operating characteristic analysis for maximizing test efficiency (minimizing misclassification) and maximizing Youden J statistic and the corresponding test performance characteristics for ELISA-A[b] and ELISA-B.[c,]*

Test	Criteria	Positive cutoff	Diagnostic sensitivity	Diagnostic specificity
ELISA-A (%Inh)	Test efficiency	>34.9	95.2 (84.2–98.7)	100.0 (93.7–100.0)
	Youden J statistic	>34.9	95.2 (84.2–98.7)	100.0 (93.7–100.0)
ELISA-B (S/N)	Test efficiency	<63.5	97.6 (87.7–99.6)†	100.0 (93.7–100.0)
	Youden J statistic	<83.0	100.0 (91.6–100.0)‡	98.2 (90.7–99.7)

* Numbers in parentheses are 95% confidence intervals. %Inh = percent inhibition; S/N = signal-to-noise ratio.
† Significant improvement over diagnostic sensitivity at manufacturer's cutoff; $p = 0.0005$.
‡ Significant improvement over diagnostic sensitivity at manufacturer's cutoff; $p < 0.0001$.

cross-reactivity of specific antibodies against other *Pestivirus* species, particularly BDV, with the assays used in this study.

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Authors' contributions

CA Evans and SR Lanyon contributed to acquisition, analysis, and interpretation of data, and drafted the manuscript. MP Reichel contributed to analysis and interpretation of data. All authors contributed to conception and design of the study, critically revised the manuscript, gave final approval, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Sources and manufacturers

a. AGID, Elizabeth Macarthur Agriculture Institute (EMAI), New South Wales, Australia.
b. PrioCHECK BVDV Ab P80 ELISA, Prionics, Martinsried, Germany.
c. BVDV p80 protein antibody test kit, IDEXX Laboratories, Rydalmere, New South Wales, Australia.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References

1. Bachofen C, et al. Persistent infections after natural transmission of bovine viral diarrhoea virus from cattle to goats and among goats. Vet Res 2013;44:32.
2. Becher P, et al. Genetic diversity of pestiviruses: identification of novel groups and implications for classification. Virology 1999; 262:64–71.
3. Brownlie J, et al. Pathogenesis and epidemiology of bovine virus diarrhoea virus infection of cattle. Ann Rech Vet 1987;18:157–166.
4. Edwards S, et al. Evidence for an immunocompromising effect of bovine pestivirus on bovid herpesvirus 1 vaccination. Vet Res Commun 1986;10:297–302.
5. Evans CA, et al. Reproductive performance in experimentally BVDV infected ewes and seroconversion rates in sheep co-mingled with BVDV PI calves. Small Rumin Res 2015;123: 314–319.
6. Goyal SM, et al. Isolation of bovine viral diarrhea virus from an alpaca. J Vet Diagn Invest 2002;14:523–525.
7. Grooms DL. Reproductive consequences of infection with bovine viral diarrhea virus. Vet Clin North Am Food Anim Pract 2004;20:5–19.
8. Häsler B, et al. An economic model to evaluate the mitigation programme for bovine viral diarrhoea in Switzerland. Prev Vet Med 2012;106:162–173.
9. Houe H. Economic impact of BVDV infection in dairies. Biologicals 2003;31:137–143.
10. Lanyon SR, et al. Validation and evaluation of a commercially available ELISA for the detection of antibodies specific to bovine viral diarrhoea virus (bovine pestivirus). Aust Vet J 2013;91:52–56.
11. Lim CF, Carnegie PR. A survey of hairy shaker disease (border disease, hypomyelinogenesis congenita) in sheep. Aust Vet J 1984;61:174–177.
12. Littlejohns IR, Horner GW. Incidence, epidemiology and control of bovine pestivirus infections and disease in Australia and New Zealand. Rev Sci Tech 1990;9:195–205.
13. Løken T, Nyberg O. Eradication of BVDV in cattle: the Norwegian project. Vet Rec 2013;172:661.
14. McFadden AM, et al. The first case of a bull persistently infected with Border disease virus in New Zealand. N Z Vet J 2012;60:290–296.
15. Nettleton PF. Pestivirus infections in ruminants other than cattle. Rev Sci Tech 1990;9:131–150.
16. Parsonson IM, et al. The effects of bovine viral diarrhoea-mucosal disease (BVD) virus on the ovine fetus. Vet Microbiol 1979;4:279–292.
17. Presi P, et al. Bovine viral diarrhea (BVD) eradication in Switzerland—experiences of the first two years. Prev Vet Med 2011;99:112–121.
18. Ridpath JF, et al. Prevalence and antigenic differences observed between *Bovine viral diarrhea virus* subgenotypes isolated from cattle in Australia and feedlots in the southwestern United States. J Vet Diagn Invest 2010;22:184–191.
19. Ridpath JF, et al. Change in predominance of *Bovine viral diarrhea virus* subgenotypes among samples submitted to a diagnostic laboratory over a 20-year time span. J Vet Diagn Invest 2011;23:185–193.
20. Saliki JT, Dubovi EJ. Laboratory diagnosis of bovine viral diarrhea virus infections. Vet Clin North Am Food Anim Pract 2004;20:69–83.
21. Scherer CF, et al. Experimental infection of pregnant ewes with bovine viral diarrhea virus type-2 (BVDV-2): effects on the pregnancy and fetus. Vet Microbiol 2001;79:285–299.
22. Snowdon WA, et al. The reaction of pregnant ewes to inoculation with mucosal disease virus of bovine origin. J Comp Pathol 1975;85:241–251.
23. Vilcek S, et al. Genetic typing of pestiviruses from New Zealand. N Z Vet J 1998;46:35–37.

2.2 Chapter discussion

Achieving a high sensitivity and specificity for diagnostic tests is required for the accurate identification of infected animals. However, concurrent one hundred percent sensitivity and specificity cannot always be achieved and sometimes one performance characteristic has to be favoured over the other. With regards to the diagnostic testing for antibodies to BVDV, the type of infection we are trying to detect, either acute or persistent infection, is important when determining which characteristic is more significant. Is it more important to detect individuals, which are antibody positive (are or have been acutely infected with BVDV) or individuals which are antibody negative (BVDV naïve or persistently BVDV infected)?

It was reported that by changing the manufacturer's cut-off for the two ELISAs investigated in this study, sensitivity of the test, and the accurate identification of antibody positive sheep, could be increased in both assays. Results of the Prionics ELISA showed that by lowering the manufacturer's cut-off from the suggested 50%Inh to 34.9%Inh, sensitivity was increased from 90.5% to 95.2% while maintaining optimal (100%) specificity (the accurate detection of antibody negative animals). Similarly, results for the IDEXX ELISA showed that by increasing the manufacturer's cut-off from 40S/N to 63.5S/N, sensitivity could be increased from 69.1% to 97.6%, while likewise maintaining optimal specificity. Optimal sensitivity could be achieved in the IDEXX ELISA, although this comes at a cost to specificity and was not considered to be a viable option due to the possibility of misidentifying persistently BVDV infected sheep as acutely infected.

From the results of this study it was decided that the IDEXX ELISA, with a positive cut-off of 63.5S/N, was most suitable for use throughout the remaining studies, due to it achieving the highest sensitivity whilst retaining 100% specificity of the three diagnostic tests. Now that a

suitable diagnostic test has been identified to detect *Pestivirus* infections in sheep, the true relationship between BVDV infections and sheep populations in Australia can be investigated.

Chapter 3: Outcomes of acute BVDV-1c infections in pregnant ewes

The control of BVDV in cattle populations has been successful in many parts of the world (Lindberg *et al.* 2006; Presi *et al.* 2011) due to the identification and removal of PI cattle (Presi *et al.* 2011). However, many of these control programs focus on cattle populations alone and ignore the potential risk posed to, and by, other non-bovine species. In Australia, cattle and sheep often live in close proximity throughout many farming regions and it has previously been reported that BVDV-1c can naturally transmit from cattle to sheep (Evans *et al.* 2015). As a result it has been questioned whether, in the absence of continual re-infection from endemically infected cattle populations, the 70.9 million sheep found within Australia (MLA 2016) could pose a risk to the spread and persistence of BVDV within Australian cattle populations.

Previously it has been reported that acute infections in pregnant ewes, with varying strains of BVDV, can result in high abortion/stillborn rates, neurological lesions, skeletal deformities and/or the birth of persistently BVDV infected lambs (Hewicker-Trautwein *et al.* 1995; Scherer *et al.* 2001). Similarly, for ewes infected with the predominant Australian strain, BVDV-1c, highly reduced lambing rates and the birth of a persistently infected lamb have been reported (Evans *et al.* 2015). However, a flaw in this study was the absence of a similarly managed, uninfected and pregnant ewe flock. Although lambing losses were severe in this study, it was not clear if the reported lambing losses were due primarily to BVDV-1c infection or other management practices. Therefore, the full reproductive effect BVDV-1c infection has on lambing rates in Australian sheep is unknown. Furthermore, the clinical signs of infection present in pregnant ewes, acutely infected with BVDV-1c, is also unknown.

In comparison, it has been well documented that animals persistently infected (PI) with BVDV are the main transmission source of BVDV and that the identification and elimination of these animals is critical when controlling the virus. Although predominantly a disease of cattle, the

birth of persistently BVDV infected lambs has been reported on a number of occasions (Snowdon *et al.* 1975; Scherer *et al.* 2001; Evans *et al.* 2015). In many cases the viability of these BVDV PI lambs has been poor however even a limited presence on farm may aid in the spread of the virus to susceptible animals. Therefore before the risk posed by BVDV-1c PI lambs in the spread of BVDV within Australia can be investigated, it first needs to be determined if viable BVDV-1c PI lambs can be produced.

It was recently reported that the experimental infection of a pregnant ewe at, approximately, 67 days gestation resulted in the birth of a BVDV-1c PI lamb (Evans *et al.* 2015). This finding is in line with the gestational age at infection (0 – 80 days) of lambs born persistently infected with Border disease virus (Nettleton 1990) and suggests an appropriate timeframe for foetal infection in order to study the successful development and viability of persistently BVDV-1c infected lambs.

The following two manuscripts report the results of an experimental trial whereby ewes were experimentally inoculated with BVDV-1c between 59 and 69 days of gestation. The timing of infection was determined such that the potential for PI lamb development was increased and abortions/absorptions decreased.

3.1 Findings in ewes

The primary outcome of this manuscript was to report on the clinical and reproductive differences between ewes infected with BVDV-1c during early/mid gestation compared to ewes which remained naïve to the virus. The following manuscript details these findings and has been published by the *Journal of Small Ruminant Research* Vol 149, Pp. 121-127.

Original article: Clinical responses and reproductive outcomes in pregnant ewes experimentally infected with bovine viral diarrhoea virus (Type-1c) between days 59 and 69 of gestation

CA Evans, MP Reichel, F Hemmatzadeh and PD Cockcroft (2017)

Clinical responses and reproductive outcomes in pregnant ewes experimentally infected with bovine viral diarrhoea virus (Type-1c) between days 59 and 69 of gestation

Journal of Small Ruminant Research Vol 149, Pp. 121-127

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Overall percentage (%)	85%
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Clinical responses and reproductive outcomes in pregnant ewes experimentally infected with bovine viral diarrhoea virus (type-1c) between days 59 and 69 of gestation

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ABSTRACT

Low lambing rates and the birth of persistently infected (PI) lambs have previously been recorded in sheep infected with BVDV. However, there is little information available in regards to the clinical profile of acute BVDV infections in sheep. As a result the aim of this study was to investigate the clinical, haematological and reproductive responses in pregnant ewes infected with the predominant Australian BVDV strain, BVDV-1c.

Twenty-two pregnant ewes were experimentally inoculated with serum derived from a BVDV PI cattle serum between 59 and 69 days gestation. A further 11 pregnant ewes were left uninfected. No clinical changes were observed in the inoculated ewe group although a mild leukopaenia and a prolonged decrease in eosinophil counts was detected. Severe foetal losses, physical and neurological abnormalities in lambs and the birth of a persistently infected lamb was also recorded in the inoculated ewe group.

Results from this study suggest that acute BVDV-1c infections in sheep are clinically in-apparent, unless infection occurs in a pregnant flock, where severe reproductive losses can be seen at lambing. To eliminate the reproductive losses associated with BVDV infection close contact between sheep and cattle, of unknown BVDV status, should be avoided during the joining and pregnancy periods.

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1. Introduction

Expressions of acute bovine viral diarrhoea (BVDV) infections in cattle can range from subclinical to severe illness, depending on the type and strain of virus present (Baker, 1995; Saliki and Dubovi, 2004). Pyrexia, mild leukopaenia, immunosuppression and a wide array of reproductive losses are commonly observed following acute BVDV outbreaks (Wellenberg et al., 2002). Reproductive dysfunction results from the ability of BVDV to cross the placenta and establish an infection within the developing foetus (Nettleton and Entrican, 1995; Niskanen and Lindberg, 2003), with the outcomes dependent of the stage of gestation at which infection took place (Grooms, 2004). A variety of reproductive outcomes including; abortion, foetal absorption, animals born with congenital or neurological abnormalities as well as animals born with persistent BVDV infections commonly occur following infection of pregnant cattle (Grooms, 2004; Broaddus et al., 2009).

In susceptible cattle populations, persistently BVDV infected (PI) animals are the main source of infection. These PI animals shed high quantities of virus in their excretions and secretions (Niskanen and Lindberg, 2003) and are capable of infecting BVDV naïve animals. Permanently infected animals arise when infection of the dam occurs prior to the foetus developing immunocompetence, during early to mid-gestation (18–125 days) (Grooms, 2004).

Previous studies have shown that BVDV-specific antibodies occur in a variety of non-bovine species, including sheep (Scherer et al., 2001), deer (Nettleton, 1990), goats (Bachofen et al., 2013), camels (Gao et al., 2013), pigs (Tao et al., 2013) and alpaca (Goyal et al., 2002). The clinical manifestations and reproductive losses associated with BVDV infection in sheep are of particular interest due to their frequent proximity to cattle in many countries and the risk of cross-species transmission. Several studies have been undertaken to determine the reproductive losses associated with maternal infection with BVDV. Results from these studies indicate high abortion rates, ranging from 52 to 100%, in ewes infected with BVDV between 25 and 100 days gestation (Snowdon et al., 1975;

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Parsonson et al., 1979; Scherer et al., 2001; Evans et al., 2015). It has also been demonstrated that the birth of persistently BVDV infected lambs may occur when infection takes place early in gestation (Snowdon et al., 1975; Scherer et al., 2001; Evans et al., 2015). However, the majority of these studies have used types or strains of virus not typically found in Australia, where infection appears to be predominated by a single type-1 strain in cattle (Ridpath et al., 2010). The clinical profile of acute infections of BVD Type 1-c in adult sheep is not known.

The aim of the study was to investigate the clinical, haematological and reproductive responses in pregnant ewes infected with the predominant Australian BVDV strain, BVDV-1c.

2. Materials and methods

2.1. Experimental animals

The study was approved by the University of Adelaide's Animal Ethics Committee prior to commencement of this project (S-2014-111A).

The Merino ewes used in this study were sourced from the University of Adelaide's commercial flock, resident at its Roseworthy Campus in South Australia. Eighty two ewes were tested for both BVDV-specific antibodies by IDEXX BVDV/MD/BDV p80 Protein Antibody Test Kit (IDEXX Laboratories Inc, Rydalmere, NSW), and BVDV-specific antigen by IDEXX Bovine Viral Diarrhoea Virus (BVDV) Antigen Test Kit/Serum Plus ELISA (IDEXX Laboratories Inc, Rydalmere, NSW). All animals were found to be negative for BVDV antibodies and antigen according to the cut-off value established for the antibody ELISA by Evans et al. (in press), and the manufacturer's cut-off for the antigen ELISA.

2.2. Breeding management of the experimental ewes

During April 2015, the oestrus cycles of the experimental sheep were synchronised using a commercially available progesterone releasing intra-vaginal device (EAZI-BREED CIDR sheep and goat device, Animal Health, Pfizer Australia Pty Ltd). The CIDR's were removed sequentially from groups of 20 ewes on days 14, 15 and 16 and from 22 ewes on day 17 post CIDR insertion. Upon CIDR removal, each group of ewes were randomly allocated to one of four paddocks. Each of these paddocks housed two or three Border Leicester or Merino rams. The ewes and rams were separated at 25 days post CIDR insertion following an 8–11 day mating period. The ewes were then left undisturbed until transabdominal ultrasound scanning at 59 days following the first CIDR removal.

2.3. Source of BVDV Type1-c inoculum

In September 2014 blood was taken from a persistently BVDV-1c infected heifer from a farm in Meningie, South Australia. This heifer was produced in an earlier study at the University of Adelaide's Roseworthy Campus, South Australia, and was confirmed to be persistently infected with BVDV-1c (Lanyon et al., 2014). Blood was collected into 10 ml collection tubes and then centrifuged at $2400 \times g$ for 10 min. Serum was decanted off and stored at −80 °C in 10 ml tubes. The serum was tested for the presence of BVDV-specific antibodies and BVDV-specific antigen by ELISAs (ibid) was thawed (at 2–8 °C overnight) once only prior to use.

2.4. The experimental groups

Ewes confirmed pregnant by ultra-sound scanning were randomly assigned to one of two treatment groups. The control group consisted of 11 ewes, pregnant with 23 foetuses while the inoculated group, consisted of 22 ewes, pregnant with 42 foetuses. Ewes in the inoculated group were subcutaneously infected with 2 ml freeze-thawed BVDV PI cattle serum, between 59 and 69 days of gestation. Each 2 ml dose of BVDV PI serum contained, equal to, 1.3×10^7 of viral genome copy numbers, based on the absolute quantification in a real-time quantitative reverse transcriptase PCR method.

Animals from both control and inoculated treatment groups were maintained in two paddocks separated by a 1.5 m gap and were fed *ad lib* feed and water. All animals, ewes and lambs from both treatment groups, were systematically observed on a daily basis using a defined checklist of clinical signs as indicators of overall health and wellbeing. Lambs were also weighed weekly from day of birth (DOB) until eight weeks of age.

Ewes had rectal temperatures taken at each blood sampling time point and were scanned for pregnancy by ultrasound on days 14, 21, 35, 49, 56 and 98 post-inoculation. Foetal losses were recorded when a ewe was found not pregnant at scanning. All ewes that carried their pregnancies to term were allowed to lamb naturally.

2.5. Sampling protocols

Blood samples were taken from all the experimental ewes on the following days where day 0 is the day on which the 'inoculated group' was inoculated: 0, 3, 5, 7, 10, 12, 14, 17 and then weekly until a week prior to lambing. Blood samples were taken into plain vacutainer serum tubes and EDTA collection tubes by venepuncture of the jugular vein.

Lambs from both treatment groups had DOB and weekly weights recorded, until 8 weeks of age. Ear notch and blood samples (plain vacutainer serum tubes and EDTA collection tubes) were also collected on DOB from all lambs born in the inoculated group. Blood samples were then collected from all live lambs weekly until eight weeks of age. Aborted foetuses, stillborn lambs and deceased lambs from the inoculated treatment group were submitted to the Veterinary Diagnostic Laboratory of the University of Adelaide for post-mortem examination and sample collection.

Serum was obtained from the blood samples by centrifuging the plain vacutainer serum tubes at $2400 \times g$ for 10 min and the serum decanted in to 1 ml storage tubes before being stored at −80 °C.

2.6. Serum and tissue analysis

Thawed serum samples from all ewes were tested for BVDV-specific antibodies using the IDEXX BVDV Ab P80 ELISA, where signal-to-noise ratios (S/N) of <63.5% were considered positive as per Evans et al. (in press). Thawed serum samples were also tested for BVD viral antigen using the IDEXX BVDV Antigen ELISA (IDEXX Laboratories, Rydalmere, NSW) whereby a sample was considered positive if it had an S-N value of <0.3, as *per* manufacturer's instructions.

All serum samples from live, inoculated treatment group lambs were tested for BVDV-specific antibodies using a competitive ELISA, the IDEXX BVDV Ab P80 protein test kit. Either serum, peritoneal fluid or ear tissue samples from all live, aborted or dead lambs, from the inoculated treatment group, were tested for BVD viral antigen using IDEXX BVDV Serum/Ag PLUS ELISA (IDEXX Laboratories Inc. Rydalmere, NSW), as *per* manufacturer's instructions.

EDTA blood samples from all ewes, on days 0–21 post inoculation, were analysed using a CellDyn Machine (model: 3700). Blood samples collected into EDTA tubes were analysed within 48 h of the samples being taken to determine the haematological parameters: total and differential leukocyte counts, haemoglobin concentration, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin concentration, and platelet counts.

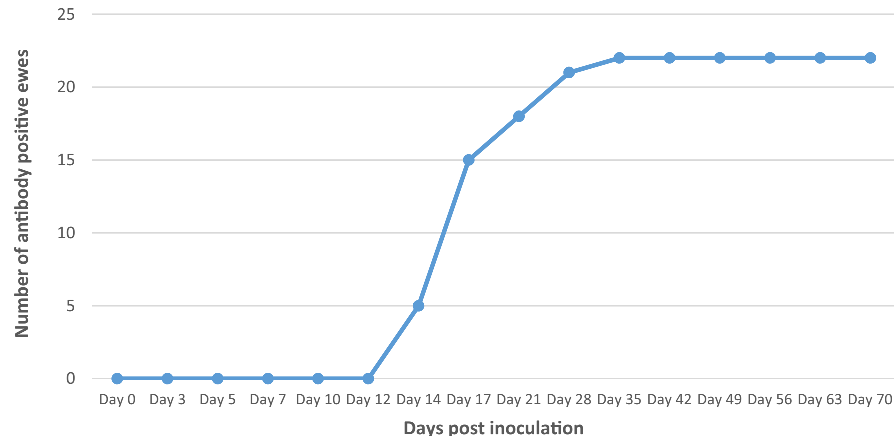

Fig. 1. The number of inoculated treatment group ewes positive for BVDV-specific antibodies on each sampling day from day of inoculation (Day 0) until 70 days post inoculation.

2.7. Reverse transcriptase-PCR

Serum samples which returned a positive result by antigen ELISA were then tested by RT-PCR to confirm result. Total viral RNA was extracted from the freeze-thawed serum samples using the QIAmp Viral RNA Mini Kit (Qiagen, Germany). Reverse transcription and PCR was then undertaken by running the viral RNA samples through the QIAGEN OneStep RT-PCR Kit (Qiagen, Germany) using the primer pair BVD-292F (CTA GCC ATG CCC TTA GTA GGA CTA), and BVD-292R (CAA CTC CAT GTG CCATGTAC AGC A). Each reaction consisted of 5x QIAGEN OneStep RT-PCR Buffer, 400 μM dNTP mix, 0.6 μM of each Primer, QIAGEN OneStep RT-PCR Enzyme Mix, RNAse Inhibitor, RNAse free water and 5 μl of template RNA in a total reaction volume of 50 μl. Reverse transcription and PCR were undertaken under the following thermal cycler conditions (per manufacturer's instructions): 30 min at 50 °C, followed by 15 min at 95 °C followed by a 40 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C. Termination of the reaction was completed with a final extension of 10 min at 72 °C. Lastly, the PCR products were analysed by electrophoresis on a 2% agarose gel and stained with Gel-Red.

2.8. Analytical statistical methods

The means and 95% confidence intervals were calculated for rectal temperature, all haematological parameters and lamb weights for both treatment groups. Where 95% confidence intervals were observed not to overlap, a significant difference was recorded

A Pearson Chi-squared test was carried out on all reproductive performance outcomes to determine if any significant differences occurred between the two treatment groups.

3. Results

3.1. Seroconversion to BVDV in the ewes

Serum samples analysed using the cut-off value established by Evans et al. (in press) of <63.5%S/N indicated that all 22 of the inoculated treatment group seroconverted between 14 and 49 days post inoculation (Fig. 1). Five ewes (23%) had seroconverted by Day 14; 15 ewes (68%) by Day 17; 18 ewes (82%) by Day 21 and 22 (100%) ewes by Day 35. Antibody titres for all infected ewes were very strong, with values falling below 20% S/N by the final sampling on Day 70 post-inoculation.

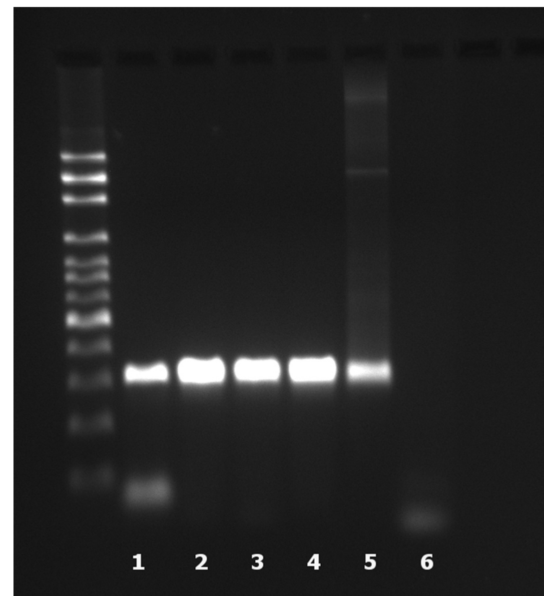

Fig. 2. RT-PCR gel for serum samples from 4 ewes taken on Day 7 post BVDV inoculation which were antigen ELISA positive. Well 1 = Ewe 55; Well 2 = Ewe 15; Well 3 = Ewe 43; Well 4 = Ewe 11; Well 5 = positive control; Well 6 = negative control.

Antigen was detected in four ewes from the inoculated ewe group (Ewes 11, 15, 43 and 55) on Day 7 post inoculation by antigen ELISA. This result was confirmed by PCR with all four ewes returning positive results by RT-PCR (Fig. 2).

None of the ewes in the control treatment group seroconverted with titres well above the cut-off value of 63.5% S/N throughout the pre-lambing study period.

3.2. Clinical parameters

No differences in daily observed clinical signs were identified between the two treatment groups. Mean rectal temperatures for both ewe treatment groups were similar throughout the study period (Fig. 3) with the majority of values falling within the reference range of 39–40 °C (Terra, 2009). However, on Day 5 post inoculation the mean rectal temperature of the inoculated group was 40.4 °C, which was marginally higher than that of the normal

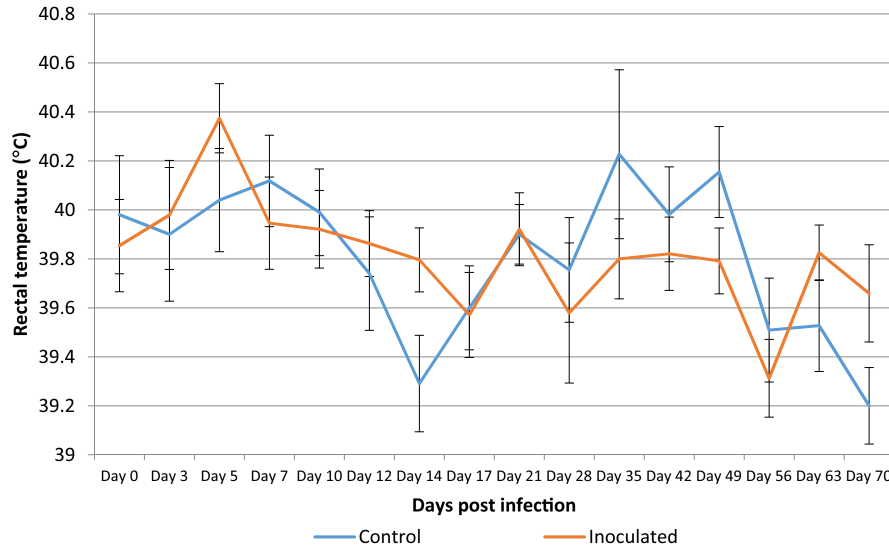

Fig. 3. Mean rectal temperature for control and inoculated ewe treatment groups from day of inoculation (Day 0) until 70 days post inoculation, 95% confidence intervals are shown.

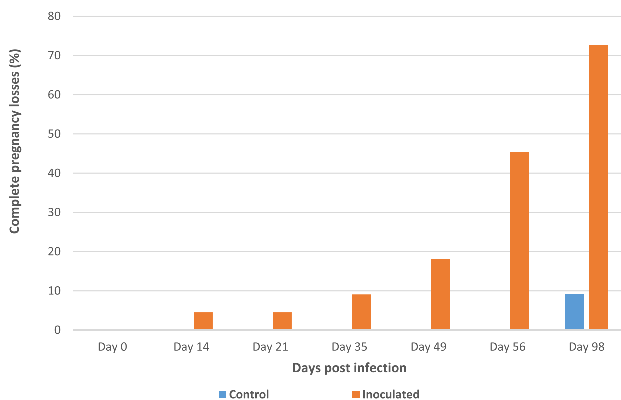

Fig. 4. Complete pregnancy losses (%) for 11 control ewes and 22 BVDV inoculated ewes, from day of inoculation (Day 0) until 98 days post inoculation.

reference range for sheep. Similarly on Days 7, 35 and 49 post inoculation the control group had mean rectal temperatures marginally higher than the normal reference range.

There were significant differences in the mean rectal temperatures between the two treatment groups on Days 14, 49 and 70 post inoculation (Fig. 3). The mean rectal temperature of the inoculated group was significantly higher (0.5 °C) than that of the control group on Days 14 and 70 while the control group was significantly higher (0.3 °C) on Day 49 post inoculation.

Mean haematology counts are given in Table 1 for both ewe treatment groups. On Day 3 post inoculation the mean white blood cell count was significantly lower in the inoculated group compared to the control group. Similarly on Day 5 lymphocyte, red blood cell, haemoglobin and haematocrit counts were all significantly lower in the inoculated group. Also on Day 5 the inoculated group had a higher average monocyte count compared to the controls. There were also consistently lower eosinophil counts in the inoculated group from Day 3 to 12 and again on Day 28 compared to the control group.

3.3. Reproductive performance

Complete pregnancy losses where no foetuses remain in the ewe for both treatment groups are given in Fig. 4. The earliest pregnancy loss recorded was at the Day 14 post inoculation scan in a ewe from the inoculated treatment group. Another ewe from the inoculated treatment group was found to be empty on the Day 35 scan; two more on Day 49 and six more on Day 56. During this time none of the control ewes were scanned as empty.

Lambing started at Day 86 post inoculation and continued until Day 98. During this period six ewes from the inoculated treatment group either aborted, gave birth to stillborn lambs only or were not seen to lamb. In comparison only one control ewe had a complete pregnancy loss, giving birth to a single stillborn lamb.

By the end of the lambing period just six of the 22 inoculated treatment group ewes had viable pregnancies (at least one live lamb born). A total of nine lambs (21.4%), from a possible 42 foetuses, were born alive in the inoculated ewe group (Fig. 5) resulting in a lambing percentage of 31.8% (Table 2). Only four of these lambs survived until 8 weeks of age resulting in a percentage lamb survival at 8 weeks of age of just 18.2% (Table 2). Of the 33 non-viable foetuses from the inoculated treatment group there were 5 (11.9%) observed abortions, 7 (16.7%) stillborn lambs and 21 (50%) unobserved abortions/absorptions/losses (Fig. 5). Post mortems of aborted, stillborn and live lambs born to inoculated ewes showed a wide variety of physical abnormalities including, anasarca, arthrogryposis, brachygnathia, porencephaly, hydrancephaly and 'hair-like' wool.

In comparison, 10 of the 11 control ewes had viable pregnancies with a total of 19 lambs (82.6%), from a possible 23 foetuses, born alive (Fig. 5). Fifteen of these lambs survived until 8 weeks of age. This gave the control ewe group a lambing percentage of 172.7% and percentage lamb survival at 8 weeks of age of 136.4% (Table 2). Of the four foetuses lost to the control group two (8.7%) were stillborn lambs and two (8.7%) were unobserved losses (Fig. 5).

Mean lamb weights from DOB until 8 weeks of age are given in Fig. 6. Lambs born to inoculated ewes tended to be lighter than lambs born to control ewes. However significant differences were only seen at DOB, week1 and week 2 time points.

Table 1
Mean haematological values for inoculated and control ewe groups from day of inoculation (Day 0) until Day 35 post infection, with 95% confidence intervals given in brackets. Values that are significantly different between the two treatment groups on any day are highlighted in grey.

Measurement	Treatment group	Day 0	Day 3	Day 5	Day 7	Day 10	Day 12	Day 14	Day 17	Day 21	Day 28	Day 35
White blood cell count (x10^9/L)	Inoculated	4.0 (±0.4)	3.8 (±0.5)	4.3 (±0.6)	3.7 (±0.6)	4.6 (±0.5)	4.9 (±0.5)	4.6 (±0.5)	4.9 (±0.5)	4.4 (±0.5)	4.2 (±0.4)	4.9 (±0.7)
	Control	4.1 (±0.5)	5.1 (±0.7)	5.4 (±0.8)	4.8 (±1.0)	5.7 (±1.4)	5.9 (±0.9)	4.6 (±0.6)	5.6 (±1.2)	4.1 (±0.7)	4.5 (±0.9)	4.2 (±0.8)
Lymphocyte count (x10^9/L)	Inoculated	1.5 (±0.2)	1.4 (±0.2)	1.6 (±0.3)	1.9 (±0.5)	1.7 (±0.3)	2.1 (±0.3)	1.9 (±0.3)	1.9 (±0.3)	2.0 (±0.4)	1.9 (±0.4)	1.9 (±0.5)
	Control	1.5 (±0.2)	2.1 (±0.5)	2.6 (±0.4)	2.3 (±0.8)	2.4 (±1.1)	2.5 (±0.5)	2.0 (±0.3)	2.3 (±0.9)	1.7 (±0.4)	1.5 (±0.3)	1.4 (±0.4)
Neutrophil count (x10^9/L)	Inoculated	1.6 (±0.3)	1.4 (±0.3)	1.5 (±0.2)	1.0 (±0.2)	1.4 (±0.2)	1.7 (±0.3)	1.6 (±0.2)	1.8 (±0.3)	1.2 (±0.2)	1.0 (±0.2)	1.6 (±0.2)
	Control	1.7 (±0.4)	1.6 (±0.3)	1.3 (±0.5)	1.0 (±0.3)	1.6 (±0.3)	1.7 (±0.3)	1.3 (±0.4)	1.8 (±0.4)	1.0 (±0.3)	1.1 (±0.3)	1.4 (±0.3)
Red blood cell count (x10^{12}/L)	Inoculated	10.5 (±0.4)	11.0 (±0.5)	10.6 (±0.4)	10.6 (±0.4)	10.3 (±0.4)	10.7 (±0.8)	10.8 (±0.8)	10.3 (±0.4)	10.2 (±0.4)	10.5 (±0.5)	10.3 (±0.5)
	Control	10.6 (±0.5)	10.9 (±0.8)	11.5 (±0.3)	10.7 (±0.8)	11.6 (±1.6)	10.7 (±0.8)	10.6 (±0.8)	10.5 (±0.5)	10.4 (±0.7)	10.4 (±0.5)	9.8 (±0.6)
Monocyte count (x10^9/L)	Inoculated	0.4 (±0.1)	0.5 (±0.1)	0.6 (±0.1)	0.4 (±0.1)	0.6 (±0.1)	0.6 (±0.1)	0.5 (±0.1)	0.6 (±0.1)	0.5 (±0.1)	0.5 (±0.1)	0.5 (±0.1)
	Control	0.3 (±0.1)	0.4 (±0.1)	0.4 (±0.1)	0.4 (±0.1)	0.5 (±0.1)	0.5 (±0.2)	0.5 (±0.1)	0.6 (±0.1)	0.5 (±0.2)	0.5 (±0.2)	0.4 (±0.1)
Eosinophil count (x10^9/L)	Inoculated	0.4 (±0.1)	0.3 (±0.1)	0.4 (±0.1)	0.2 (±0.1)	0.4 (±0.1)	0.4 (±0.1)	0.4 (±0.1)	0.5 (±0.1)	0.6 (±0.1)	0.6 (±0.1)	0.6 (±0.1)
	Control	0.6 (±0.1)	0.9 (±0.3)	1.1 (±0.4)	1.0 (±0.4)	1.0 (±0.3)	0.8 (±0.3)	0.7 (±0.2)	0.7 (±0.2)	0.7 (±0.2)	1.2 (±0.3)	0.9 (±0.2)
Basophil count (x10^9/L)	Inoculated	0.1 (±0.0)	0.2 (±0.0)	0.2 (±0.1)	0.2 (±0.1)	0.2 (±0.1)	0.2 (±0.0)	0.2 (±0.1)	0.2 (±0.0)	0.2 (±0.1)	0.2 (±0.1)	0.3 (±0.1)
	Control	0.1 (±0.0)	0.2 (±0.1)	0.2 (±0.1)	0.1 (±0.0)	0.1 (±0.1)	0.2 (±0.1)	0.2 (±0.2)	0.2 (±0.1)	0.2 (±0.1)	0.2 (±0.2)	0.1 (±0.1)
Haemoglobin count (g/L)	Inoculated	129.5 (±3.5)	133.6 (±5.1)	128.4 (±3.3)	128.3 (±4.1)	124.5 (±3.3)	128.5 (±7.8)	129.5 (±8.5)	124.8 (±3.7)	123.6 (±3.7)	128.0 (±5.2)	124.9 (±4.6)
	Control	129.6 (±5.5)	129.9 (±7.8)	139.1 (±5.8)	129.5 (±7.3)	138.8 (±15.6)	129.1 (±7.6)	127.9 (±7.4)	126.6 (±6.3)	127.4 (±5.9)	125.5 (±6.0)	121.1 (±6.8)
Haematocrit count (L/L)	Inoculated	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)
	Control	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)	0.4 (±0.0)
MCV (fL)	Inoculated	35.4 (±0.9)	34.1 (±0.8)	34.1 (±0.9)	34.5 (±0.9)	34.5 (±0.8)	34.2 (±0.9)	35.0 (±0.9)	34.3 (±0.8)	35.1 (±0.8)	35.2 (±0.9)	35.0 (±0.8)
	Control	35.5 (±1.3)	33.9 (±1.1)	34.1 (±1.1)	35.2 (±1.4)	34.0 (±1.5)	34.4 (±1.2)	35.2 (±1.2)	34.5 (±1.1)	35.1 (±1.1)	35.4 (±1.2)	35.2 (±1.2)
MCH (pg)	Inoculated	12.4 (±0.3)	12.1 (±0.3)	12.1 (±0.3)	12.2 (±0.2)	12.1 (±0.2)	12.1 (±0.3)	12.0 (±0.2)	12.1 (±0.2)	12.1 (±0.2)	12.2 (±0.3)	12.1 (±0.3)
	Control	12.2 (±0.3)	12.0 (±0.3)	12.1 (±0.3)	12.2 (±0.3)	12.1 (±0.3)	12.1 (±0.3)	12.1 (±0.3)	12.0 (±0.3)	12.3 (±0.3)	12.1 (±0.4)	12.4 (±0.3)
MCHC (g/L)	Inoculated	348.9 (±4.3)	356.1 (±4.2)	356.0 (±5.4)	349.7 (±4.9)	352.2 (±4.4)	354.5 (±5.4)	343.9 (±5.3)	353.4 (±5.2)	346.0 (±4.1)	346.5 (±5.5)	347.4 (±6.2)
	Control	346.1 (±9.3)	354.3 (±7.3)	355.9 (±8.3)	346.8 (±9.7)	356.1 (±8.6)	351.3 (±8.0)	343.8 (±6.7)	349.0 (±10.5)	349.4 (±6.2)	343.1 (±9.2)	351.3 (±6.6)
Platelet count (x10^9/L)	Inoculated	339.7 (±62.9)	259.6 (±77.8)	226.3 (±48.6)	195.4 (±48.2)	217.5 (±49.4)	226.1 (±55.7)	231.0 (±50.2)	193.3 (±53.0)	245.6 (±55.0)	272.4 (±66.0)	296.3 (±68.5)
	Control	301.7 (±86.9)	327.9 (±118.6)	310.9 (±92.4)	239.3 (±73.7)	303.1 (±89.7)	233.9 (±82.2)	174.0 (±51.8)	152.4 (±54.5)	244.2 (±74.2)	199.2 (±74.0)	197.3 (±60.4)

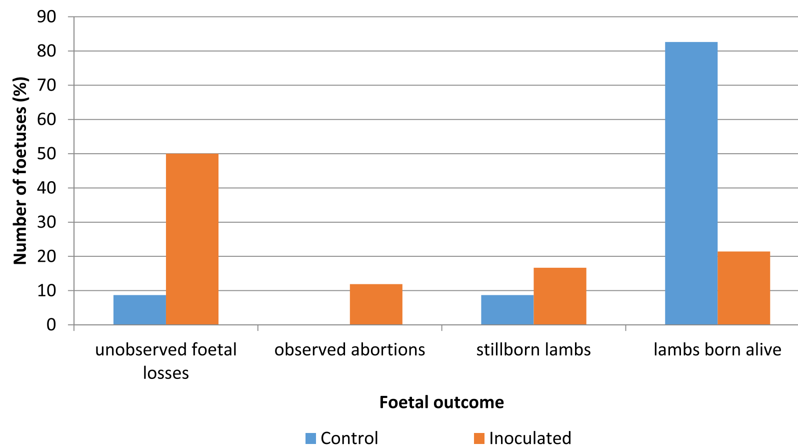

Fig. 5. Breakdown of foetal outcomes from 22 BVDV inoculated ewes, pregnant with 42 foetuses, and 11 control ewes, pregnant with 23 foetuses.

3.4. BVDV status of lambs

Of the nine live lambs born to the inoculated treatment group five of them were positive for BVDV-specific antibodies but negative for BVDV antigen. Four of these antibody positive lambs survived until 8 weeks of age. The remaining four live lambs were negative for BVDV-specific antibodies and positive for BVDV antigen. Three of these lambs died within 24 h of birth with the remaining lamb surviving the 8 week post lambing study period. Pre-colostral serum and weekly serum samples from week 2 to week 8 from this lamb returned a positive ELISA antigen confirming that the lamb was persistently infected with BVDV.

Table 2
Reproductive performance outcomes for 11 control ewes and 22 ewes inoculated with BVDV between 59 and 69 days gestation.

	Number of Ewes	Number of ewes with total pregnancy loss	Number of ewes birthing live lambs	Lambing percentage	Percentage lamb survival at 8 weeks of age
Control	11	1 (9.1%)	10 (90.9%)	172.7%	136.4%
Inoculated	22	16 (72.7%)	6 (27.3%)	31.8%	18.2%
Pearson Chi-Square		P = 0.001	P = 0.001	P = 0.000	P = 0.000

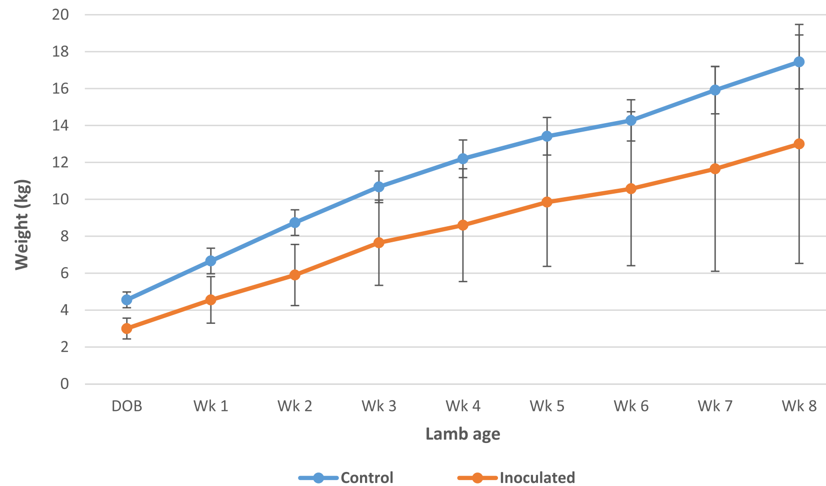

Fig. 6. Mean weekly lamb weights from day of birth (DOB) to 8 weeks of age, for lambs from control (n = 15) and inoculated (n = 4) treatment group ewes. 95% confidence intervals are shown.

Six of the stillborn/aborted lambs from the inoculated treatment group were negative for BVDV-specific antibodies and positive for BVDV antigen. A further three stillborn/aborted lambs were positive for BVDV-specific antibodies and negative for BVDV antigen. The remaining three foetuses were unable to be collected and thus BVDV status on these lambs is not defined.

All lambs (live and stillborn) from the control ewe group were negative for both BVDV-specific antibodies and BVDV antigen.

4. Discussion

No changes in food consumption, alertness or faecal consistency have been recorded in ewes infected with BDV (Garcia-Perez et al., 2009) while effects of BVDV infections in cattle can range from subclinical to severe illness depending on the type and strain of virus present (Baker, 1995; Saliki and Dubovi, 2004). Results from the present study are consistent with previous findings in sheep with no obvious clinical signs of infection identified (Garcia-Perez et al., 2009).

Despite the absence of any obvious clinical signs, differences in average rectal temperature were recorded between the two treatment groups. Inoculated treatment group ewes recorded the highest mean rectal temperature of 40.4 °C on Day 5, 0.4 °C higher than the normal reference range for sheep (Terra, 2009). From previous studies it is understood that pyrexia can occur any time between 5 and 14 days post infection with both BVDV in cattle and BDV in sheep (Tråvén et al., 1991; Nettleton et al., 1998; Garcia-Perez et al., 2009). Although the timing of two out of the three temperature increases recorded in this study are in line with previous findings, the temperatures recorded in the inoculated ewes are either not statistically different from those of the control ewes or are not consistently high enough to be categorised as a pyrexia according to reference values.

Haematologically there were mild differences between the control and inoculated ewes. The leukopaenia observed in the inoculated ewe group at Day 3 is in line with previous work relating to BDV infection in sheep. It has been well documented that during the short-lived viraemic period experienced in acutely infected sheep, mild leukopaenia can be observed between 2 and 11 days post infection (Nettleton et al., 1998; Garcia-Perez et al., 2009). Similarly in cattle, leukopaenia has been associated with viraemia in acutely BVDV infected animals (Baker, 1995; Muller-Doblies et al., 2004). Although viraemia was not detected, by antigen ELISA, in any of the inoculated ewes on Day 3, antigen was detected in four ewes on Day 7. These results were confirmed with the use of RT-PCR and indicate a viraemia beginning 4–7 days post infection in these ewes. This is temporally consistent with the leukopaenia results seen on Day 3.

However, the most significant haematological difference between the two treatment groups was the substantially lower eosinophil counts seen in the inoculated treatment group from Day 3 to Day 12. Low eosinophil counts in ruminants often occurs in response to stress or corticosteroid increases and inflammatory responses, where parturition and adverse weather conditions have been linked to corticosteroid induced eosinopenia (Morris, 2009). It is not understood why low eosinophil counts were observed for such a prolonged period of time in the inoculated treatment group but it is possible that acute BVDV infections cause a higher level of stress on animals than previously recognised.

There was a high and severe impact on the reproductive performance of the ewes infected with BVDV. There was a substantially lower lambing percentage observed in the inoculated treatment group (31.8%) compared to the control ewe group (172.7%). This low lambing percentage was due to 16 of the 22 inoculated ewes aborting or giving birth to only dead lambs. These results are in line with previous findings with abortion rates in pregnant ewes ranging from 43% (Evans et al., 2015) to 100% (Parsonson et al., 1979), following acute BVDV infections.

Monitoring of pregnancy losses was achieved by trans-abdominal ultra-sound scanning every seven to 14 days from Day 0 to 56 post inoculation and a post lambing scan at Day 98. Losses were detected from 14 days on-wards in the inoculated treatment group. These losses would not normally be observed until lambing in a commercial setting, when a high number of barren ewes would be identified. Severe foetal abnormalities were seen in aborted and stillborn lambs from the inoculated treatment group which is consistent with previous reports of both BVDV and BDV infection in sheep (Hewicker-Trautwein et al., 1995; Garcia-Perez et al., 2009).

Another loss to producers is the reduced lamb weights seen in lambs from ewes infected with BVDV. Significant differences in mean lamb weights were seen on DOB, week 1 and week 2 although there was an overall trend that lambs from inoculated ewes were smaller than those from control ewes. The lack of significance between the two lamb treatment groups, from week 3 onwards, may be due to the low number of lambs, and the wide spread of weights, from the inoculated treatment group (n = 4)

surviving until 8 weeks of age. However, these findings are in comparison to what has been previously reported in cattle infected with BVDV, and sheep infected with BDV, such that maternal infection with BVDV/BDV causes intrauterine growth restriction and subsequent low birth weights and growth retardation of offspring (Done et al., 1980; Caffrey et al., 1997). Further work in this area needs to be undertaken in order to understand the effect BVDV infection has on the growth of lambs.

Also of importance in this study, was the birth of a viable BVDV-1c PI lamb. This lamb was determined to be persistently infected with BVDV-1c due to consecutive samples testing positive for BVDV antigen and negative for BVDV antibodies, by ELISA. Although a number of lambs, aborted/stillborn or born alive, were positive for virus and negative for BVDV antibodies, only one lamb survived past 24 h of age and on to the end of the study. This indicates that although survival of PI lambs is poor they can persist past a few hours of age, and as such have the potential, like that of PI cattle, to infect any naïve and susceptible animals they come in to contact with. The risk of infection posed by a BVDV PI sheep as a reservoir of infection for cattle or sheep has not been defined.

5. Conclusion

Infection of pregnant ewes with BVDV-Type 1c by experimental inoculation between 59 and 69 days gestation caused severe reproductive losses and a viable PI lamb. Apart from the reproductive losses the infection in the ewe caused no observable clinical changes in the ewes with rectal temperatures remaining within normal temperatures or marginally high. A mild leukopaenia and a prolonged decrease in eosinophil counts was detected. To eliminate the risks associated with BVDV infection in pregnant ewes, close contact between sheep and cattle of unknown BVDV status should be avoided during the joining and pregnancy periods. It also suggests that in cases where abortion storms and physical abnormalities in lambs are seen, BVDV infection should be considered as a possible cause, particularly in areas where sheep and cattle regularly come in to contact with one another.

Role of the funding source

The University of Adelaide and Meat and Livestock Australia had no part in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References

Bachofen, C., Vogt, H.R., Stalder, H., Mathys, T., Zanoni, R., Hilbe, M., Schweizer, M., Peterhans, E., 2013. Persisitent infections after natural transmission of bovine viral diarrhoea virus from cattle to goats among goats. Vet. Res. 44, 32–42.

Baker, J.C., 1995. The clinical manifestations of bovine viral diarrhea infection. Vet. Clin. North Am. Food Anim. Pract. 11, 425–445.

Broaddus, C.C., Lamm, C.G., Kapil, S., Dawson, L., Holyoak, G.R., 2009. Bovine viral diarrhea virus abortion in goats housed with persistently infected cattle. Vet. Pathol. 46, 45–53.

Caffrey, J.F., Dudgeon, A.M., Donnelly, W.J., Sheahan, B.J., Atkins, G.J., 1997. Morphometric analysis of growth retardation in fetal lambs following experimental infection of pregnant ewes with border disease virus. Res. Vet. Sci. 62, 245–248.

Done, J.T., Terlecki, S., Richardson, C., Harkness, J.W., Sands, J.J., Patterson, D.S., Sweasey, D., Shaw, I.G., Winkler, C.E., Duffell, S.J., 1980. Bovine virus diarrhoea-mucosal disease virus: pathogenicity for the fetal calf following maternal infection. Vet. Rec. 106, 473–479.

Evans, C.A., Lanyon, S.R., Sims, S.K., Reichel, M.P., 2015. Reproductive performance in experimentally BVDV infected ewes and seroconversion rates in sheep co-mingled with BVDV PI calves. Small Rumin. Res. 123, 314–319.

Evans, C.A., Lanyon, S.R., Reichel, M.P., 2017. Investigation of AGID and two commercial ELISAs for the detection of Bovine viral diarrhea virus-specific antibodies in sheep serum. J. Vet. Diagn. Invest., in press.

Gao, S.D., Luo, J.H., Du, J.Z., Lang, Y.F., Cong, G.Z., Shao, J.J., Lin, T., Zhao, F.R., Belak, S., Liu, L.H., Chang, H.Y., Yin, H., 2013. Serological and molecular evidence for natural infection of bactrian camels with multiple subgenotypes of bovine viral diarrhea virus in Western China. Vet. Microbiol. 163, 172–176.

Garcia-Perez, A.L., Minguijon, E., Estevez, L., Barandika, J.F., Aduriz, G., Juste, R.A., Hurtado, A., 2009. Clinical and laboratorial findings in pregnant ewes and their progeny infected with border disease virus (BDV-4 genotype). Res. Vet. Sci. 86, 345–352.

Goyal, S.M., Boujihad, M., Haugerud, S., Ridpath, J.F., 2002. Isolation of bovine viral diarrhea virus from an alpaca. J. Vet. Diagn. Invest. 14, 523–525.

Grooms, D.L., 2004. Reproductive consequences of infection with bovine viral diarrhea virus. Vet. Clin. Food Anim. Pract. 20, 5–19.

Hewicker-Trautwein, M., Trautwein, G., Frey, H.R., Liess, B., 1995. Variation in neuropathogenicity in sheep fetuses transplacentally infected with non-cytopathogenic and cytopathogenic biotypes of bovine-virus diarrhoea virus. Zentralbl. Veterinarmed. B 42, 557–567.

Lanyon, S.R., Sims, S.K., Cockcroft, P.D., Reichel, M.P., 2014. Comparison of serum, ear notches, and nasal and saliva swabs for Bovine viral diarrhea virus antigen detection in colostrum-fed persistently infected (PI) calves and non-PI calves. J. Vet. Diagn. Invest. 26, 783–787.

Morris, D.D., 2009. Alterations in the leukogram. In: Smith, B.P. (Ed.), Large Animal Internal Medicine. Mosby Elesvier, St Loius, Missouri, pp. 405–410.

Muller-Doblies, D., Arquint, A., Schaller, P., Heegaard, P.M., Hilbe, M., Albini, S., Abril, C., Tobler, K., Ehrensperger, F., Peterhans, E., Ackermann, M., Metzler, A., 2004. Innate immune responses of calves during transient infection with a noncytopathic strain of bovine viral diarrhea virus. Clin. Diagn. Lab. Immunol. 11, 302–312.

Nettleton, P.F., Entrican, G., 1995. Ruminant pestiviruses. Br. Vet. J. 151, 615–642.

Nettleton, P.F., Gilray, J.A., Russo, P., Dlissi, E., 1998. Border disease in sheep and goats. Vet. Res. 29, 327–340.

Nettleton, P.F., 1990. Pestivirus infections in ruminants other than cattle. Rev. Sci. Technol. 9, 131–150.

Niskanen, R., Lindberg, A., 2003. Transmission of bovine viral diarrhoea virus by unhygienic vaccination procedures, ambient air, and from contaminated pens. Vet. J. 165, 125–130.

Parsonson, I.M., Ohalloran, M.L., Zee, Y.C., Snowdon, W.A., 1979. Effects of bovine viral diarrhoea-mucosal disease (BVD) virus on the ovine fetus. Vet. Microbiol. 4, 279–292.

Ridpath, J.F., Fulton, R.W., Kirkland, P.D., Neill, J.D., 2010. Prevalence and antigenic differences observed between Bovine viral diarrhea virus subgenotypes isolated from cattle in Australia and feedlots in the southwestern United States. J. Vet. Diagn. Invest. 22, 184–191.

Saliki, J.T., Dubovi, E.J., 2004. Laboratory diagnosis of bovine viral diarrhea virus infections. Vet. Clin. North Am. Food Anim. Pract. 20, 69–83.

Scherer, C.F.C., Flores, E.F., Weiblen, R., Caron, L., Irigoyen, L.F., Neves, J.P., Maciel, M.N., 2001. Experimental infection of pregnant ewes with bovine viral diarrhoea virus type-2 (BVDV-2): effects on the pregnancy and fetus. Vet. Microbiol. 79, 285–299.

Snowdon, W.A., Parsonson, I.M., Broun, M.L., 1975. Reaction of pregnant ewes to innoculation with mucosal disease virus of bovine origin. J. Comp. Pathol. 85, 241–251.

Tao, J., Liao, J.H., Wang, Y., Zhang, X.J., Wang, J.Y., Zhu, G.Q., 2013. Bovine viral diarrhea virus (BVDV) infections in pigs. Vet. Microbiol. 165, 185–189.

Terra, R.L., 2009. Ruminant history, physical examination and records. In: Smith, B.P. (Ed.), Large Animal Internal Medicine. Mosby Elesvier, St Louis, Missouri, pp. 3–14.

Tråvén, M., Alenius, S., Fossum, C., Larsson, B., 1991. Primary bovine viral diarrhoea virus infection in calves following direct contact with a persistently viraemic calf. Zentralbl. Veterinarmed. B 38, 453–462.

Wellenberg, G.J., van der Poel, W.H.M., Van Oirschot, J.T., 2002. Viral infections and bovine mastitis: a review. Vet. Microbiol. 88, 27–45.

3.2 Findings in lambs

The primary objective of this manuscript was to report on the developmental outcomes seen in aborted and live born lambs following BVDV-1c infections *in utero*. This manuscript reports on the extensive pathological findings observed in fifteen lambs born during this trial and has been prepared for submission in the Journal of *Veterinary Pathology* (submitted).

Original article: Pathological outcomes in lambs born to ewes experimentally infected with bovine viral diarrhea virus Type-1c (BVDV-1c) between 59 and 69 days of gestation

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Veterinary Pathology (submitted)

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Caitlin A Evans
Contribution to the Paper	Researched, designed and implemented animal trial, managed animal trial, undertook data analysis and interpretation, drafted and edited manuscript.
Overall percentage (%)	80%
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper
Signature	Date 24/7/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Lucy Woolford
Contribution to the Paper	Helped with sample collection and analysis, interpretation of data and drafting and editing manuscript.
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Contribution to the Paper	Helped with research, design and implementation of animal trial, interpreting data and editing manuscript.
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Contribution to the Paper	Helped with research, design and implementation of animal trial, interpreting data and editing manuscript
Signature	Date 30/07/2017

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Pathological outcomes in lambs born to ewes experimentally infected with bovine viral diarrhea virus Type 1c (BVDV-1c) between 59 and 69 days of gestation†

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Abstract

Acute bovine viral diarrhea virus (BVDV) infections in sheep are largely clinically in-apparent although BVDV infection in pregnant ewe flocks has been shown to result in severe lambing losses, the development of persistently infected lambs and lambs born with developmental malformations. The predominant Australian BVDV strain (BVDV-1c) has previously been shown to cause substantial lambing losses, however the pathological outcomes in lambs following gestational infection with BVDV-1c are unknown. Following experimental infection of pregnant ewes with BVDV-1c between 59 and 69 days of gestation, fifteen aborted, stillborn, dead or euthanized lambs were examined. Six lambs were identified to be BVDV antibody positive and BVDV antigen negative lambs while nine lambs were identified as BVDV antibody negative and BVDV antigen positive. A wide range of pathological lesions were identified in both subsets of lambs; anasarca, intrahepatic cholestasis, neurological malformations (hydrancephaly, porencephaly, cerebellar dysplasia) and skeletal malformations (arthrogryposis, facial asymmetry, brachygnathia) were most commonly observed. Anasarca in conjunction with a cholestatic hepatopathy was observed in four/nine antibody negative and antigen positive lambs. A hairy coat associated with primary follicular dysplasia, a developmental lesion normally identified with BDV infected lambs, was also identified in one lamb presenting with tremors and cerebellar dysplasia ("hairy shaker"). Findings from this study indicate that early-mid gestational infection of lambs with BVDV-1c can cause severe developmental malformations.

Keywords: Bovine Viral Diarrhea; Sheep; Border Disease Virus; Fetal development; Pathology

Bovine viral diarrhea virus (BVDV) is a highly prevalent disease of cattle which, along with Border disease virus (BDV) in sheep and Classical swine fever virus (CSFV) in pigs, belongs to the *Pestivirus* genus[18]. Both BVDV and BDV have been shown to easily cross between several species including a wide range of ruminants [17]. Previous experimental infections of pregnant ewes with BVDV-2 have led to high abortion/stillborn rates, but no developmental deformities in lambs[23], while experimental infections of pregnant ewes with other BVDV subtypes have led to neurological lesions and skeletal deformities[11,12]. Immunotolerant and persistently infected lambs have also been shown to occur following infection of the ewe between 55 and 67 days of gestation[6,23].

In Australia, there is a predominance of a single BVDV Type-1 strain (BVDV-1c) reported[22]. Experimental infection with BVDV-1c in pregnant ewes has led to severe fetal losses and the birth of a persistently infected lamb[6], however the pathological outcomes seen in lambs following gestational infections with this and other strains of BVDV has not been well recorded. As a result, the pathological effects of BVDV-1c infection on the foetal development of lambs, particularly during the period of organogenesis, is not fully understood. The aim of this study was therefore to characterise the pathological consequences seen in lambs born to ewes experimentally infected with BVDV-1c between 59 and 69 days gestation.

Materials and Methods

All animal experiments were approved by the University of Adelaide's Animal Ethics Committee prior to this project (S-2014-111A).

Animals

Merino and Merino x Border Leicester lambs were produced following the synchronisation and natural mating of a group of 22 Merino ewes. All the ewes were experimentally inoculated with serum from a BVDV-1c persistently infected heifer by subcutaneous injection, between 59 and 69 days gestation and housed and sampled as per Evans, et al. [7]. The time to the first detection of antibodies to BVDV ranged from 14-35 days post inoculation. All the ewes were kept in an outside paddock with *ad lib* feed and water. Ewes were left to lamb naturally and lambs remained with their mothers until either death or euthanasia at 8 weeks of age occurred.

Sample collection

The lambs born to the infected ewes were blood sampled on the day of birth. Further blood samples were taken weekly until either death or euthanasia. Blood samples were taken into plain evacuated serum tubes and spun at 2,400 x g for 10 minutes. Serum was then decanted and stored in 1ml tubes at -80°C. In place of serum samples, peritoneal fluid was collected from aborted and stillborn lambs and also stored at -80°C.

Fifteen aborted, stillborn, dead or euthanized lambs were submitted to the Veterinary Diagnostic Laboratory of the University of Adelaide and underwent post-mortem examination and sample collection within 6 hours of death or abortion/stillbirth. Full post mortem investigations were unable to be carried out on 2 submitted lambs/fetuses due to the effects of severe predation. Fetuses aborted/absorbed outside of the lambing period (79 and 88 days post inoculation) were not submitted for post mortem examination. Brain, liver, lung, lymph node, spleen, thymus and kidney samples were taken from all lambs, and skin samples were also taken from lambs that presented with ‘hair-like’ wool. Duplicate samples of each tissue type were

taken, one sample was stored at -80°C and the other fixed in 10% neutral-buffered formalin.

BVD antibody testing

Serum samples from live born lambs, and peritoneal fluid samples from stillborn/aborted lambs, were tested for BVDV-specific antibodies using the IDEXX BVDV/MD/BDV p80 Protein Antibody Test Kit (IDEXX Laboratories Inc, Rydalmere, NSW). Samples which returned a signal-to-noise ratio (S/N) of ≤63.5% were classified as positive for BVDV-specific antibodies as determined by Evans, et al. [5].

BVD virus testing

Serum samples from live born lambs, and peritoneal fluid samples from stillborn/aborted lambs, were tested for BVD virus using the IDEXX Bovine Viral Diarrhea Virus (BVDV) Antigen Test Kit/Serum Plus ELISA (IDEXX Laboratories Inc, Rydalmere, NSW). Samples with S-N values >0.300 were classified as positive for BVDV antigen as per the manufacturer's instructions.

Nucleotide sequencing

Total viral RNA was extracted from freeze-thawed serum samples for Lamb 15 and the originating PI cattle serum which was used to inoculate ewes. Reverse transcription and PCR was then undertaken by running the viral RNA samples through the QIAGEN OneStep RT-PCR Kit (Qiagen, Germany) using the primer pair BVD-292F (CTA GCC ATG CCC TTA GTA GGA CTA), and BVD-292R (CAA CTC CAT GTG CCATGTAC AGC A). Each reaction consisted of 5x QIAGEN OneStep RT-PCR Buffer, 400µM dNTP mix, 0.6 µM of each Primer, QIAGEN OneStep RT-PCR Enzyme Mix, RNAse Inhibitor, RNAse free water and 5µL of template RNA in a total reaction volume of 50µL. The reaction was run under the following thermal

cycler conditions (per manufacturer's instructions): 30min at 50°C for cDNA synthesis, followed by 15min at 95°C then 40 cycles of 1min at 94°C, 1 min at 52°C and 1min at 72°C before termination with a final extension of 10min at 72°C.

Both RT-PCR products were purified and submitted to the Australian Genome Research Facility Ltd for sanger-sequencing of the 5'-UTR region in both directions, using the same primers as mentioned above. The sequences were then aligned against one of the Australian type specific strains (accession number AB300674.1) to create a 292bp block of the 5'-UTR region of the BDVD genome, for each sample. Nucleotide sequences for BVDV-1, BVDV-2 and BDV reference strains were obtained from GenBank for nucleotide alignments using Clustal-W program[13]. A phylogenetic tree based on the 5'-UTR sequences was constructed using the neighbour-joining method within the MEGA program (Version 6.06).

Histopathologic analysis

Formalin-fixed tissues were trimmed to 5 mm thick sections and embedded in paraffin blocks by routine histopathological technique. Tissues were sectioned at 5 µm and stained with Harris's haematoxylin and eosin (HE). Selected sections were also stained with Fouchet's and Luxol Fast Blue using standard techniques. Images of sections were taken using an Olympus DP25 camera mounted on an Olympus BX43 microscope and captured with labSensTM software (Olympus Corporation, Philadelphia).

Results

Seroconversion of ewes

The time to seroconversion for each of the 22 ewes, the gestational age of the fetuses at which seroconversion was detected and the fetal outcomes are listed in

Table 1. Antibodies to BVDV were first detected in ewes as early as 14 days post inoculation and as late as 35 days post inoculation. Lambing occurred between 79 and 88 days post inoculation. Fifteen aborted, stillborn or live lambs were submitted for gross pathology. They consisted of six BVDV antibody positive and BVDV antigen negative lambs and nine BVDV antibody negative and BVDV antigen positive lambs.

Table 1 (a) Reported pregnancy losses in 22 ewes experimentally inoculated with BVDV-1c between 59 and 69 days of gestation compared to time to seroconversion;

Seroconversion in ewes detected		Total number of ewes	No. of ewes with aborted/stillborn lambs	No. of ewes with lambs surviving <24hrs	No. of ewes with surviving lambs
Days after inoculation	**Fetal age in days (±5 days)**				
14	78	5	4	1	-
17	81	9	6	2	1
21	85	4	3	1	1
28	92	3	2	0	1
35	99	1	1	-	-
Total		22	16	4	3

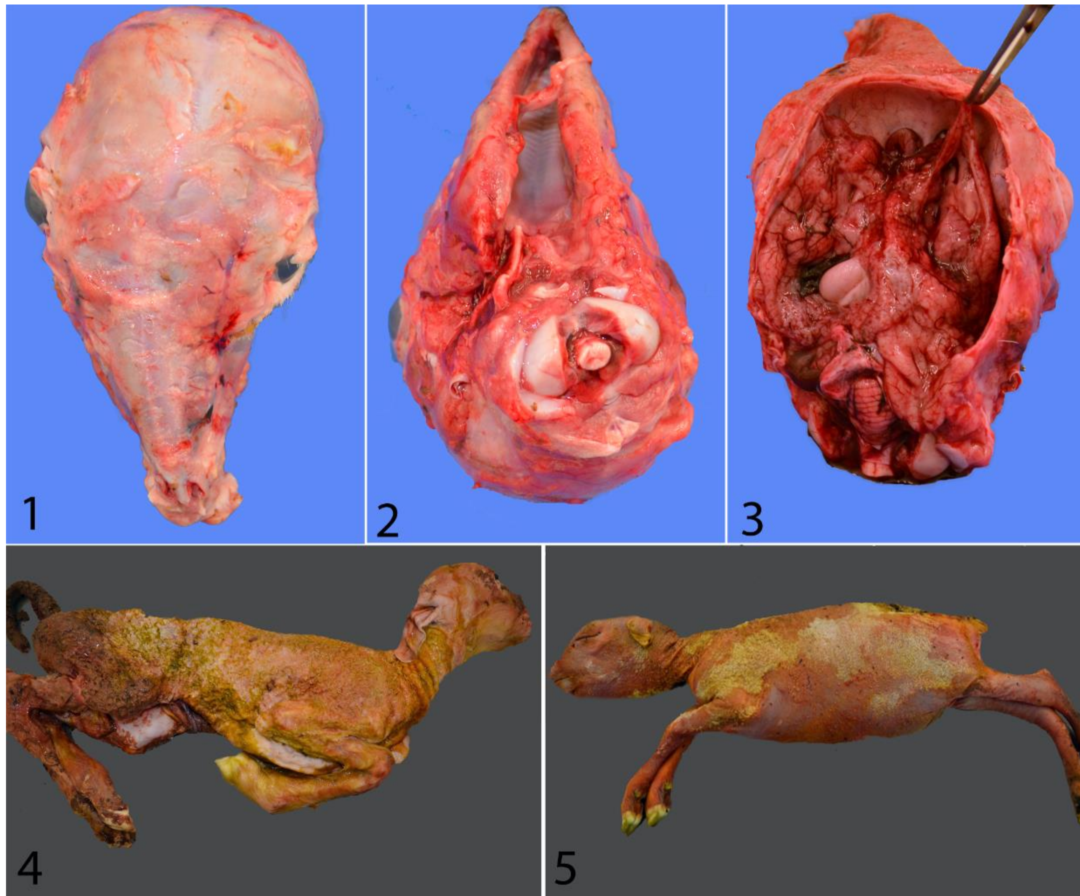
(b) Fetal losses, and corresponding lamb IDs, in lambs born to ewes experimentally inoculated with BVDV-1c between 59 and 69 days of gestation compared to time to seroconversion in ewes

Seroconversion in ewes detected		Total number of fetuses	Aborted/stillborn lambs		Lambs surviving <24hrs		Surviving lambs	
Days after inoculation	**Fetal age in days (±5 days)**		**Total number**	***Lamb ID***	**Total number**	***Lamb ID***	**Total number**	***Lamb ID***
14	78	9	7	-	2	*1 and 12*	-	-
17	81	19	14	*8, 9 and 10*	3	*13 and 14*	2	*4 and 15*
21	85	7	5	*2 and 7*	1	*6*	1	*5*
28	92	6	5	*11*	-	-	1	*3*
35	99	1	1	-	-	-	-	-
Total		42	32	-	6	-	4	-

Antibody positive and Antigen negative lambs

The post mortem findings in six antibody positive and antigen negative lambs are detailed in Table 2. Five of these lambs were born alive and all but one (Lamb No. 3) were undersized when compared to normal birth weight ranges for this breed (weight range 3.5 – 6.0 kg; Oldham, et al. [20]). Two lambs died within 24 hours of birth, and the remaining three lambs survived until eight weeks of age.

Skeletal deformities were observed in three of these lambs, including arthrogryposis and severe rotational deformity and convexity of the skull (frontal bones, occipital bones) and nasal bones (n = 1, figures 1 and 2), facial asymmetry (n = 2), and brachygnathia superior (n=1). Hydrancephaly (Figure 3) with cystic septum pellucidum was also observed in the lamb with arthrogryposis (Figure 4). Mild to moderate mesenteric lymphadenomegaly was observed in three lambs. The gastrointestinal tract distal to the abomasum had not formed (intestinal aplasia) in the lamb with arthrogryposis and severe skull deformities. Suppurative bronchopneumonia was present in one lamb that survived until eight weeks of age.

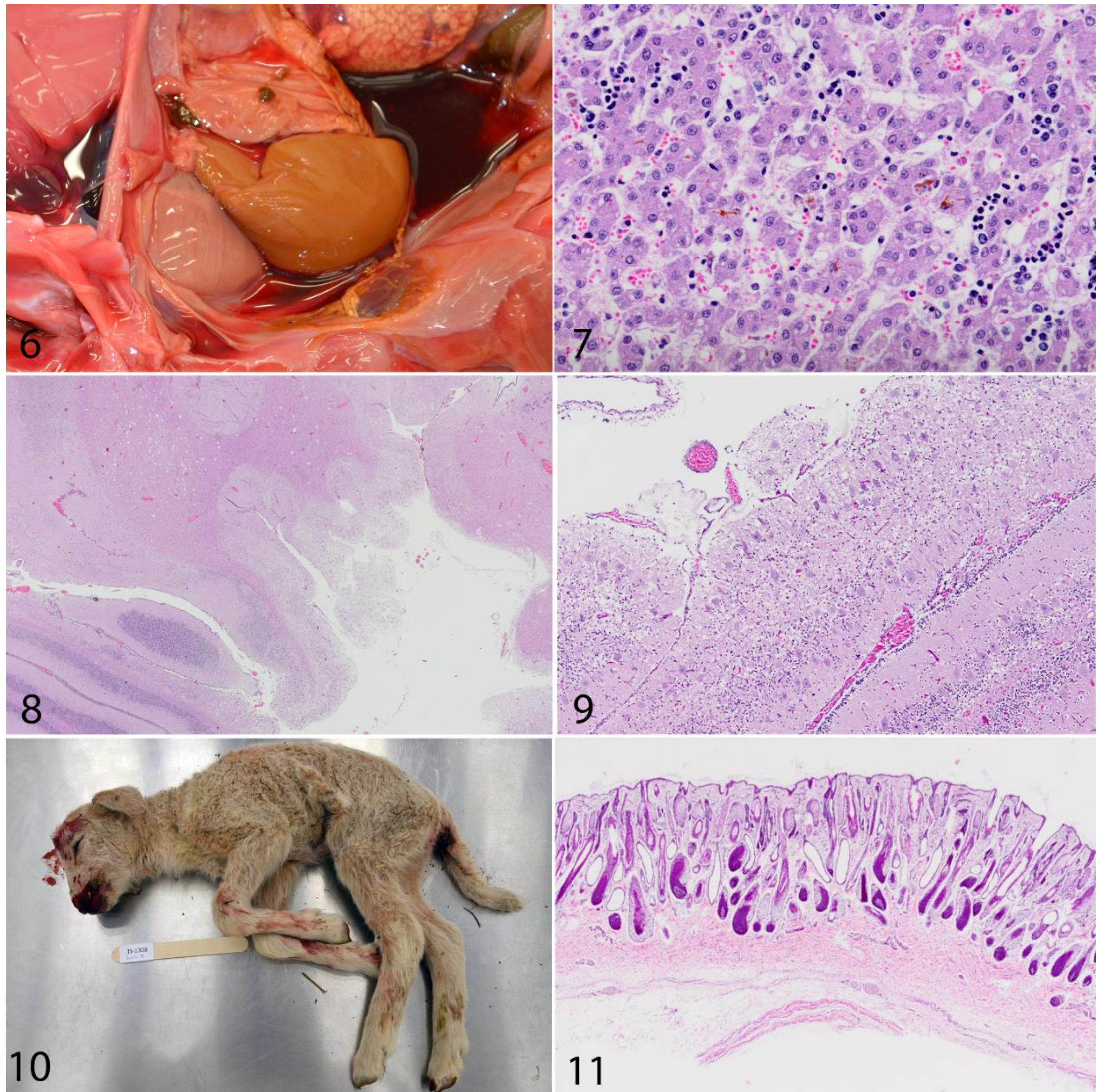


Figures 1 – 3, fetal lesions associated with experimental infection with BVDV-1c, antibody positive-antigen negative lambs. **Figure 1 and 2**, skull, lamb No. 2. Rotational deformity and convexity of the skull, most notable in frontal, nasal and occipital bones**.** **Figure 3**, lamb No. 2, brain. Hydrancephaly. **Figure 4.** Lamb No. 2. Arthrogryposis

Antibody negative and Antigen positive lambs

The post mortem findings for nine antibody negative and antigen positive lambs are detailed in Table 2. Four lambs were born alive but died within 24 hours of birth; only one lamb (Lamb No. 15) survived until eight weeks of age, which was subsequently confirmed to be persistently infected. All lambs in this group were undersized when compared to normal birth weight ranges (weight range 3.5 – 6.0 kg; Oldham, et al. [20]). Of the lambs that were aborted, gestational age was estimated to be between 115 and 125 days and the lambs weighed between 1.17 and 2.31 kg; sex and age were unable to be determined for one lamb due to severe predation.

Four of the lambs (No. 7 - 10) were affected by a distinct subset of lesions characterised by generalised anasarca (Figure 5) cholestatic hepatopathy (Figures 6 and 7), meconium staining of the skin and fleece (interpreted as fetal distress *in utero*) and advanced fetal autolysis. Histologically, there was widespread distension of canaliculi by bile plugs and mild to moderate hepatic extramedullary hematopoiesis. Examination of lung from these fetuses identified meconium and fetal squames within alveoli, consistent with distress *in utero*. Aside from this subset of lambs, a cholestatic hepatopathy and pronounced extramedullary haematopoiesis were also observed in one lamb born alive that presented with cerebellar dysplasia and hair-like wool (Lamb No. 13). Autolysis in lambs with cholestatic hepatopathy precluded detailed hepatic assessment in all bar lamb No 13; aside from widespread intrahepatic cholestasis, no further hepatobiliary lesions were noted in this lamb.



Figures 5 – 11, fetal lesions associated with experimental infection with BVDV-1c, antibody negative-antigen positive lambs. **Figure 5**, lamb No. 7: Fetal anasarca. **Figure 6**, lamb No. 8 (15-1299), liver. The liver has rounded lobar borders and is discoloured orange with friable parenchyma (cholestatic hepatopathy, fetal autolysis). **Figures 7**: Lamb No. 13, liver. Canaliculi are distended by bile plugs. Sinusoids contain haematopoietic precursors. Hematoxylin and Eosin. **Figures 8 and 9.** Lamb No. 13, cerebellum. Irregular folia atrophy, folial oedema, depletion and necrosis of granular cells, and ectopia and degeneration of Purkinje cells. **Figure 10.** Lamb No. 13. Fleece is hair-like. **Figure 11.** Lamb No. 13, haired skin from dorsolateral hind limb. A predominance of primary hair follicles is observed throughout the dermis.

Gross neurological lesions were observed in three of eight lambs in this group for which the brain was available for examination, comprising porencephaly (n = 2), cerebellar hypoplasia and dysplasia (n = 1, Figures 8 and 9) and white matter rarefaction in cerebellar peduncles (n =1). Cerebellar lesions were characterised by irregular folia atrophy, folial oedema, depletion and necrosis of granular cells, ectopia and degeneration of Purkinje cells, and mild non-suppurative meningoencephalitis in most affected regions. The lamb with cerebellar dysplasia and white matter rarefaction presented clinically with dysstasia, and the fleece was found to be hair-like (Figure 10) with a predominance of primary follicles seen histologically (Figure 11). Histological examination of the brain in lambs with porencephaly identified thinning of the corticies, neuronal loss and malacia, and focal infiltration by pigment laden mononuclear cells in the region adjacent to the pore. Other findings in this group included bilateral hydronephrosis (n = 2), non-fusion and laxity at growth plates of long and flat bones (n = 1), mild brachygnathia superior (n = 1), and moderate unilateral thyroid gland enlargement. Bone and thyroid lesions were not further examined by histopathology. Thymus was examined from the four lambs that survived until eight weeks of age, however there were no significant findings.

Table 2: A summary of the gross and histopathological post-mortem lesions seen in 15 sheep fetuses and lambs from 22 ewes experimentally inoculated with bovine viral diarrhea virus (-1c) between 59 and 69 days gestation

Lamb ID	Sex	Body weight at birth (kg)	Crown Rump Length[‡] (cm)	Age at death[↑]	Clinical, Gross and Histopathological Findings
Antibody positive and antigen negative lambs					
1	F	2.6	44	<24hrs	Mild ataxia
2	M	3.5	52	Born dead	Hydrancephaly, arthrogryposis, severe skull deformity, intestinal aplasia
3	M	3.6	-	8 weeks	Brachygnathia superior, mild facial asymmetry, nasal convexity, mesenteric lymphadenomegaly
4	M	2.7	-	8 weeks	Mild facial asymmetry, mesenteric lymphadenomegaly
5	M	3.2	-	8 weeks	Bronchopneumonia, mesenteric lymphoid hyperplasia,
6	*	1.3	42	<1hr	None
Antibody negative and antigen positive lambs					
7	F	1.3	34	115 days gestation	Anasarca, porencephaly, dislocation and laxity at growth plates of all long and flat bones, right thyroid enlargement, cholestatic hepatopathy
8[†]	M	2.3	46	125 days gestation	Anasarca, cholestatic hepatopathy
9	M	1.5	38	118 days gestation	Anasarca, porencephaly, brachygnathia superior, bilateral hydronephrosis, cholestatic hepatopathy
10[†]	*	2.1	*	*	Anasarca, cholestatic hepatopathy
11	F	1.2	34	115 days gestation	None
12	F	2.1	44	<1hr	None

Table 2 cont.

Lamb ID	Sex	Body weight at birth (kg)	Crown Rump Length‡ (cm)	Age at death↑	Clinical, Gross and Histopathological Findings
13	F	2.3	50	<24hrs	Cerebellar dysplasia, dysstasia, 'hair-like' wool covering body (primary follicular dysplasia), cholestatic hepatopathy
14	F	1.6	43	<24hrs	Ataxia, 'hair-like' wool over shoulders and dorsolateral hind limbs, bilateral hydronephrosis
15	F	2.5	-	8 weeks	Mesenteric lymphadenomegaly

* = unknown
† = brain/spinal and/or reproductive tract missing due to predation
‡ = CRL of lambs which died <24hrs after birth only
↑ = approximate gestational age at death calculated from CRL[8,16]

Sequence alignment and phylogenetic tree

Phylogenetic analysis revealed both the inoculating cattle serum and lamb 15 were 100% homologous with one another and were located within the BVDV-1 clade. The most closely related BVDV-1 species were those of the Australian and New Zealand BVDV-1c strains (Figure 12).

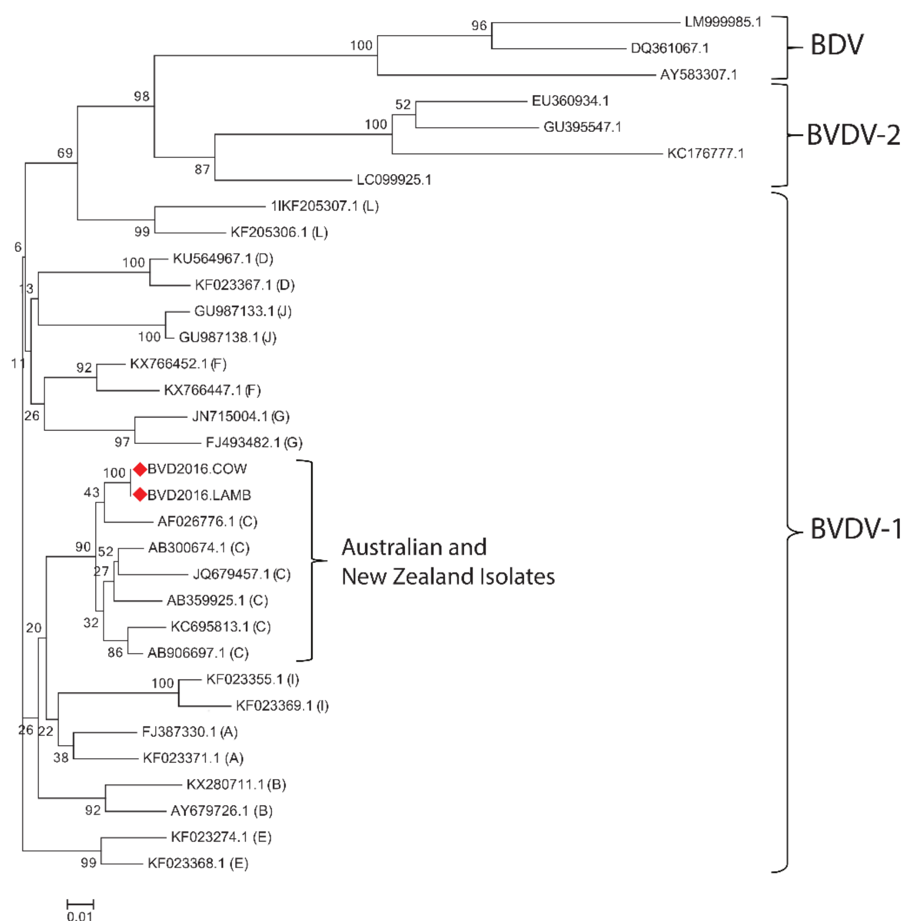


Figure 12: Neighbour-joining phylogenetic tree using MEGA program (Version 6.06) comparing the 5'-UTR nucleotide region from the inoculating cattle serum and Lamb 15 (BVD2016.Cow and BVD2016.Lamb) with known BDV, BVDV-2 and BVDV-1 reference strains. Ten of the recognized BVDV-1 strains are identified by a bracketed letter following the accession number.

Discussion

The ovine fetus is unable to respond to an antigenic stimuli until 60 to 80 days of gestation and is deemed immunotolerant during this time[17]. When an infection, such as BVDV, takes place prior to this gestational period the infective status of the fetus is unpredictable [17]. In the current study two subsets of lambs (antigen negative and antibody positive; antigen positive and antibody negative) were identified. It is likely that these two subsets of lambs occurred due to the inoculation of ewes between 59 and 69 days of gestation, during this ‘unpredictable’ fetal period. Within these two subsets a wide range of pathological malformations were identified which again are thought to be due to the gestational age of the fetuses at inoculation. Similar pathological malformations as those identified in this study have been recorded following BVDV and BDV infections in pregnant ewes between 16 and 105 days gestation[9,14,23]. However it is also possible that the variation in malformations observed between lambs can be attributed to the different lengths of time between inoculation and fetal infection, suggested by the variation in time to seroconversion observed in the ewes.

Previously studies have identified that following the natural infection of pregnant ewes with Border disease virus, the virus quickly spreads to the placenta and infects the fetus within one week of infection[17]. If the same principle applies to BVDV infection in pregnant ewes then it can be assumed that infection of the fetuses in this study occurred between 66 and 76 days of gestation. However, it is unclear if the ‘one week following infection’ applies from when the ewes contracted the virus or from when they themselves were infectious (viraemic). Irrespective of this, seroconversion was first detected in the ewes of the current study between 14 and 35 days post inoculation (73 – 114 days of gestation). It is likely that this wide gestational window could explain the significant variation in lesions and fetal

outcomes identified in the lambs from this study. However, the true age at infection for each of the lambs is unknown and estimates can only be made based on the size of the lamb at post mortem, or birth, and the physical/neurological abnormalities each lamb presented with.

In general, the pathological findings in antigen negative and antibody positive lambs were less severe to those seen in antigen positive and antibody negative lambs. Five of the 6 antigen negative lambs were born alive, with 3 of these surviving until 8 weeks of age, at which time they were euthanized. In these 6 lambs, a range of developmental abnormalities were observed, including hydrancephaly, arthrogryposis, facial asymmetry and brachygnathia. While the exact timing of fetal infection for each lamb in the present study is unknown, the pathological malformations in the present study have previously been identified following fetal infections with BVDV and BDV in cattle and sheep respectively[1,9,11]. The differences in dose, infection route and the virulence of the viruses used in these previous studies does make direct comparisons difficult. Nevertheless, previous studies investigating BVDV infection in cattle have documented the association of calves presenting with hydrancephaly, porencephaly, brachygnathia and arthrogryposis following maternal infection during early to mid-gestation (80-150 days of gestation) [2,10]. Similarly in sheep, pathological malformations such as arthrogryposis, brachygnathia and hydrancephaly have been associated with BDV infection in sheep between 55 and 76 days of gestation [9,11], a similar time frame to the infection period in the present study.

Bronchopneumonia was identified in one lamb from the antigen negative subset of lambs, which may or may not be reflective of immunosuppression due to BVDV infection. Although a positive relationship between bronchopneumonia and BVDV

has previously been identified [4] the cause of the presenting bronchopneumonia in this lamb is unknown.

In the subset of antigen positive lambs, advanced foetal autolysis and small foetal size was identified in the majority of aborted lambs. These findings suggest that these lambs died soon after BVDV infection had occurred, possibly due to overwhelming viral replication. It has been previously specified that the most dangerous time for the ovine fetus to be infected with BDV is in the first 60 days of gestation as viral infection is uncontrolled and foetal death is common[17]. It has also been identified that foetal deaths relating to both BVDV or BDV infections, in cattle or sheep, can either occur rapidly, leading to reabsorption of the fetus, or may not occur until many weeks or months later, when small fetuses are aborted [1,17]. The majority of the small, aborted fetuses examined in the current study were not observed until lambing had begun, well after the predicted time of death (Figure 2).

Fetal anasarca (hydrops fetalis) and intrahepatic cholestatic hepatopathy were identified in 5 of the 9 antigen positive lambs. Fetal anasarca is the result of impaired fluid exchange and proposed and known causes include genetic etiologies, fetal infections, fetal anaemia, cardiac or renal disease and malformations of the placenta [15,21,24]. Placentae were not examined in this study. In cattle, the detection of BVDV antigen in aborted fetuses has previously been associated with anasarca and hydrocephalus [4]. Similarly in sheep anasarca has been reported in two fetuses from ewes experimentally infected with a non-cytopathic strain of BVDV[12]. In regards to the present study, there appears to be a strong association between mid-gestational infection with BVDV-1c and the development of antigen positive and antibody negative lambs presenting with fetal anasarca and a cholestatic hepatopathy. The pathogenesis of the cholestasis is unclear; however, given the absence of

hepatocellular degeneration, necrosis or significant parenchymal inflammation or fibrosis, intrahepatic cholestasis as a result of haemolysis or increased fetal RBC turnover must be considered. Although extramedullary hematopoiesis is a considered a normal finding in the fetus, haematopoietic infiltrates appeared more pronounced in lambs with intrahepetaic cholestasis than other lambs examined in this study, which may support haemolysis as the cause for observed cholestasis.

Additional lesions in antigen negative lambs included porencephaly in two lambs, and cerebellar dysplasia and white matter rarefaction in one lamb which also presented with muscular tremors, ‘hair like’ wool and an increased predominance of primary wool follicles usually associated with BDV or ‘hairy-shaker’ disease in sheep [19]. The presentation of ‘hairy’ lambs has previously been described following infection with BVDV at 54 days of gestation[3] however as far as the authors are aware the predominance of primary hair follicles has not previously been described in BVDV affected lambs.

Mild skeletal malformations were seen in only two lambs, one which presented with non-fusion of growth plates in long and flat bones and the other with mild brachygnathia superior. Previously bone lesions associated with BVDV infection have been observed in persistently infected bovine fetuses, characterised by impaired trabecular modelling, thought to be due to decreased osteoclastic activity[25]. However in the present study bone lesions were not examined histologically limiting further interpretation and discussion on pathogenesis. Lastly, unilateral thyroid enlargement, bilateral hydronephrosis and mesenteric lymphadenomegaly were also identified in individual antigen negative lambs.

Generally with BVDV and BDV infections in ruminants, immunotolerant and persistently infected animals are born apparently healthy [1] without the range of pathological malformations seen during this study. It is likely that the malformations seen in the current subset of lambs developed due to the timing of the infection. In many of the ewes there was an extended length of time to seroconversion possibly resulting in fetal infection occurring during the period of organogensis. Due to the majority of antigen positive lambs dying either *in utero* or as young neonates concurrent testing could not be undertaken, and the accurate identification of which of these lambs were truly persistently infected with BVDV-1c could not be achieved. Only one antigen positive lamb survived to 8 weeks of age and by sequencing of the 5'-UTR region was determined to be persistently infected with BVDV-1c.

In summary, this study details the gross and histopathological lesions identified in lambs, infected in utero by the predominant BVDV strain in Australia, BVDV-1c. Anasarca and neurological and skeletal malformations were the most common consequences following fetal infection with anasarca associated only with lambs antibody negative and antigen positive. This study also reported primary follicular dysplasia in sheep infected with BVDV, a development normally identified with BDV infected lambs.

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References

1 Baker JC: Clinical aspects of bovine virus diarrhoea virus infection. *Rev Sci Tech* 1990:9(1):25-41.

2 Baker JC: The clinical manifestations of bovine viral diarrhea infection. *Vet Clin North Am Food Anim Pract* 1995:11(3):425-445.

3 Barlow RM, Rennie JC, Gardiner AC, Vantsis JT: Infection of pregnant sheep with the NADL strain of bovine virus diarrhoea virus and their subsequent challenge with Border disease IIB pool. *J Comp Pathol* 1980:90(1):67-72.

4 Baszler TV, Evermann JF, Kaylor PS, Byington TC, Dilbeck PM: Diagnosis of naturally occurring bovine viral diarrhea virus infections in ruminants using monoclonal antibody-based immunohistochemistry. *Vet Pathol* 1995:32(6):609-618.

5 Evans CA, Lanyon SR, Reichel MP: Investigation of AGID and two commercial ELISAs for the detection of Bovine viral diarrhea virus-specific antibodies in sheep serum. *Journal of Veterinary Diagnostic Investigation* 2017:29(2):181-185.

6 Evans CA, Lanyon SR, Sims SK, Reichel MP: Reproductive performance in experimentally BVDV infected ewes and seroconversion rates in sheep co-mingled with BVDV PI calves. *Small Ruminant Research* 2015:123(2-3):314-319.

7 Evans CA, Reichel MP, Hemmatzadeh F, Cockcroft PD: Clinical responses and reproductive outcomes in pregnant ewes experimentally infected with bovine viral diarrhoea virus (Type-1c) between days 59 and 69 of gestation. *Small Ruminant Research* 2017.

8 Galpin N: The pre-natal development of the coat of the New Zealand Romney lamb. *The Journal of Agricultural Science* 1935:25(3):344-360.

9 Garcia-Perez AL, Minguijon E, Estevez L, Barandika JF, Aduriz G, Juste RA, et al.: Clinical and laboratorial findings in pregnant ewes and their progeny infected with Border disease virus (BDV-4 genotype). *Research in Veterinary Science* 2009:86(2):345-352.

10 Grooms DL: Reproductive consequences of infection with bovine viral diarrhea virus. *Veterinary Clinics Food Animal Practice* 2004:20:5-19.

11 Hewicker-Trautwein M, Liess B, Frey HR, Trautwein G: Virological and pathological findings in sheep fetuses following experimental infection of pregnant ewes with cytopathogenic-bovine-virus diarrhoea virus. *Zentralbl Veterinarmed B* 1994:41(4):264-276.

12 Hewicker-Trautwein M, Trautwein G, Frey HR, Liess B: Variation in neuropathogenicity in sheep fetuses transplacentally infected with non-cytopathogenic and cytopathogenic biotypes of bovine-virus diarrhoea virus. *Zentralbl Veterinarmed B* 1995:42(9):557-567.

13 Higgins DG, Thompson JD, Gibson TJ: Using CLUSTAL for multiple sequence alignments. *Methods Enzymol* 1996:266:383-402.

14 Loken T: BORDER DISEASE IN SHEEP. *Veterinary Clinics of North America-Food Animal Practice* 1995:11(3):579-&.

15 Monteagudo L, Lujan L, Tejedor T, Climent S, Acin C, Navarro A, et al.: Fetal anasarca (hydrops fetalis) associated with lymphoid tissue agenesis possibly due to an autosomal recessive gene defect in sheep. *Theriogenology* 2002:58:1219-1228.

16 Mufti AM, Wani GM, Wani NA, Buchoo BA, Khan MZ: Prenatal development of ovine fetus. *Small Rumin Res* 2000:38(1):87-89.

17 Nettleton PF: Pestivirus infections in ruminants other than cattle. *Rev Sci Tech* 1990:9(1)**:**131-150.

18 Nettleton PF, Entrican G: Ruminant pestiviruses *British Veterinary Journal* 1995:151**:**615-642.

19 Nettleton PF, Gilray JA, Russo P, Dlissi E: Border disease in sheep and goats. *Veterinary Research* 1998:29**:**327-340.

20 Oldham CM, Thompson AN, Ferguson MB, Gordon DJ, Kearney GA, Paganoni BL: The birthweight and survival of Merino lambs can be predicted from the profile of liveweight change of their mothers during pregnancy. *Animal Production Science* 2011:51**:**776-783.

21 Plant JW, Lomas ST, Harper PA, Duncan DW, Carroll SN: Hydrops foetalis in sheep. *Aust Vet J* 1987:64(10)**:**308-310.

22 Ridpath JF, Fulton RW, Kirkland PD, Neill JD: Prevalence and antigenic differences observed between Bovine viral diarrhea virus subgenotypes isolated from cattle in Australia and feedlots in the southwestern United States. *Journal of Veterinary Diagnostic Investigation* 2010:22(2)**:**184-191.

23 Scherer CFC, Flores EF, Weiblen R, Caron.L., Irigoyen.L.F., Neves.J.P., et al.: Experimental infection of pregnant ewes with bovine viral diarrhoea virus type-2 (BVDV-2): effects on the pregnancy and fetus. *Veterinary Microbiology* 2001:79**:**285-299.

24 Selvaraju M, Palanisamy M, Ravikumar K, Prabaharan V, Ravi R, Ezakial Napolean R, et al.: Dystocia due to foetal anasarca with ascites in a sheep - a case report. *J Vet Anim Sci* 2008:39**:**62-63.

25 Webb BT, Norrdin RW, Smirnova NP, Van Campen H, Weiner CM, Antoniazzi AQ, et al.: Bovine Viral Diarrhea Virus Cyclically Impairs Long Bone Trabecular

Modelling in Experimental Persistetnly Infected Fetuses. *Vet Pathol* 2012:49(6):930-94

3.3 Chapter discussion

An original objective of this trial was to produce as many viable BVDV-1c PI lambs as possible while limiting the number of foetal losses. Previously it had been reported that the development of persistently BDV infected lambs results from the infection of ewes prior to 80 days of gestation (Nettleton 1990; Schweizer and Peterhans 2014). However it has also been reported that infection with BDV in the first 60 days of gestation can result in a high rate of foetal death, as during this time virus replication is uncontrolled and resulting foetal death is common (Nettleton 1990). As artificial insemination was not an option in the design of this experiment the closest gestational window that could be achieved between ewes was 10 days. As such the final gestational window of 59-69 days was chosen as it fell within the optimal period for viable PI lamb development, and decreased the chance of high foetal death/low lambing rates occurring.

The inclusion of an uninfected, control ewe group within this study allowed for the comparison of a number of clinical, haematological and reproductive effects to be recorded in relation to acute BVDV infection of pregnant ewes. Results from this study revealed that, overall, acute BVDV-1c infections in sheep are clinically in-apparent with only a mild rise in temperature, a mild leukopaenia and a prolonged decrease in eosinophil counts being recorded. These clinically in-apparent findings are in line with what has previously been reported in both, cattle acutely infected with BVDV (Nettleton and Entrican 1995) and sheep acutely infected with BDV (Nettleton 1990). As such results indicate that acute BVDV-1c infections are clinically inapparent in non-pregnant sheep making them difficult to detect in a commercial flock.

In contrast to the literature indicating that fewer foetal deaths occur during acute BDV infections in pregnant ewes, acute BVDV-1c infection between 59 and 69 days gestation in pregnant ewes in this study resulted in a high foetal death rate. A lambing percentage of just

31.8% was recorded from infected ewes in this study. In comparison, pregnant ewes of the same gestational stage and managed under the same conditions, but naïve to BVDV, reported a lambing percentage of 172.7%. These results support previous findings by Evans *et al.* (2015) that reported that severe lambing losses are possible when acute BVDV-1c infections develop within a pregnant ewe flock. However due to the inclusion of an uninfected and similarly managed pregnant ewe flock in the current study, it can now be confirmed that the high lamb losses are due to BVDV-1c infection rather than other poor management practices.

High lamb losses were not the only reproductive outcome observed in BVDV-1c infected ewes in this study. A wide range of pathological lesions were seen in both live-born and aborted lambs from infected ewes. These pathological lesions included anasarca, skeletal deformities, hydranencephaly, porencephaly and primary follicular dysplasia, many of which have previously been observed in lambs born to ewes infected with either BVDV (Hewicker-Trautwein *et al.* 1995; Scherer *et al.* 2001) or BDV (Nettleton *et al.* 1998; Garcia-Perez *et al.* 2009) during mid-gestation. However as far as the authors are aware this is the first report of histologically confirmed primary follicular dysplasia associated with BVDV infection in sheep.

Despite the confirmation that acute BVDV-1c infections in pregnant ewes can severely impact lambing rates and lamb survival, an important objective for this study was to produce viable persistently BVDV-1c infected lambs. While nine lambs/foetuses were confirmed to be BVDV-antibody negative and BVDV-antigen positive, only one of these lambs survived to an age where consecutive testing could be carried out to confirm persistent infection. The remainder of the antigen positive lambs either died soon after birth or were non-viable, aborted foetuses. These results suggest that persistently BVDV-1c infected ovine foetuses are unlikely to develop to term and if they do, survival past a few days of age is poor. However the birth of a single, viable BVDV-1c PI lamb during this study still highlights the need to fully understand the role sheep populations play in the spread of BVDV throughout Australia as even a single PI animal

may have the potential to infect large numbers of susceptible individuals. Currently however, it is unknown if a BVDV-1c PI ovine is able to infect susceptible sheep and cattle.

Chapter 4: Transmission of BVDV-1c in sheep

The effective epidemiological control of a virus, such as BVDV, cannot be achieved unless all significant sources of infection are identified. As BVDV has been shown to readily infect many non-bovine species (Nettleton 1990), cattle populations may not be the only source of infection of the virus. The persistence of BVDV may also be due to interactions between different species of infected and susceptible livestock, such as cattle and sheep. While BVDV infections have been shown to occur naturally in sheep (Pratelli *et al.* 2001; Evans *et al.* 2015), it is unknown whether BVDV infected sheep are able to maintain an infection within a population and/or transmit the virus back to cattle.

Previous studies, including those in Chapter 3, have shown that the development and survival of BVDV-1c PI lambs does not occur readily. However, a single BVDV-1c PI lamb was shown to survive to euthanasia, at eight weeks of age. Understanding the ability of a BVDV-1c PI lamb to infect susceptible animals is crucial in determining the importance of sheep in the epidemiology of BVDV infections and their inclusion in control programs for BVDV both in Australia and elsewhere.

The identification and removal of PI cattle having been shown to effectively control BVDV in many parts of the world (Lindberg *et al.* 2006; Presi *et al.* 2011). However, BVDV has been reported to circulate within a herd for up to two and a half years in the absence of, or following no direct contact with, PI cattle (Barber and Nettleton 1993; Moerman *et al.* 1993). It is believed that the persistence of the virus under these circumstances may be due to a short period of viral excretion in animals undergoing acute BVDV infections.

In Chapter 3 it was demonstrated that BVDV naïve control ewes did not become infected when separated from ewes undergoing acute BVDV infections by a 1.5m, double fenced gap. This

1.5m gap between paddocks is shorter in distance than current minimum buffer zone recommendations (3m) for limiting disease spread (Boardman 2015). Although the distance between treatment groups of ewes was less than recommended, transmission of BVDV-1c did not occur between infected and control ewes.

This leads to question whether sheep undergoing an acute BVDV-1c infection are able to infect *Pestivirus* naive animals. Understanding the potential for acutely BVDV-1c infected sheep to spread the virus to susceptible animals needs to be determined in order to fully understand the role acutely infected sheep play in the epidemiology of BVDV within Australia.

The following two manuscripts report on the results of a series of natural infection trials from sheep persistently or acutely infected with BVDV-1c to pestivirus naïve sheep and cattle.

To investigate the infectivity of a BVDV-1c PI lamb ≤8 weeks of age, three simultaneous experimental trials were undertaken. The aim of these trials was to investigate the infectivity of the BVDV-1c PI with regards to naïve cattle and sheep using sero-conversion as an indication of infection. The three trials consisted of: ewes and lambs co-paddocked with the PI lamb for an extended period of time, steers housed adjacent to the PI lamb and steers exposed directly to the PI lamb.

To investigate the infectivity of acutely BVDV infected sheep, five *Pestivirus* naïve wethers were co-paddocked with five wethers undergoing an acute BVDV-1c infection and were monitored for sero-conversion.

4.1 Transmission from persistently infected sheep

The manuscript presented in this section reports on the infective nature of a persistently BVDV infected, neonatal lamb to pestivirus naïve sheep and cattle. This manuscript has been prepared for submission to the *Veterinary Record* (submitted).

Original article: Natural transmission of BVDV-1c from a persistently infected, neonate lamb to naïve sheep and cattle

CA Evans, F Hemmatzadeh, MP Reichel and PD Cockcroft

Natural transmission of BVDV-1c from a persistently infected, neonate lamb to naïve sheep and cattle

Veterinary Record (submitted)

Statement of Authorship

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Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper
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By signing the Statement of Authorship, each author certifies that:

i the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii the sum of all co-author contributions is equal to 100% less the candidate's stated contribution

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Natural transmission of BVDV-1c from a persistently infected, neonate lamb to naïve sheep and cattle

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Abstract

This study investigated the transmission of BVDV-1c from a neonatal PI lamb to naïve sheep and cattle using three treatment groups: four naïve ewes and their 5 lambs, which were co-paddocked with the PI lamb, five steers, which were housed in a paddock adjacent to the PI lamb and five steers, which were directly exposed to the PI lamb. Serum samples were collected and tested for BVDV-specific antibodies. Serum samples from the PI lamb, from day of birth to 8 weeks of age, were tested for BVDV-specific antibodies and antigen and submitted for real time-PCR to determine the viral load present at each week of age.

Only one lamb from the co-paddocked group developed BVDV-specific antibodies following co-mingling while all the steers in both the cattle treatment groups remained BVDV antibody negative. Real time-PCR results from the PI lamb showed lower viral loads from day of birth to 6 weeks of age, compared to results at 7 and 8 weeks of age. This may reflect maternal colostral BVDV antibodies concentrations in the neonate lamb or other viral properties.

Keywords: BVDV, transmission, sheep, cattle, neonate lamb

Introduction

Bovine viral diarrhoea virus (BVDV) belongs to the *Pestivirus* genus, along with Border disease virus (BDV) in sheep and Clinical swine fever virus in pigs and is an important reproductive and immunosuppressive disease of cattle (Edwards and others 1986; Grooms 2004).

Previous studies have shown that BVDV infections may also occur in a wide range of non-bovine ruminant species, including sheep (Scherer and others 2001), goats (Bachofen and others 2013), deer (Nettleton 1990) and alpacas (Goyal and others 2002). Many of the reproductive losses seen in cattle infected with BVDV have also been reproduced in sheep, with high abortion rates, the birth of congenitally and neurologically abnormal lambs and the birth of persistently BVDV infected lambs (Evans and others 2017b; Hewicker-Trautwein and others 1995; Scherer and others 2001) being previously reported.

In Australia, there is a predominance of a single BVDV type 1 strain, BVDV-1c (Ridpath and others 2010). While the survival of BVDV-1c PI has been shown to be poor (Evans and other 2015; Evans and others 2017b) even the limited survival of these animals indicates a need to understand if young PI lambs are capable of transmitting BVDV-1c to susceptible animals. As such, the aim of this study was to determine if a BVDV-1c PI, neonate lamb was capable of infecting naïve sheep and cattle by either direct exposure or through close proximity in adjacent paddocks.

Materials and Methods

The study was approved by the University of Adelaide's Animal Ethics Committee (S-2015-124 and S-2014-111B).

Experimental animals

A BVDV PI lamb was produced during a preceding study undertaken at the University of Adelaide (Evans and others 2017b). The dam of this lamb had been experimentally inoculated with BVDV-1c PI cattle serum between 59 and 69 days of gestation and was confirmed to be antibody positive and virus negative. The resultant lamb had consumed colostrum and as such was positive for BVDV-specific antibodies at all time periods. An ear notch sample, taken on the day of birth, tested positive for BVDV-specific antigen by IDEXX Bovine Viral Diarrhoea Virus (BVDV) Antigen Test Kit/Serum Plus ELISA (IDEXX Laboratories Inc, Rydalmere, NSW). Phylogenetic analysis of the PI lamb and the inoculating PI cattle serum were found to be 100% homologous and were most closely related to the BVDV-1 species of Australia and New Zealand, BVDV-1c strains (unpublished data).

The PI lamb was housed with its dam in a BVDV-1c antibody-positive flock. This flock consisted of ewes which had previously undergone acute infections to BVDV-1c and four antibody positive and antigen negative lambs, produced by these ewes during the preceding study (Evans and others 2017b). All lambs, including the PI, were aged <1 week of age at the commencement of the study.

The naïve animals used in this study were weaned Angus steers, Merino ewes and Merino-X lambs sourced from the University of Adelaide's commercial farm, resident at its Roseworthy Campus in South Australia. All lambs were aged <1 week of age at the commencement of the study. All animals were tested prior to inclusion in this study for both antibodies to BVDV and BDV.

Treatment groups

Three treatment groups were used to study transmission of BVDV-1c from a neonate PI lamb to naïve sheep and cattle.

Treatment group A – Comingled sheep

Four confirmed BVDV naïve ewes and their five BVDV naïve lambs were co-paddocked with the neonate PI lamb, and its BVDV-1c seroconverted flock, in a small, outdoor yard (size 30 x 25 m) for a period of eight weeks. Co-paddocking commenced on the day of birth (DOB) of the PI lamb and when all naïve lambs were <1 week old.

Treatment group B – Adjacent paddock steers

Five BVDV naïve steers were housed in an adjoining yard to the neonate PI lamb (and its flock) for a period of four weeks. These yards were situated outdoors but allowed for direct contact interactions along the common fence line, particularly during feeding, when feed for both groups was fed out along this common fence line. Separate water troughs were used for each group.

Treatment group C – Direct exposure steers

Five BVDV naïve steers were housed in a small paddock, >100m from both treatment groups A and B. On the day of exposure each steer was restrained in a cattle crush and exposed to the PI lamb for a period of 5 minute. The lamb was restrained in a 1m x 0.5m tub which was placed in close proximity to the steer's face (Figure 1). Direct contact between the steers and the PI lamb did occur, as well as inhalation of the air surrounding the PI lamb. This process was repeated 4 weeks later.

Figure 1: Close proximity of the neonate PI lamb and steers from treatment group C

Sampling

Blood was collected into plain 5ml vacutainer serum tubes by venepuncture from the jugular or caudal vein.

Treatment group A

Animals from treatment group A were sampled weekly from Day 0 (the day the trial started) until Day 56.

Treatment groups B and C

Animals from both treatment groups were sampled three times only, on Days 0, 28 and 56.

Serum analysis

Thawed serum samples from all sheep, including the PI lamb, were tested for BVDV-specific antibodies using the IDEXX BVDV Ab P80 ELISA, with interpretation as per Evans and others (2017a) for sheep, otherwise as per manufacturer's instructions for cattle. Samples were also

tested for BVD viral antigen using the IDEXX BVDV Antigen ELISA as per manufacturer's instructions.

Quantitative-PCR

Quantitative polymerase chain reaction (qPCR) was carried out on all serum samples collected from the PI lamb. Firstly, total viral RNA was extracted using the QIAmp Viral RNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions. Viral RNA was then used to quantify the viral load in selected serum samples using the Power SYBR® Green RNA-to C_T™ 1-Step Kit (Applied Biosystems, USA), as per the manufacturer's instructions.

A 229 bp fragment of the 5'UTR (un-translated region) of the BVD virus was amplified by an in-house real time-PCR using primer pair BVD-292F (CTA GCC ATG CCC TTA GTA GGA CTA), and BVD-292R (CAA CTC CAT GTG CCATGTAC AGC A). The purified PCR product was cloned into pGEM T-easy vector (Promega Inc., USA) through A-T ligation. The resulted 3307bp construct was transformed into E. coli DH5α competent cells. Ten positive clones were identified by blue/white screening. Plasmids were extracted using QIAmp Spin Miniprep Kit (Qiagen, Germany) from these ten clones and were confirmed by sequencing using forward and reverse M13 primers. No diversity was observed in any of the sequences. The pGEM-T easy vector containing the 5'UTR fragment was named pGEM-BVD-UTR.

DNA concentration for the extracted plasmid was measured using a NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, USA). The plasmid copy number per microlitre was calculated using an online calculator (http://cels.uri.edu/gsc/cndna.html). A ten-fold serial dilution of the pGEM-BVD-UTR plasmid DNA was used for all Q-PCR assays.

To generate standard curves for the pGEM-BVD-UTR plasmid, each dilution was tested in triplicate and used as quantification standards to set up the standard curve. At the same time

0.66µl of viral RNA from each serum sample was tested in an aliquot of the same reaction mix as the standards. These samples were run in triplicate and the Ct values for the samples and standards were compared. The plasmid copy number logarithm was plotted against the measured Ct values for each sample.

Results

PI lamb

This lamb tested positive for BVDV-specific antibodies at all sampling time points and positive for BVDV antigen from Day 21 to Day 56 (Table 1). Real time-PCR revealed BVD viral copy numbers/ml for this lamb were consistent from DOB to Day 42 and were highest at Day 49 and 56 with 1.3 x 10^6 and 1.3 x 10^5 viral genome copies/ml recorded, respectively.

Treatment group A

Ewes and lambs from treatment group A were all negative for BVDV antibodies and BVDV-specific viral antigen at all sampling time points, except one lamb. Serum samples from this lamb, Lamb 5, were positive for BVDV antibodies at Day 49 and Day 56 post co-paddocking, with antibody S/N ratios of 18.9% and 17.1% recorded respectively.

Treatment groups B and C

All steers from treatment groups B and C remained negative for BVDV antibodies and BVDV-specific viral antigen at all sampling time points.

Tables

Table 1: Antibody ELISA, antigen ELISA and qPCR titres for the persistently BVDV infected neonate lamb from day of birth (DOB) to 8 weeks of age.

Antibody ELISA S/N ratios <63.5% are classified as positive; antigen ELISA results >0.3 are classified as positive.

	Units	DOB (ear notch)	Serum								
			DOB	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 35**	**Day 42**	**Day 49**	**Day 56**
Antibody	S/N%	N/A	15.92	16.57	15.02	18.45	19.33	20.12	19.91	21.14	18.66
Antigen	Corrected optical density	3.26	3.46	0.15	0.25	1.86	2.59	2.19	2.15	2.45	2.7
qPCR	copy number/ml	N/A	1.3×10^5	1.3×10^5	1.3×10^5	1.3×10^5	1.3×10^5	1.3×10^5	1.3×10^5	1.3×10^6	1.3×10^6

Discussion

Consistently strong serum antigen results indicate that, despite the positive antibody results, this lamb was persistently infected with BVDV. Pre-colostral blood samples were not obtained from this lamb and explain why a positive antibody result was recorded, in serum samples, on Day 0 and thereafter. The ingestion of colostrum, and associated colostrum derived antibodies to BVDV-1c, is believed to be the reason behind the negative antigen results recorded on Days 7 and 14. This phenomenon has previously been documented and is often called the 'colostral diagnostic gap' and relates to maternal antibodies inhibiting diagnostic testing and the accurate detection of the virus, in PI animals, for up to several weeks (Brock and others 1998; Fux and Wolf 2012; Lanyon and others 2014; Zimmer and others 2004).

In comparison, qPCR results showed that viral copy numbers were consistently detected, at 10^5 copies/ml, from Day 0 – 42 and then increased to 10^6 copies/ml at Day 49. Whether these initially lower viral loads are due to colostrum ingestion (and hence antibody interference) is not well understood. Previously mean qPCR values from serum collected from PI calves indicated a viral load of $10^{6.1}$ copies of BVDV RNA per 5ul from newborn calves (pre-colostral), which then dropped to $10^{2.7}$ after colostral ingestion before rising to $10^{5.9}$ by 70 days of age (Fux and Wolf 2012).

Direct contact with a BVDV PI animal is thought to be the main source of transmission between animals, due to the large quantities of virus shed in bodily fluids such as nasal/saliva excretions and the urine of PIs (Brownlie and others 1987; Meyling and others 1990; Lindberg and Houe 2005). Similar to the 'diagnostic gap' phenomenon, it is possible that colostral antibodies may temporarily suppress virus replications in PI animals, thus reducing the quantity of virus being shed by these animals during the first 4-6 weeks of life (Meyling and others 1990). In the present

study, the viral loads reported for the PI lamb were obtained for serum samples only however and the viral loads of nasal and saliva secretions were not determined.

Only a single lamb from treatment group A developed an antibody response to BVDV. Antibodies were detected in this lamb at both the 49 and 56 day sampling time points. Although the time to seroconversion in the lamb in this study falls within previously recorded time frames for infection, it is unknown when exactly infection occurred, but the rate of transmission appears low.

Similarly, none of the steers from either treatment group B or C showed indications of BVDV antibodies at any of the sampling time points, after exposure to a PI lamb. This suggests that the transmission of BVDV-1c from an antibody-positive PI neonate lamb up to the age of 8 weeks, to naïve cattle, does not occur readily either after 5 minutes of close exposure or by way of a shared fence line. Transmission of BVDV from cattle to sheep has been reported on a number of occasions from PI cattle aged 6 months to 2 years of age (Carlsson 1991; Fulton and others 2005; Niskanen and others 2000). Similarly studies relating to the transmission of Pestiviruses from sheep to cattle, although limited to two studies, used PI sheep over one year of age (Carlsson and Belak 1994; Braun and others 2014). While transmission was shown to occur in these previous studies, the current study showed a low rate of transmission only.

The only other study, as far as the authors are aware, which used a neonatal PI animal investigated the transmission of BVDV to susceptible sheep from a PI lamb aged between 0 and 227 days (Plant and others 1977). Antibody development occurred in both lambs and adult sheep. This study did suggest that lambs were infected more readily than adult sheep, when the PI lamb was <4 weeks of age (Plant and others 1977), a finidng which is similar to what was reported in the current study.

Although the pathogenicity of BVDV-1c is unknown, it is likely to be of a low virulence, such as the other BVDV-1 strains 1b, 1e and 1k (Ridpath and others 2007), and thus results in low viral shedding in infected animals. However, differences in virulence of the infecting virus strain, as well as differences in housing, the age and number of PI animals used, the presence of colostral derived antibodies in PI animals and trial design do make establishing comparisons with previous studies difficult.

In summary, this study has demonstrated that transmission of BVDV-1c from a neonatal BVDV-1c PI lamb, which had successful passive transfer of maternal antibodies, to naive sheep and cattle does not occur readily. Although this study investigates the transmission of BVDV to susceptible animals after a species transfer, suppression of virus replication due to colostral antibody ingestion is believed to explain the low transmission of BVDV-1c reported in the current study. Findings of this study have also indicated a need for further work to be undertaken to; determine if weaned BVDV PI sheep are able to infect susceptible animals; determine the full effect of colostral antibodies on the infectivity of young BVDV PI animals, including cattle; and determine if serum viral load relates to the amount of virus excreted by a PI animal in nasal or saliva secretions.

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The University of Adelaide and Meat and Livestock Australia had no part in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication

Conflict of Interest Statement

None

References

BACHOFEN, C., VOGT, H. R., STALDER, H., MATHYS, T., ZANONI, R., HILBE, M., SCHWEIZER, M. & PETERHANS, E. (2013) Persistent infections after natural transmission of bovine viral diarrhoea virus from cattle to goats among goats. Veterinary Research 44, 32-42

BRAUN, U., REICHLE, S. F., REICHERT, C., HASSIG, M., STALDER, H. P., BACHOFEN, C. & PETERHANS, E. (2014) Sheep persistently infected with Border disease readily transmit virus to calves seronegative to BVD virus. Veterinary Microbiology 168, 98-104

BROCK, K. V., GROOMS, D. L., RIDPATH, J. & BOLIN, S. R. (1998) Changes in levels of viremia in cattle persistently infected with bovine viral diarrhea virus. J Vet Diagn Invest 10, 22-26

BROWNLIE, J., CLARKE, M. C., HOWARD, C. J. & POCOCK, D. H. (1987) Pathogenesis and epidemiology of bovine virus diarrhoea virus infection of cattle. Ann Rech Vet 18, 157-166

CARLSSON, U. (1991) Border Disease in Sheep Caused by Transmission of Virus from Cattle Persistently Infected with Bovine Virus Diarrhea Virus. Veterinary Record 128, 145-147

CARLSSON, U. & BELAK, K. (1994) Border Disease Virus Transmitted to Sheep and Cattle by a Persistently Infected Ewe - Epidemiology and Control. Acta Veterinaria Scandinavica 35, 79-88

EDWARDS, S., WOOD, L., HEWITT-TAYLOR, C. & DREW, T. W. (1986) Evidence for an Immunocompromising Effect of Bovine Pestivirus on Bovid Herpesvirus-1 Vaccination. Veterinary Research Communications 10, 297-302

EVANS, C. A., LANYON, S. R. & REICHEL, M. P. (2017a) Investigation of AGID and two commercial ELISAs for the detection of Bovine viral diarrhea virus-specific antibodies in sheep serum. Journal of Veterinary Diagnostic Investigation 29, 181-185

EVANS, C. A., LANYON, S. R., SIMS, S. K. & REICHEL, M. P. (2015) Reproductive performance in experimentally BVDV infected ewes and seroconversion rates in sheep co-mingled with BVDV PI calves. Small Ruminant Research 123, 314-319

EVANS, C. A., REICHEL, M. P., HEMMATZADEH, F. & COCKCROFT, P. D. (2017b) Clinical responses and reproductive outcomes in pregnant ewes experimentally infected with bovine viral diarrhoea virus (Type-1c) between days 59 and 69 of gestation. Small Ruminant Research

FULTON, R. W., BRIGGS, R. E., RIDPATH, J. F., SALIKI, J. T., CONFER, A. W., PAYTON, M. E., DUFF, G. C., STEP, D. L. & WALKER, D. A. (2005) Transmission of bovine viral diarrhea virus 1b to susceptible and vaccinated calves by exposure to persistently infected calves. Can J Vet Res 69, 161-169

FULTON, R. W., RIDPATH, J. F., SALIKI, J. T., BRIGGS, R. E., CONFER, A. W., BURGE, L. J., PURDY, C. W., LOAN, R. W., DUFF, G. C. & PAYTON, M. E. (2002) Bovine viral diarrhea virus (BVDV) 1b: predominant BVDV subtype in calves with respiratory disease. Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire 66, 181-190

FUX, R. & WOLF, G. (2012) Transient elimination of circulating bovine viral diarrhoea virus by colostral antibodies in persistently infected calves: a pitfall for BVDV-eradication programs? Vet Microbiol 161, 13-19

GOYAL, S. M., BOUJIHAD, M., HAUGERUD, S. & RIDPATH, J. F. (2002) Isolation of bovine viral diarrhea virus from an alpaca. Journal of Veterinary Diagnostic Investigation 14, 523-525

GROOMS, D. L. (2004) Reproductive consequences of infection with bovine viral diarrhea virus. Veterinary Clinics Food Animal Practice 20, 5-19

HAMERS, C., COUVREUR, B., DEHAN, P., LETELLIER, C., LEWALLE, P., PASTORET, P.P, KERKHOFS, P. (2000) Diffrerences in experimental virulence of bovine viral diarrhoea viral strains isolated from haemorrhagic syndromes. Veterinary Journal 3, 250-258

HEWICKER-TRAUTWEIN, M., TRAUTWEIN, G., FREY, H. R. & LIESS, B. (1995) Variation in neuropathogenicity in sheep fetuses transplacentally infected with non-cytopathogenic and cytopathogenic biotypes of bovine-virus diarrhoea virus. Zentralbl Veterinarmed B 42, 557-567

LANYON, S. R., SIMS, S. K., COCKCROFT, P. D. & REICHEL, M. P. (2014) Comparison of serum, ear notches, and nasal and saliva swabs for Bovine viral diarrhea virus antigen detection in colostrum-fed persistently infected (PI) calves and non-PI calves. Journal of Veterinary Diagnostic Investigation 26, 783-787

LINDBERG, A. & HOUE, H. (2005) Characteristics in the epidemiology of bovine viral diarrhea virus (BVDV) of relevance to control. Prev Vet Med 72, 55-73; discussion 215-219

MEYLING, A., HOUE, H. & JENSEN, A. M. (1990) Epidemiology of bovine virus diarrhoea virus. Rev Sci Tech 9, 75-93

NETTLETON, P. F. (1990) Pestivirus infections in ruminants other than cattle. Rev Sci Tech 9, 131-150

NETTLETON, P. F. & ENTRICAN, G. (1995) Ruminant pestiviruses British Veterinary Journal 151, 615-642

NISKANEN, R., LINDBERG, A., LARSSON, B. & ALENIUS, S. (2000) Lack of virus transmission from bovine viral diarrhoea virus infected calves to susceptible peers. Acta Vet Scand 41, 93-99

PLANT, J. W., GARD, G. P. & ACLAND, H. M. (1977) Transmission of a mucosal disease virus infection between sheep. Aust Vet J 53, 574-577

RIDPATH, J. F., FULTON, R. W., KIRKLAND, P. D. & NEILL, J. D. (2010) Prevalence and antigenic differences observed between Bovine viral diarrhea virus subgenotypes isolated from cattle in Australia and feedlots in the southwestern United States. Journal of Veterinary Diagnostic Investigation 22, 184-191

RIDPATH, J. F., NEILL, J. D. & PETERHANS, E. (2007) Impact of variation in acute virulence of BVDV1 strains on design of better vaccine efficacy challenge models. Vaccine 25, 8058-8066

SCHERER, C. F. C., FLORES, E. F., WEIBLEN, R., CARON.L., IRIGOYEN.L.F., NEVES.J.P. & MACIEL.M.N. (2001) Experimental infection of pregnant ewes with bovine viral diarrhoea virus type-2 (BVDV-2): effects on the pregnancy and fetus. Veterinary Microbiology 79, 285-299

WELLENBERG, G. J., VAN DER POEL, W. H. M. & VAN OIRSCHOT, J. T. (2002) Viral infections and bovine mastitis: a review. Veterinary Microbiology 88, 27-45

ZIMMER, G. M., VAN MAANEN, C., DE GOEY, I., BRINKHOF, J. & WENTINK, G. H. (2004) The effect of maternal antibodies on the detection of bovine virus diarrhoea virus in peripheral blood samples. Veterinary Microbiology 100, 145-149

4.2 Transmission from acutely infected sheep

The manuscript presented in this section reports on the infective nature of acutely BVDV-1c infected sheep to pestivirus naïve sheep. It has been accepted for publication by the *Journal of Small Ruminant Research* Vol 153 Pp. 5-8.

Short communication: The risk of transmission from sheep experimentally infected with BVDV-1c during the acute phase to BVDV naïve sheep

CA Evans, JL Moffat, F Hemmatzadeh and PD Cockcroft

The risk of transmission from sheep experimentally infected with BVDV-1c during the acute phase to BVDV naïve sheep

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Contribution to the Paper	Researched, designed and implemented animal trial, managed animal trial, analysed samples, undertook data analysis, drafted and edited manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Short communication

The risk of transmission from sheep experimentally infected with BVDV-1c during the acute phase to BVDV naïve sheep

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ABSTRACT

Bovine viral diarrhoea virus (BVDV) is an economically important disease of cattle known to cause immunosuppression and reproductive dysfunction in infected animals. Although predominantly a disease of cattle other ruminant species, such as sheep, have been shown to undergo infection with similar outcomes as those observed in cattle.

The main source of BVDV transmission is known to be persistently infected (PI) animals which shed large quantities of virus through all secretions and excretions. However, it is believed that BVDV is able to persist within herds even after the removal of these PI animals. Although not proven, this persistence is thought to be due to the short period of viral excretion shown to occur in acutely infected animals. As a result the aim of this study was to determine if transmission of BVDV could occur between sheep undergoing an acute BVDV infection and sheep naïve to BVDV.

Five wethers, experimentally infected with BVDV-1c, were co-mingled with five pestivirus naïve wethers for a period of seven weeks. Weekly blood samples were collected and tested for the presence of BVDV-specific antibodies and BVDV antigen. Acute infection was successful in the experimentally inoculated animals, with antibodies detected in all five wethers by Day 35 post inoculation. Transmission of BVDV-1c was not shown to occur between these acutely BVDV-1c infected wethers and the naïve wethers co-mingled with them.

Bovine viral diarrhoea virus (BVDV) is a highly prevalent and economically important disease of cattle (Nettleton and Entrican, 1995) where infections with the virus can lead to both immunosuppression (Wilhelmsen et al., 1990) and reduced reproductive performance (Grooms, 2004). Abortions, stillbirths as well as the birth of calves with physical abnormalities and those persistently infected with BVDV are all common outcomes following BVDV infection of a pregnant cow (Nettleton and Entrican, 1995; Grooms, 2004). The reproductive outcomes seen are a result of the virus's ability to cross the placenta and establish an infection within the developing foetus (Nettleton and Entrican, 1995) and are dependent on the stage of gestation when infection occurred (Grooms, 2004; Broaddus et al., 2009).

The spread and persistence of BVDV in cattle populations is due to presence of the highly infectious, persistently infected (PI) animals. It has been well documented that these PI animals shed large quantities of virus through all secretions and excretions and as such are the main source of BVDV transmission in cattle (Brownlie et al., 1987; Lindberg and Alenius, 1999; Lindberg and Houe, 2005). As a result many BVDV control programmes target the identification and elimination of these animals (Lindberg and Alenius, 1999) in order to reduce the prevalence of the disease.

However it is believed by some that these PI animals are not the only source of BVDV infection. It has been documented that cattle acutely infected with BVDV also undergo a short viraemic period prior to the development of BVDV antibodies (Brownlie et al., 1987). In cattle this viraemic period is identifiable by the detection of BVD virus in serum, blood, buffy coat cells and/or nasal secretions between 3 and 10 days after infection has taken place (Brownlie et al., 1987; Brownlie, 1990; Collins et al., 2009). The presence of detectable virus during this period brings forth the idea that acutely infected animals could be a source for further infections, in the absence of PI animals. Although it has been indicated that PI animals are essential for BVDV survival, the virus has been shown to circulate in herds for up to a two and a half years in the absence of, or no direct contact with, PI cattle (Barber and Nettleton, 1993; Moerman et al., 1993).

Although typically a disease of cattle, BVDV has been shown to infect a range of other ruminant species including goats (Bachofen et al., 2013), deer (Nettleton, 1990) and sheep (Scherer et al., 2001). A number of studies have investigated the reproductive effects associated with BVDV infection in sheep, with high abortion rates, neonatal lamb

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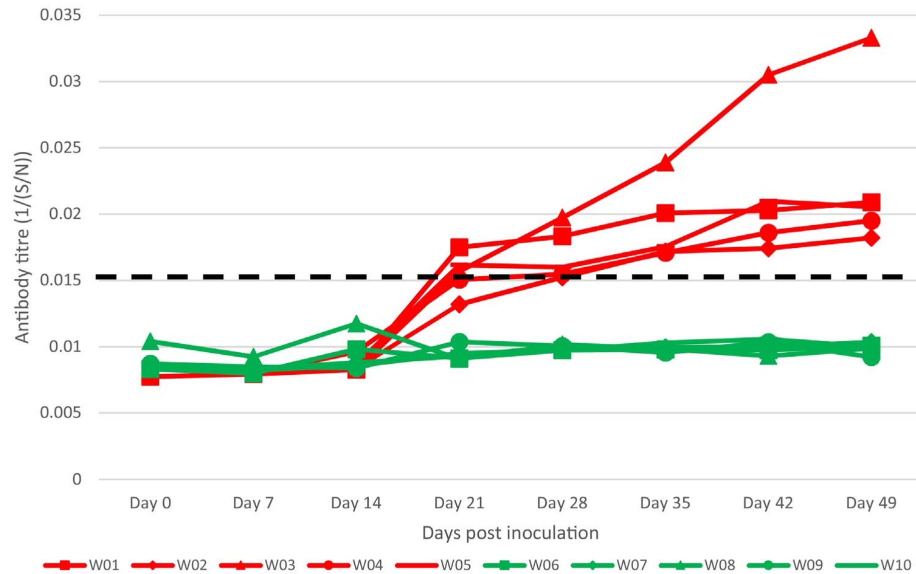

Fig. 1. Individual antibody ELISA titres for the ten experimental sheep. Antibody titres are given as 1/(S/N%) due to ELISA used being a competitive ELISA. Animals in red were experimentally inoculated with BVDV PI cattle serum, while those in green were naïve animals, housed with those undergoing acute infection. Cut-off, as established by Evans et al. (2017), is indicated by the dashed line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

deaths and the birth of BVDV PI lambs being recorded (Parsonson et al., 1979; Scherer et al., 2001; Evans et al., 2015). However transmission rates of BVDV from infected sheep, both acutely infected and PI, have not been quantified. Recently it has been shown that BVDV PI sheep are capable of infecting naïve cattle (Braun et al., 2014) however transmission of BVDV from acutely infected sheep has not been studied. The aim of this study was to determine if naïve sheep would become infected and sero-convert when co-mingled and housed with sheep undergoing an acute BVDV infection.

Five merino cross adult sheep (wethers) were experimentally infected by inoculation of 2 ml of BVDV PI cattle serum by subcutaneous injection in the neck. The BVDV PI cattle serum was collected from a confirmed persistently BVDV-1c infected heifer that was produced during earlier experimental work undertaken at the University of Adelaide (Lanyon et al., 2014) and had been stored at −80 °C for approximately 12 months. Each 2 ml dose of BVDV PI serum contained, equal to, 1.3×10^6 of viral genome copy numbers, based on the absolute quantification in a real-time quantitative reverse transcriptase PCR method.

The five experimentally infected sheep were then co-mingled and kept as a single group with five pestivirus naïve adult sheep (wethers) for seven weeks in a small paddock (size 30 × 25 m) with shared feed and water sources. All the animals were blood sampled weekly from the day of BVDV inoculation (Day 0) until 49 days post inoculation. Blood samples were collected into plain evacuated tubes by venepuncture of the jugular vein. All animals were assessed daily for observational signs of ill health and were condition scored at weekly intervals at the time of blood sampling. All animal experiments were approved by the University of Adelaide's Animal Ethics Committee prior to this project (S-2016-013).

Serum was harvested by centrifuging the blood filled plain evacuated tubes at 2400 g for 10 min. Serum was poured off in to 1 ml storage tubes and stored at −80 °C. Thawed serum samples were then tested for BVDV-specific antibodies using the IDEXX BVDV/MD/BDV p80 Protein Antibody Test Kit (IDEXX Laboratories, Rydalmere, NSW), following the manufacturer's instructions developed for sheep serum. Samples which returned a signal-to-noise ratio (S/N) of ≤63.5%, as determined by Evans et al. (2017), were considered positive for BVDV-specific antibodies. Serum samples from Days 42 and 49 post infection were also submitted to the Elizabeth McArthur Agricultural Institute (New South Wales, Australia), where they were tested by an agar gel immunodiffusion (AGID[a]) assay for BVDV antibodies. This AGID assay uses the C24V reference strain as the antigen, with a score of 0 considered negative and scores of 1–3 considered positive.

Thawed serum samples were also tested for BVDV by Antigen Test Kit/Serum Plus ELISA (IDEXX Laboratories Inc, Rydalmere, NSW). Samples which returned an S-N ratio of > 0.300 were considered positive for BVDV virus, as per the manufacturer's instructions.

Total viral RNA was extracted from freeze-thawed serum samples of all inoculated sheep (Days 7, 14 and 21) using the QIAmp Viral RNA Mini Kit (Qiagen, Germany). A serum sample volume of 280 μl, instead of 140 μl, was used with manufacturer's instructions altered as instructed. Reverse transcriptase polymerase chain reaction (RT-PCR) was then performed, where the viral RNA samples were run through the QIAGEN OneStep RT-PCR Kit (Qiagen, Germany) using the primer pair BVD-292F (CTA GCC ATG CCC TTA GTA GGA CTA), and BVD-292R (CAA CTC CAT GTG CCATGTAC AGC A). Each reaction consisted of 5 x QIAGEN OneStep RT-PCR Buffer, 400 μM dNTP mix, 0.6 μM of each Primer, QIAGEN OneStep RT-PCR Enzyme Mix, RNAse Inhibitor, RNAse free water and 5 μl of template RNA in a total reaction volume of 50 μl. Reverse transcriptase and PCR were undertaken under the following thermal cycler conditions (per manufacturer's instructions): 30 min at 50 °C, followed by 15 min at 95 °C followed by a 40 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C. A final extension of 10 min at 72 °C terminated the reaction before the PCR products were analysed by electrophoresis on a 2% agarose gel and stained with Gel-Red.

The earliest positive result for the presence of BVDV-specific antibodies in the inoculated sheep was on Day 21 post inoculation. By Day 35 post inoculation all five experimentally inoculated sheep returned positive results for BVDV antibodies (Fig. 1). None of the five naïve sheep showed any change in antibody status throughout the study period (Fig. 1).

Results from the AGID assay confirmed that all 5 infected sheep were positive for BVDV antibodies on Days 42 and 49. A score of 3 was recorded for W03 on both Day 42 and 49 while the remaining four sheep achieved scores of 1 on each of these days. AGID assay also confirmed that the five naïve sheep had remained negative, with a score of 0 being recorded for all samples submitted.

Antigen was not detected in any of the infected or naïve animals by

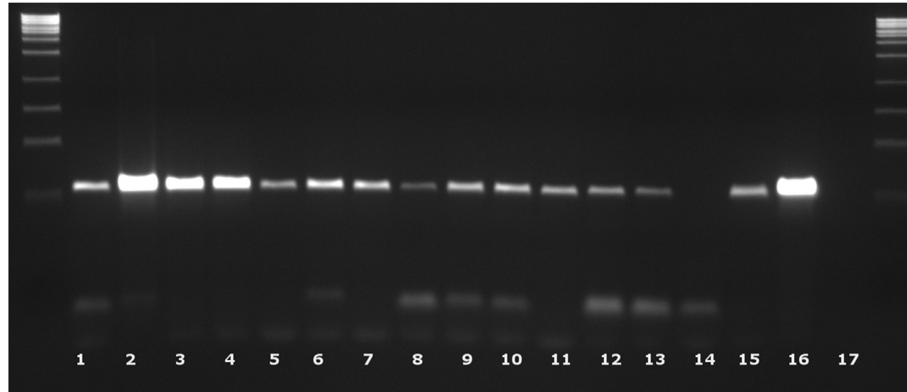

Fig. 2. RT-PCR gel for the five inoculated sheep on Days 7. 14 and 21 post inoculation. Well 1 = W01, Day 7; Well 2 = W02, Day 7; Well 3 = W03, Day 7; Well 4 = W04, Day 7; Well 5 = W05, Day 7; Well 6 = W01, Day 14; Well 7 = W02, Day 14; Well 8 = W03, Day 14; Well 9 = W04, Day 14; Well 10 = W05, Day 14; Well 11 = W01, Day 21; Well 12 = W02, Day 21; Well 13 = W03, Day 21; Well 14 = W04, Day 21; Well 15 = W05, Day 21; Well 16 = positive control; Well 17 = negative control.

antigen ELISA with all samples returning a score of less than 0.3% (S-N). However, RT-PCR confirmed that all five inoculated sheep were positive for virus on one or more time points from Day 7–21 post infection (Fig. 2).

The results of this study indicate that BVDV transmission did not occur between the acutely BVDV infected sheep and the co-housed BVDV naïve sheep. The antibody titres for all five naïve sheep remained negative throughout the study period despite the first of the infected sheep sero-converting by Day 21 post infection. In support of these findings, previous experimental infection trials have shown no transmission of BVDV to fully susceptible in-contact cattle (Radostits and Littlejohns, 1988; Uttenthal et al., 2006) despite accounts of circulating BVDV in herds previously cleared of PI animals (Barber and Nettleton, 1993; Moerman et al., 1993). It has been thought that the current lack of transmission from acutely infected sheep could be due to the virus strain used, its virulence and/or the resulting amount of virus shed by the animal following infection (Bolin and Grooms, 2004; Ridpath, 2010).

In 2007 Ridpath et al. studied three strains of BVDV-1 (1b, 1e and 1k) and discovered that, according to the three clinical criteria, none of these strains could be classified as high virulence. This study also discovered that although there were differences in virulence between the three strains it appeared that there is a continuum of virulence associated with BVDV-1 strains, unlike what is seen with BVDV-2 strains (Ridpath et al., 2007). It has also been previously recorded that there is a positive relationship between the amount of virus shed by an animal and the virulence of the infecting BVDV strain (Hamers et al., 2000). Although the virulence for the strain used in this study (BVDV-1c) has not been determined it is probable that it is of a low virulence, such as the 1b, 1e and 1k strains and there is low viral shedding in these animals as a result.

In addition, the absence of transmission reported in this trial could also be due to the low viral loads excreted by acutely infected animals. It has been documented that acutely infected animals undergo a period of viral detection and subsequent virus shedding (Brownlie et al., 1987) however the viral loads excreted by acutely infected animals are substantially lower than those of persistently infected animals (Young et al., 2006; Kosinova et al., 2007). Quantitative analysis for the amount of virus present in the inoculated sheep was not undertaken during this study.

In summary this study found that experimental sheep infected using serum from a bovine heifer PI with BVDV 1c strain by subcutaneous inoculation did not transmit the infection to co-mingled BVDV naïve sheep during a 7 week study period. The results of this study indicate that infected sheep in the acute phase of infection with the BVDV 1c strain have very low transmission rates and that persistence of BVDV in sheep populations is unlikely to be due to acutely infected individuals.

Role of the funding source

The University of Adelaide and Meat and Livestock Australia had no part in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Conflict of interest statement

None.

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References

Bachofen, C., Vogt, H.R., Stalder, H., Mathys, T., Zanoni, R., Hilbe, M., Schweizer, M., Peterhans, E., 2013. Persisitent infections after natural transmission of bovine viral diarrhoea virus from cattle to goats among goats. Vet. Res. 44, 32–42.
Barber, D.M.L., Nettleton, P.F., 1993. Investigations into bovine viral diarrhoea virus in a dairy herd. Vet. Rec. 133, 549–550.
Bolin, S.R., Grooms, D.L., 2004. Origination and consequences of bovine viral diarrhea virus diversity. Vet. Clin. North Am. Food Anim. Pract. 20, 51–68.
Braun, U., Reichle, S.F., Reichert, C., Hassig, M., Stalder, H.P., Bachofen, C., Peterhans, E., 2014. Sheep persistently infected with Border disease readily transmit virus to calves seronegative to BVD virus. Vet. Microbiol. 168, 98–104.
Broaddus, C.C., Lamm, C.G., Kapil, S., Dawson, L., Holyoak, G.R., 2009. Bovine viral diarrhea virus abortion in goats housed with persistently infected cattle. Vet. Pathol. 46, 45–53.
Brownlie, J., Clarke, M.C., Howard, C.J., Pocock, D.H., 1987. Pathogenesis and epidemiology of bovine virus diarrhoea virus infection of cattle. Ann. Rech. Vet. Ann. Vet. Res. 18, 157–166.
Brownlie, J., 1990. The pathogenesis of bovine viral diarrhoea virus infections. Rev. Sci. Tech. 9, 43–59.
Collins, M.E., Heaney, J., Thomas, C.J., Brownlie, J., 2009. Infectivity of pestivirus following persistence of acute infection. Vet. Microbiol. 138, 289–296.
Evans, C.A., Lanyon, S.R., Sims, S.K., Reichel, M.P., 2015. Reproductive performance in experimentally BVDV infected ewes and seroconversion rates in sheep co-mingled with BVDV PI calves. Small Rumin. Res. 123, 314–319.
Evans, C.A., Lanyon, S.R., Reichel, M.P., 2017. Investigation of AGID and two commercial ELISAs for the detection of Bovine viral diarrhea virus-specific antibodies in sheep serum. J. Vet. Diagn. Invest. 29, 181–185.
Grooms, D.L., 2004. Reproductive consequences of infection with bovine viral diarrhea virus. Vet. Clin. Food Anim. Pract. 20, 5–19.
Hamers, C., Couvreur, B., Dehan, P., Letellier, C., Lewalle, P., Pastoret, P.P., Kerkhofs, P., 2000. Diffrerences in experimental virulence of bovine viral diarrhoea viral strains isolated from haemorrhagic syndromes. Vet. J. 3, 250–258.

Kosinova, E., Psikal, I., Robesova, B., Kovarcik, K., 2007. Reat-time PCR for quantification of bovine viral diarrhea virus RNA using SYBR Green I fluorimetry. Vet. Med. 52, 253–261.
Lanyon, S.R., Sims, S.K., Cockcroft, P.D., Reichel, M.P., 2014. Comparison of serum, ear notches, and nasal and saliva swabs for Bovine viral diarrhea virus antigen detection in colostrum-fed persistently infected (PI) calves and non-PI calves. J. Vet. Diagn. Invest. 26, 783–787.
Lindberg, A., Alenius, S., 1999. Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. Vet. Microbiol. 64.
Lindberg, A., Houe, H., 2005. Characteristics in the epidemiology of bovine viral diarrhea virus (BVDV) of relevance to control. Prev. Vet. Med. 72, 55–73 discussion 215-219.
Moerman, A., Straver, P.J., de Jong, M.C.M., Quak, J., Baanvinger, Th., van Oirschot, J.T., 1993. A long term epidemiological study of bovine viral diarrhoea infections in a large herd of dairy cattle. Vet. Rec. 132, 622–626.
Nettleton, P.F., Entrican, G., 1995. Ruminant pestiviruses. Br. Vet. J. 151, 615–642.
Nettleton, P.F., 1990. Pestivirus infections in ruminants other than cattle. Rev. Sci. Tech. 9, 131–150.
Parsonson, I.M., Ohalloran, M.L., Zee, Y.C., Snowdon, W.A., 1979. Effects of bovine viral diarrhoea-mucosal disease (BVD) virus on the ovine fetus. Vet. Microbiol. 4, 279–292.
Radostits, O.M., Littlejohns, I.R., 1988. New concepts in the pathogenesis, diagnosis and control of diseases caused by the bovine viral diarrhea virus. Can. Vet. J. 29, 513–528.
Ridpath, J.F., Neill, J.D., Peterhans, E., 2007. Impact of variation in acute virulence of BVDV1 strains on design of better vaccine efficacy challenge models. Vaccine 25, 8058–8066.
Ridpath, J., 2010. The contribution of infections with bovine viral diarrhea viruses to bovine respiratory disease. Vet. Clin. North Am.: Food Anim. Pract. 26, 335–348.
Scherer, C.F.C., Flores, E.F., Weiblen, R., Caron, L., Irigoyen, L.F., Neves, J.P., Maciel, M.N., 2001. Experimental infection of pregnant ewes with bovine viral diarrhoea virus type-2 (BVDV-2): effects on the pregnancy and fetus. Vet. Microbiol. 79, 285–299.
Uttenthal, A., Hoyer, M.J., Grondahl, C., Houe, H., van Maanen, C., Rasmussen, T.B., Larsen, L.E., 2006. Vertical transmission of bovine viral diarrhoea virus (BVDV) in mousedeer (Tragulus javanicus) and spread to domestic cattle. Arch. Virol. 151, 2377–2387.
Wilhelmsen, C.L., Bolin, S.R., Ridpath, J.F., Cheville, N.F., Kluge, J.P., 1990. Experimental primary postnatal bovine viral diarrhea viral infections in six-month-old calves. Vet. Pathol. 27, 235–243.
Young, N.J., Thomas, C.J., Collins, M.E., Brownlie, J., 2006. Real-time RT-PCR detection of Bovine viral diarrhoea virus in whole blood using an external RNA reference. J. Virol. Methods 138, 218–222.

4.3 Chapter discussion

The development and survival of a persistently BVDV-1c infected lamb highlights the importance of determining the role of sheep populations in the persistence of BVDV within Australia. Although the PI lamb in this study was <8 weeks of age and subsequently had not been weaned, it was important to understand if this animal was capable of transmitting BVDV-1c to *Pestivirus* naive sheep and cattle.

Results from this study identified that cattle subjected to either direct contact with or housed adjacent to a persistently BVDV-1c infected lamb, did not develop antibodies to BVDV. However, one lamb, co-paddocked in the group which contained the PI lamb, was confirmed to have developed BVDV-specific antibodies by the end of the sampling period. These results indicated that the rate of transmission from a pre-weaned BVDV PI lambs, which have had successful passive transfer of maternal antibodies, to susceptible animals is low. It also suggests that transmission from these PI lambs appears to occur more readily to sheep than between species, or that a prolonged period of close contact is required.

The low rate of transmission reported in this study may however be due to the persistency of colostrum derived maternal BVDV antibodies. In young PI calves it has been previously reported that colostral antibodies can bind to the circulating virus and inhibit the accurate detection of these animals (Fux and Wolf 2012). This phenomenon is known as the ‘colostral diagnostic gap’ (Brock *et al.* 1998; Fux and Wolf 2012). Whether this phenomenon also influences the amount of virus excreted by a PI animal, after colostral ingestion, is unknown.

Quantitative PCR results from this study indicated that the amount of virus detected in serum from the PI lamb on weeks 7 and 8 of age were 10 times greater than what was detected over the previous 6 weeks. This is similar to what has previously been observed in newborn calves

where serum viral copies dropped substantially after colostrum ingestion, before rising again by 70 days of age (Fux and Wolf 2012). However, whether a decrease in serum viral copy numbers coincide with a decrease in the amount of virus a PI animal is excreting is unknown. Furthermore, the transmission of BVDV from a neonatal PI lamb was shown to be low, it cannot be assumed that transmission rates from an older BVDV PI ovine will be the same. In this study the PI lamb was euthanized at 8 weeks of age, at the end of the experimental period, and as such further work with an older PI animal could not be undertaken.

Another means by which the virus is believed to be maintained within a population is through animals undergoing acute infections. It has been reported that prior to the development of antibodies in acutely BVDV infected cattle a short vireamic period occurs (Brownlie *et al.* 1987). It has therefore been assumed that when susceptible individuals come in to contact with these acutely infected and vireamic animals, transmission of BVDV may occur (Barber and Nettleton 1993; Moerman *et al.* 1993). In order to fully understand the role sheep populations play in the spread and persistence of BVDV, it was important to look at all possible sources of infection in sheep, including the risk posed by those acutely infected.

Experimental infection of five wethers with BVDV-1c resulted in virus being detected by RT-PCR in the serum of all five animals, between Day 7 and Day 21 post infection. Although virus was detected in all acutely infected wethers, none of the co-housed and *Pestivirus* naïve wethers showed any antibody development to BVDV. The five naïve animals remained *Pestivirus* naïve throughout the trial which indicates that the infectivity of BVDV-1c from acutely infected sheep to naïve sheep is low. Whether the lack of transmission is due to infection occurring in a non-bovine host or the low virulence and associated low viral shedding of the BVDV-1c strain or just that acute infections do not result in transmission, is unknown.

Overall, results from these studies suggest that there is low risk of BVDV-1c being transmitted from acutely BVDV-1c infected sheep or from pre-weaned BVDV PI lambs which have had successful passive transfer of maternal antibodies. This low transmission rate further suggests that the persistence of BVDV-1c throughout Australia is unlikely to be due to sheep populations, particularly due to the apparent poor survival rate of BVDV-1c PI lambs (Evans *et al.* 2015; Evans *et al.* 2017). While the risks posed to and by sheep infected with BVDV-1c on the persistence of the virus within Australia is becoming more quantified, it remains unknown what other commonly farmed livestock species are at risk of BVDV-1c infection and what role they may play in the spread of the disease.

Chapter 5: Acute BVDV-1c infections in alpacas

In order for an effective BVDV control program to be developed in Australia, identifying the livestock species at risk of BVDV infection and which of these have the potential to act as a reservoir source of infection needs to be determined. Due to the large number of sheep in Australia it was critical to first understand the role sheep play in the spread and persistence of BVDV. However, sheep are not the only non-bovine species which have been identified as susceptible to infection with BVDV. Alpacas have been shown to develop BVDV-specific antibodies following exposure to the virus, with BVDV infected alpacas previously being identified in the USA (Kim *et al.* 2009), UK (Foster *et al.* 2005), Australia (Cockcroft *et al.* 2015) and Switzerland (Danuser *et al.* 2009). Foetal losses and the birth of persistently BVDV infected crias have also been reported (Carman *et al.* 2005; Mattson *et al.* 2006) and indicates that alpacas may be a potential reservoir source of BVD infection.

In Australia, alpacas have increased in popularity due to their high quality fleece, desirability as a companion animal and ability to guard sheep from foxes. As alpacas are often kept in close proximity with sheep in Australia, and susceptible to BVDV infection, it was considered important to understand the susceptibility of alpacas to infection with the predominant Australian BVDV strain (BVDV-1c), in particular when naturally transmitted from BVDV-1c PI cattle. Antibodies to BVDV have previously been reported in South Australian alpacas at a prevalence of 2.7% (Cockcroft *et al.* 2015), however the clinical effects associated with acute BVDV infections are unknown.

5.1 Findings in alpacas

The following manuscript reports on the clinical, haematological and selected biochemical changes in acutely BVDV-1c infected alpacas following natural transmission from a PI heifer. This manuscript has been accepted for publication (on 30/05/17) by the *Australian Veterinary Journal* (in press).

Original article: BVDV in Australian alpacas: Natural infection and clinical profiles following co-mingling with a persistently infected heifer

CA Evans, E Erregger, F Hemmatzadeh and PD Cockcroft

BVDV in Australian alpacas: Natural infection and clinical profiles following co-mingling with a persistently infected heifer

Australian Veterinary Journal (in press)

Statement of Authorship

Title of Paper	BVDV in Australian alpacas: Natural infection and clinical profiles following co-mingling with a persistently infected heifer
Publication Status	☐ Published ☑ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	CA Evans, E Erregger, F Hemmatzadeh and PD Cockcroft BVDV in Australian alpacas: Natural infection and clinical profiles following co-mingling with a persistently infected heifer Australian Veterinary Journal (in press)

Principal Author

Name of Principal Author (Candidate)	Caitlin A Evans
Contribution to the Paper	Researched, designed and implemented animal trial, managed animal trial, collected and analysed samples, undertook data analysis, drafted and edited manuscript.
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 24/7/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Elke Erregger
Contribution to the Paper	Helped manage animal trial, collected samples and edited manuscript.
Signature	Date 11/07/2017

Name of Co-Author	Farhid Hemmatzadeh
Contribution to the Paper	Helped with analysis of samples by PCR, interpreting data and editing manuscript.
Signature	Date 24.7.17

Name of Co-Author	Peter D Cockcroft		
Contribution to the Paper	Helped with funding the study, the research, design and implementation of animal trial, interpreting data and editing thesis.		
Signature		Date	30/07/2017

Please cut and paste additional co-author panels here as required.

BVDV in Australian alpacas; Natural infection and clinical profiles following co-mingling with a persistently infected heifer

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Abstract

Although predominantly a disease of cattle, bovine viral diarrhoea virus (BVDV) is known to infect other ruminant and camelid species such as sheep and alpacas. The aims of this study were to determine if BVDV naïve alpacas would become acutely infected and seroconvert to the predominant Australian strain of BVDV following co-mingling with a persistently BVDV-1c infected heifer; and to determine what, if any, clinical signs, haematological responses and selected biochemical changes can be seen due to acute BVDV-1c infections in alpacas. A persistently infected heifer and four alpacas were co-mingled and housed together for a period of two weeks. Weekly blood samples were collected and twice weekly clinical examinations were performed on the 4 alpacas.

Serum analysis by antibody ELISA indicated that all four alpacas were positive for BVDV-specific antibodies between 35 and 54 days post mixing with the persistently BVDV-1c infected heifer. Viral antigen was detected by antigen ELISA in two alpacas on Days 21 and 35 post initial mixing.

In general all the physical clinical parameters measured were normal. Serum biochemical and haematological analysis in 2 of the alpacas revealed marginally low sodium, chloride and elevated potassium concentrations, a lymphocytosis, a monocytosis and a neutrophilia at some point during the study period either in one or both of the alpacas.

This study has shown that infection in Australian alpacas readily occurs when a persistently BVDV-1c infected bovine animal is co-mingled with naïve alpacas and that acute infections are clinically mild and undetectable without serological testing.

*Keywords***:** Alpaca; Antibody; Bovine viral diarrhoea virus; Clinical; ELISA

Introduction

Alpacas are increasing in popularity as a farmed livestock species in Australia and superior quality animals can command high prices. Their value lies predominantly in the quality of their fleece and their desirability as a companion animal. They also have a utility in guarding sheep from foxes and as a meat animal for exclusive restaurants. Numbers in Australia were estimated, in 2013, at 160,000 registered animals [1] although the population is now thought to be approximately 200,000 (Jane Vaughan, personal communication)

Bovine viral diarrhoea virus (BVDV) is a highly prevalent disease of cattle widely distributed throughout the world [2]. Although predominantly a disease of cattle, BVDV is known to be able to cross species barriers and infect other ruminant species including sheep [3], goats [4], deer [5] and camelid species including alpacas [6].

It has been shown that alpacas can develop BVDV-specific antibodies following exposure to the virus and BVDV infected alpacas have been identified in many countries including the USA [7], UK [8], Australia [9] and Switzerland [10]. Usually it is the persistent form of BVDV infection in alpacas that is reported with common signs including chronic ill-thrift, poor weight gain or being underweight, severe nasal discharge, high rectal temperatures and pneumonia [11, 12]. In the acute form of the disease clinical signs are often mild or non-existent, although they can include lethargy, anorexia and a break in the fleece [13]. It has also been noted that diarrhoea is not a consistent feature of BVDV infection in alpacas [12] and that infections during pregnancy can lead to similar reproductive losses to those seen in cattle including early embryonic death, abortion, stillbirth, premature birth [12, 14] and the birth of persistently BVDV infected cria [11, 15].

In a survey of just over 12,000 North American and Canadian alpacas, 46 BVDV isolates were identified. Once genotyped these 46 isolates were identified as belonging to the same

BVDV genotype, type 1b [7] despite the presence of types 1a, 1b and BVDV type-2 being identified in cattle from the same areas [7]. However in Australia it is only the BVDV-type 1 species that has been identified in cattle, with an overwhelming predominance (>96%) of the BVDV-1c genotype [16]. Although BVDV-specific antibodies have been identified in Australian alpacas [9] it is unclear which genotype this is. Similarly it is unknown what clinical signs manifest in alpacas acutely infected with BVDV-1c.

The aims of this study were to determine if BVDV naïve alpacas would become acutely infected and seroconvert following co-mingling with a persistently BVDV-1c infected heifer and to determine the clinical signs, haematological responses and selected biochemical changes associated with acute BVDV-1c infections in the alpacas.

Materials and Methods

Animals

Four alpacas (three adults and one cria born on Day 4 of the trial) were bought from a farm in South Australia, Australia and were housed in a roofed 98m^2 pen, at the University of Adelaide's Roseworthy Campus, South Australia from November 2015 to January 2016.

Blood samples collected into serum vacutainer tubes on the day of arrival from the farm. Serum samples were tested to confirm their BVDV negative antibody and antigen status by a competitive antibody ELISA, IDEXX BVDV/MD/BDV p80 Protein Antibody Test Kit (IDEXX Laboratories, Rydalmere, NSW), and antigen ELISA, IDEXX BVDV Ag Serum Plus Test (IDEXX Laboratories, Rydalmere, NSW), respectively.

The BVDV PI heifer used in this study was confirmed to be persistently infected with BVDV-1c by genotyping [17].

All animal experiments were approved by the University of Adelaide's Animal Ethics Committee prior to this project (S-2015-123) being undertaken.

Trial Design

The alpacas and the PI heifer were housed in a roofed 98m^2 pen enclosed on three sides with an open front. The animals were allowed to co-mingle for two weeks (Figure 1). The heifer was removed from the pen after this time. The alpacas remained housed in their pen for a further six weeks.

Figure 1: Housing of alpacas and PI heifer in roofed 98m^2 pen enclosed on three sides.

The alpacas were checked daily and clinical examinations were performed on days 0 (the day of mingling), 4, 7, 11, 14, 18, 21, 25, 28, 32, 35, 39 and 42. Blood samples were collected on 9 occasions, on day 0 and then again on days 7, 14, 21, 28, 35, 42, 49 and 54. Blood samples were collected into 5ml EDTA and 5ml serum tubes by venepuncture of the jugular vein.

Antibody and antigen testing

Serum was collected by centrifuging the blood filled plain vacutainer serum tubes at 2400 g for 10 minutes. Serum was then poured off in to 1ml storage tubes and stored at -80°C.

All the thawed serum samples from the alpacas were tested for BVDV-specific antibodies using the competitive IDEXX BVDV/MD/BDV p80 Protein Antibody Test Kit (IDEXX Laboratories, Rydalmere, NSW) following the manufacturer's instructions developed for sheep serum. The manufacturers cut-off and the cut-off reported by Evans et al. (in press) [18] were used to determine sero-positive samples; signal-to-noise ratios (S/N) of ≤40% were considered positive as per manufacturer's recommendations while S/N of ≤63.5% were considered positive by Evans et al. (in press) [18]. Serum samples were also tested for BVDV antigen using the IDEXX BVDV Antigen ELISA (IDEXX Laboratories, Rydalmere, NSW) whereby a sample was considered positive if it had an S-N value >0.3, as *per* manufacturer's instructions.

Testing by PCR

Serum samples that returned a positive or elevated (≥0.2 S-N) result on antigen ELISA were tested by reverse transcriptase polymerase chain reaction (RT-PCR) to determine BVDV status. Total viral RNA was extracted from the freeze-thawed serum samples using the QIAmp Viral RNA Mini Kit (Qiagen, Germany). Viral RNA samples were then run through the QIAGEN OneStep RT-PCR Kit (Qiagen, Germany) so as to undertake reverse transcription and PCR in the same tube. Primer pair BVD-292F (CTA GCC ATG CCC TTA GTA GGA CTA), and BVD-292R (CAA CTC CAT GTG CCATGTAC AGC A), were used to amplify a 292 bp block of highly conserved region of the BVDV genome from the position of 102 to 394 of the 5′ UTR of NADL strain genome, GeneBank Accession number: BVI133739) Each reaction consisted of 5x QIAGEN OneStep RT-PCR Buffer, 400µM dNTP mix, 0.6 µM of each Primer, QIAGEN OneStep RT-PCR Enzyme Mix, RNAse Inhibitor, RNAse free water and 5µL of template RNA in a total reaction volume of 50µL. Reverse transcription and PCR were undertaken under the following thermal cycler conditions (per manufacturer's instructions): 30min at 50°C, followed by 15min at 95°C followed by a 40

cycles of 1min at 94°C, 1 min at 52°C and 1min at 72°C. The reaction was terminated with a final extension of 10min at 72°C. The PCR products were analysed by electrophoresis on a 2% agarose gel and stained with Gel-Red.

Clinical signs

A clinical examination was performed on each of the 3 adult alpacas and the following clinical parameters were evaluated: demeanour (alertness and reactiveness), temperature, heart rate (HR), respiration rate (RR), capillary refill time (CPR), mucosal membrane colour (MM), faecal consistency, oro-nasal discharge, lacrimation and number of compartment 1 (C1) movements in two minutes. In comparison, the cria only had information about demeanour, temperature, HR, RR, CPR and MM recorded. The descriptors and normal ranges used for each clinical sign are detailed in Table 1 and 2.

Table 1: The descriptors used for the clinical signs; Alertness, Reactiveness, Oro-nasal discharge, Lacrimation, MM and Faecal score

Clinical Sign	Score						
Alertness	normal	mild dullness	moderate dullness	severe depression	N/A	N/A	N/A
Reactiveness	normal	slow to react	very slow to react	unreactive	N/A	N/A	N/A
Oro-nasal discharge	normal	mild	moderate	severe	N/A	N/A	N/A
Lacrimation	normal	mild	moderate	severe	N/A	N/A	N/A
MM	pale	pink	red	cyanotic	jaundiced	N/A	N/A
Faecal consistency	dry pellets	normal pellets	soft pellets	soft not pelleted	mild diarrhoea	moderate diarrhoea	severe diarrhoea

Table 2: Normal reference ranges for the clinical signs; Temperature, Heart rate, Respiration rate, Capillary refill time and C1 movements. Normal ranges are given for both adult alpacas and crias.

Clinical Sign	Normal range (adult)	Normal range (cria)	References
Temperature (°C)	37.5 – 38.9	37.8 – 38.9	Adult [26] Cria [24]
Heart rate (beats/min)	60 – 90	60-120	Adult [26] Cria [24]
Respiration rate (breathes/min)	10 – 30	10-30	Adult [26] Cria [24]
Capillary refill time (sec)	<2	<2	Adult [27] Cria [24]
C1 movements/min	0-5	N/A	Adult [26]

Blood biochemistry and haematology

Thawed serum samples from two adult alpacas (#3 and #5) were submitted to the Veterinary Diagnostics Laboratory, Roseworthy Campus for serum biochemistry to measure the values of the following analytes; sodium, urea, potassium, total protein and chloride. The results were compared to the reference intervals identified for alpacas by Hengrave, Tschudi, Martig, Liesegang and Meylan [18] and are given in Table 3.

Table 3: Normal reference ranges for blood biochemistry and white blood cell counts in adult alpacas as determined by Hengrave, Tschudi, Martig, Liesegang and Meylan [21]

	Blood parameter	Normal range
Serum biochemistry	*Sodium*	148-155 (mmol/L)
	Urea	4.5-9.1 (mmol/L)
	Potassium	4.0-5.2 (mmol/L)
	Total protein	56.2-70.4 (g/L)
	Chloride	111-146 (mmol/L)
Haematology	*Lymphocytes*	1.1-5.2 (10^9/L)
	Monocytes	0.2-0.9 (10^9/L)
	Neutrophils	3.4-9.1 (10^9/L)
	Eosinophils	0.8-3.4 (10^9/L)

Blood smears were prepared with fresh blood for both alpacas #3 and #5, at all sampling time points, and air dried. Differential white blood cell (WBC) counts were performed on smears. The number of lymphocytes, monocytes, neutrophils and eosinophils were determined by manual counting of Diff Quick stained blood smears using the battlement technique.[19] Manual 100-cell differential cell counts for WBC[20] were undertaken to calculate the absolute WBC counts for both alpacas. These values were compared to the reference ranges determined by Hengrave, Tschudi, Martig, Liesegang and Meylan [21].

Results

Antibody and antigen testing

Serum samples analysed using the manufacturer's cut-off of <40 S/N% indicated that all four alpacas seroconverted to BVDV antibody positive status between 35 and 54 days from initial exposure to the PI heifer (Figure 2). Alpacas #3 and #5 were the first to seroconvert with antibodies detected at Day 35 while Alpaca #1 was the last to show seroconversion with antibodies detected on the final day of testing (Day 54). Alpaca #6, born on day 4, showed seroconversion at Day 42, however this was actually Day 38 from its initial exposure with the PI heifer. The dam (Alpaca #5) of the cria did not develop an antibody response to BVDV until 24 days after the cria was born. Therefore the antibodies detected in the cria were as a response to acute infection and not due to gastro-intestinal absorption of maternal colostral immunoglobulins.

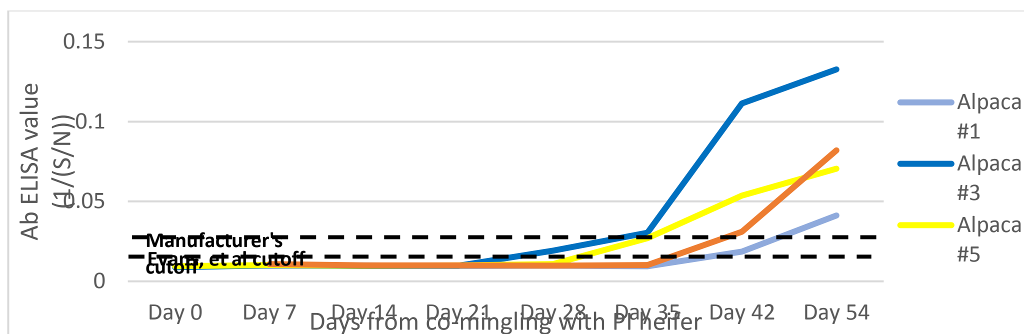

Figure 2: Individual antibody ELISA results for four alpacas, naturally exposed to a BVDV PI heifer for two weeks, from day of initial exposure (Day 0) until 54 days post initial exposure. Cut-offs for both the manufacturer's cut-off and the cut-off established by Evans et al. (in press) [18] are identified.

A positive antigen response according to the manufacturer's cut-off threshold was detected in one of the four alpacas using an ELISA. Figure 3 indicates a peak in antigen optical density in Alpaca #6 on Day 35, 7 days before seroconversion was shown to occur (Figure 2). One other alpaca (#5) showed elevated antigen levels of 0.2 S-N on Day 21, although this result was not high enough to be classified as positive. As a result samples from these two alpacas were tested by RT-PCR and a positive result was obtained in both animals (Figure 4).

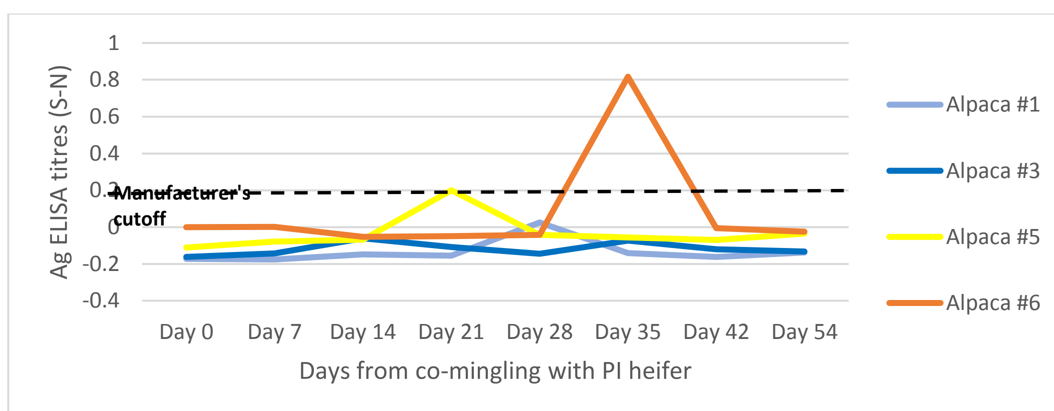

Figure 3: Individual antigen ELISA optical density values for four alpacas, naturally exposed to a BVDV PI heifer for two weeks, from day of initial exposure (Day 0) until 54 days post initial exposure. Manufacturer's cut off (0.3 S-N) is shown by the dashed line.

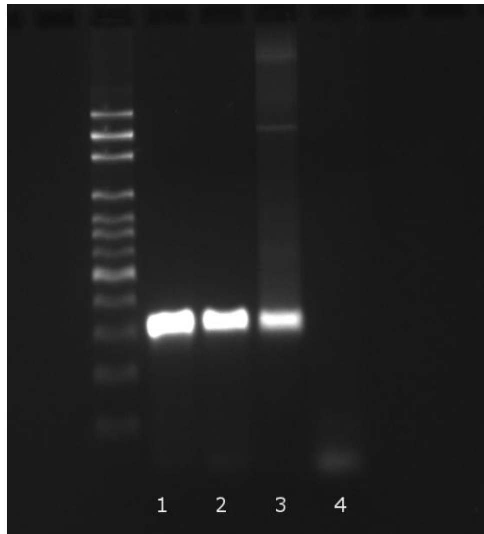

Figure 4: RT-PCR gel. Well 1 = Alpaca #5, Day 21; Well 2 = Alpaca #6, Day 35; Well 3 = positive control; Well 4 = negative control

Clinical signs

According to the classifications used (Tables 1 and 2) all values for alertness, reactiveness, oro-nasal discharge, lacrimation, mucosal membrane colour, capillary refill time and C1 movements were classified as normal throughout the study period. All the abnormal signs are underlined in Table 4.

Table 4: Recorded temperature, respiration rate, heart rate and faecal consistency for all four alpacas from day of co-mingling with a PI heifer (Day 0) until 42 days later. Note: faecal consistency was not recorded for the cria (alpaca #6). Double underlined values are those which fall below normal reference classifications, while single underlined values are those which fall above the normal reference classification. N = normal faecal consistency

	Alpaca	Day 0	Day 4	Day 7	Day 11	Day 14	Day 18	Day 21	Day 25	Day 28	Day 32	Day 35	Day 39	Day 42
TEMPERATURE (°C)	#1	36.9	37.9	37.3	36.3	37.1	36.8	38.5	37.1	36.9	37.1	37.7	36.7	37.1
	#3	37.6	38.6	38.2	36.8	37.8	37.2	37.8	37.2	36	37	37.9	37.4	37.1
	#5	37.5	38.1	38.1	37.4	-	38	38.1	38.2	37.6	37.9	38.2	37.6	37.9
	#6			39.1	39.1	-	39	39.2	39.6	39.4	38.9	39.3	38.6	38.9
RESPIRATION (breathes/min)	#1	16	32	20	20	24	20	16	20	16	30	30	20	24
	#3	36	24	16	16	20	28	20	24	20	16	30	20	16
	#5	24	-	32	20	-	20	28	36	20	20	40	16	20
	#6			28	56	40	52	32	40	24	20	30	36	24
HEART RATE (beats/min)	#1	72	80	76	80	60	76	116	72	72	64	64	76	56
	#3	56	76	72	68	60	84	48	88	48	68	66	60	52
	#5	56	72	72	48	-	52	48	44	44	40	60	48	40
	#6			144	176	120	240	192	164	200	148	80	132	180
FAECAL CONSISTENCY	#1	soft pellet	N	N	soft pellet	soft pellet	soft pellet	N	N	N	soft pellet	soft pellet	-	N
	#3	soft pellet	soft pellet	N	N	N	N	N	N	N	soft pellet	N	N	N
	#5	N	dry pellet	soft pellet	N	-	N	N	N	N	N	N	N	N

The range of temperatures seen in the adult alpacas (#1, #3 and #5) was from 36.0°C to 38.6°C however there was no pyrexia in any of the animals. In comparison the temperature range seen in the cria (#6) was from 38.6°C to 39.6°C with a constant elevated body temperature from Days 7 - 28. Respiration rates in the adult alpacas predominantly were normal however they did range from 16 – 36 (breathes/min). In the cria respiration rates were more elevated with a range of 20 – 56 (breathes/min). In adult alpacas the heart rates ranged from 40 – 88 (beats/min) with one anomaly of 116 (beats/min) in Alpaca #1 on Day 21. For the cria heart rate was much more elevated with a range of 80 – 240 (beats/min). Although faecal consistency was predominantly normal in all adult alpacas there were the occasional soft, or dry, pellets observed.

Blood biochemistry and haematology

The blood biochemistry analyte values for alpacas #3 and #5 are given in Table 5. Compared to reference ranges given in table 3, Alpaca #3 had marginally low sodium, total protein and chloride concentrations from Day 7 through to Day 42. Marginally low Urea concentrations were also seen from Day 21 to 49 while potassium concentrations were above normal reference ranges on all days.

Table 5: Weekly blood biochemistry values for Alpacas #3 and #5 from Day 7 to Day 49 post initial mixing with a BVDV PI heifer. Double underlined values are those which fall below normal reference classifications, while single underlined values are those which fall above the normal reference range.

		Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49
Alpaca #3	Sodium (mmol/L)	140.3	145.5	161.4	146.7	146.2	139.1	157.0
	Urea (mmol/L)	4.6	6.0	4.2	3.1	3.6	3.1	3.0
	Potassium (mmol/L)	5.9	6.5	6.8	5.8	5.3	5.7	6.1
	Total Protein (g/L)	50.1	53.9	60.1	52.4	52.5	50.7	60.5
	Chloride (mmol/L)	103.9	108.5	118.9	109.9	110.7	106.3	119.7
Alpaca #5	Sodium (mmol/L)	142.3	139.6	153.4	145.3	155.3	153.9	126.9
	Urea (mmol/L)	5.9	3.6	2.9	3.2	2.1	2.1	1.6
	Potassium (mmol/L)	5.5	5.5	5.4	5.0	4.7	5.4	4.0
	Total Protein (g/L)	56.3	52.1	57.6	56.0	57.4	56.6	38.2
	Chloride (mmol/L)	105.1	105.3	114.5	111.7	115.6	115.8	100.0

Similarly, Alpaca #5 had marginally different low serum concentrations of sodium on Days 7, 14, 28 and 49; total protein on Days 14, 28 and 49, chloride on Days 7, 14 and 49 and urea on Days 14 to 49. Potassium concentrations for Alpaca #5 were elevated on Days 7, 14, 21 and 42.

White blood cell counts for alpacas #3 and #5 are shown in figures 5 and 6 respectively. Compared to the reference ranges given in table 3, Alpaca #3 had a mild lymphocytosis beginning on Day 21, peaking at Day 42 followed by a decline by Day 49 with a maximum recorded value of 7.72 x 10^9 cells/L. There was also a mild monocytosis beginning on Day 35, peaking at Day 42 and declining by Day 49 with a maximum value recorded of 1.78 x 10^9/L.

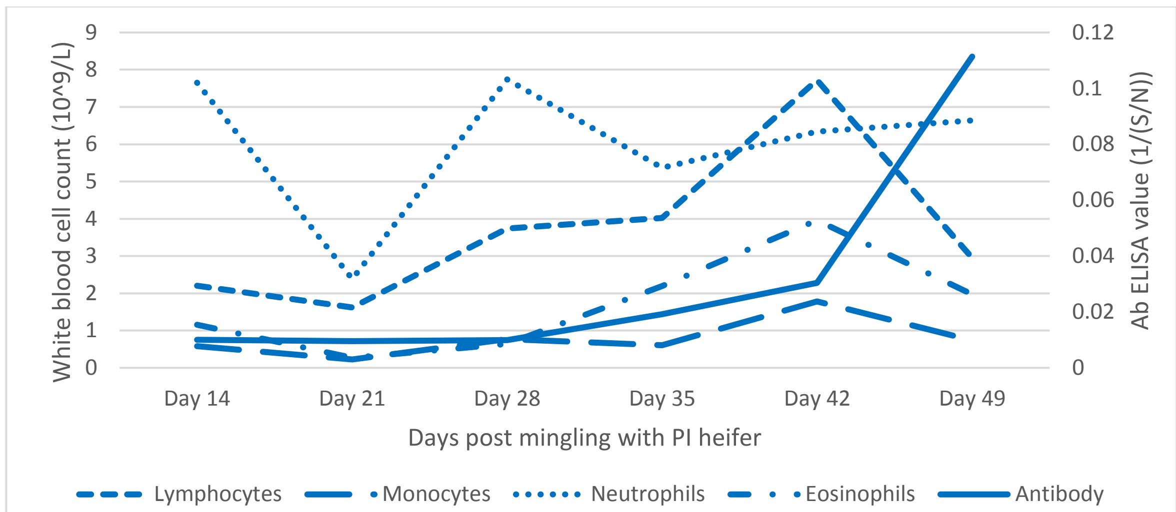

Figure 5: Lymphocyte, monocyte, neutrophil and eosinophil cell counts for Alpaca #3 from 14-49 days post mingling with a BVDV PI heifer. Antibody ELISA results (1/(S/N)) for Alpaca #3 on the corresponding days is shown as the solid line.

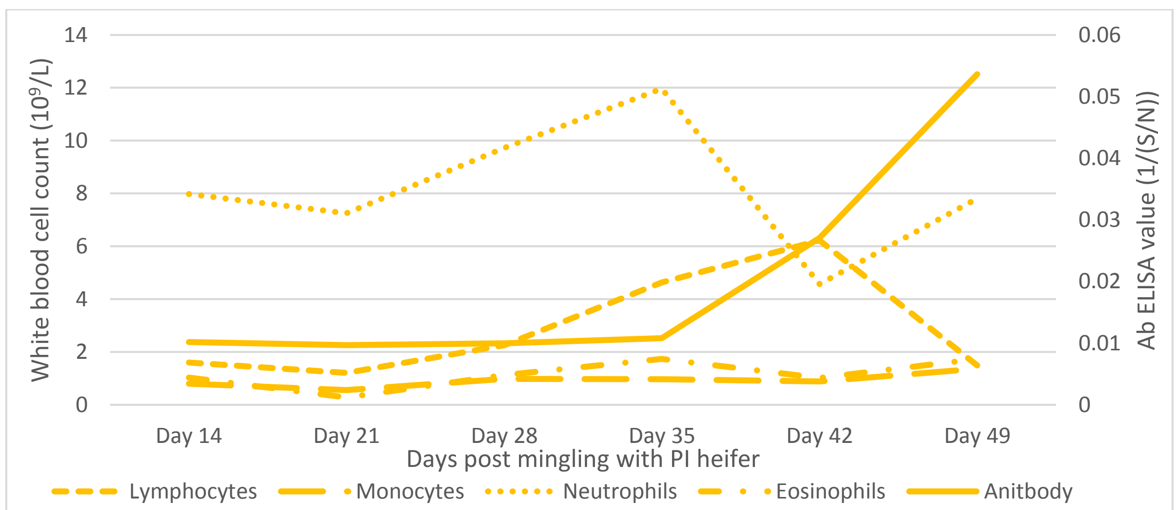

Figure 6: Lymphocyte, monocyte, neutrophil and eosinophil cell counts for Alpaca #5 from 14-49 days post mingling with a BVDV PI heifer. Antibody ELISA results (1/(S/N)) for Alpaca #5 on the corresponding days is shown as the solid line.

Similarly Alpaca #5 had a mild neutrophilia beginning on Day 21, peaking at Day 35 followed by a decline by Day 42 with a maximum recorded value of 11.97 x 10^9 cells/L.

There was also a mild lymphocytosis beginning on Day 21, peaking at Day 42 followed by a decline by Day 49 with a maximum value of 6.22 x 10^9 cells/L.

Discussion

Seroconversion in llamas following experimental infection with BVDV-1a, -1b and an unknown gentoype, via nasal aerosolisation, has been detected between 14 and 30 days post infection [14]. In comparison, the present study found that following a two week mixing period with a BVDV-1c PI heifer, all four alpacas had seroconverted (using the manufacturer's cut-off value for the ELISA) between 35 and 54 days post day of initial mixing. It is likely that this 'delay' in seroconversion is due to transmission not occurring on the initial day of mixing but rather sometime during the following 14 days. Due to the nature of the study it is hard to determine precisely when infection took place in each of the four alpacas. What can be determined however, is that the comingling of a BVDV-1c PI heifer with naïve alpacas can result in acute BVDV infection with the subsequent development of antibodies to BVDV-1c and seroconversion.

When using the cut off for antibody positive samples of ≤63.5 S/N% as determined for sheep by Evans et al. (in press)[18] seroconversion was detected 7 and 14 days earlier in alpacas #3 and #1 respectively, compared to with the manufacturer's cut-off. Using this higher threshold of ≤63.5 S/N% increases the sensitivity of this ELISA and resulted in one alpaca (#3) being identified as positive as early as Day 28 post initial mixing with the PI heifer (Figure 2). Irrespective of which cut-off is used, the results from this ELISA show a rise in antibody levels and indicates that seroconversion had occurred in each of the four alpacas.
In order to confirm that infection with BVDV had taken place, serum samples were tested for the presence of BVD virus. Viraemia was detected in the cria (#6) on Day 35 (31 days post initial mixing), 7 days before seroconversion was observed, by both antigen ELISA and RT-PCR. Similarly there was an increase in virus detected in Alpaca #5 on day 21, 14 days before

seroconversion was detected. Although this increase in virus was not enough to confirm a positive reading on ELISA the sample did read positive on RT-PCR (along with Alpaca #6) and that virus was present and BVDV infection had occurred. These results are similar to what has been identified in llamas whereby BVD virus can be detected between 6 and 27 days prior to antibody detection [14].

In animals undergoing an acute infection to BVDV it is not uncommon for pyrexia to be detected [22, 23] however this was not the case in this study. Although variations in temperature were detected they were only slight variations on what is considered normal (Table 2) and not dramatic changes. Majority of the abnormal temperatures seen in alpacas 1, 3 and 5 were marginally lower than normal body temperatures. These lower temperatures cannot be explained by cold weather as the project was undertaken in summer in Australia where temperatures at sampling were >20°C at all time points. In comparison, the cria (#6) showed consistent higher than normal body temperatures. Although consistent, these higher temperatures start from 3 days after birth and as such are more likely to be linked to the warmer weather, the stress associated with the study and that younger animals have higher body temperatures to adults [24], and not pyrexia due to BVDV infection. There were no physical abnormalities observed in any of the infected alpacas and the normal clinical appearance of the animals would invariably mean that the infection would go undetected during the acute phase of the disease.

Blood biochemistry results showed a trend towards lower than normal ranges for sodium, blood urea, potassium, total protein and chloride for both alpacas sampled, across the majority of days tested. The marginally low serum concentrations of sodium, urea, potassium and total protein, as compared to the reference ranges in table 3, could not be attributed to any detectable pathophysiological process including diarrhoea as the animals were clinically

normal based upon physical signs. These marginal variations in blood biochemistry were transient and could be diet related.

From the results of this study it has been shown that all WBC parameters remained within normal limits in alpacas acutely infected with BVDV-1c. Although leucopaenia has previously been identified between 3 and 14 days post infection in cattle both naturally and experimentally infected with BVDV [22, 23] this was not the case in this study. In the two alpacas which underwent haematological analysis in this study there was a lymphocytosis recorded in both alpacas. In addition there was a neutrophilia in one of the alpacas and a monocytosis in the other.

Although some abnormalities in blood biochemistry, haematology and temperature were seen in this study these results are inconclusive, in general all the clinical parameters measured were normal. The acute BVDV-1c infections in alpacas are likely to go unnoticed by a farmer or veterinarian.

Conclusion

This study has shown that acute infections in alpacas, with the predominant Australian strain of BVDV (BVDV-1c), naturally occur when a PI bovine animal is co-mingled with naïve alpacas. Results have also shown that these acute infections are clinically mild and undetectable without serological testing. As previous studies have indicated the reproductive losses that can occur in alpacas due to acute BVDV infections during pregnancy, the close association of pregnant alpacas and cattle of unknown BVDV status should be carefully considered in Australia in view of the results of this study.

Conflict of Interest

None

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References

1. Bell N. Australian alpaca industry celebrating 25 years. *The Weekly Times*, 2015.
2. Ridpath JF. Bovine viral diarrhea virus: global status. *The Veterinary Clinics of North America Food Animal Practice* 2010;26:105-121.
3. Evans CA, Lanyon SR, Sims SK, Reichel MP. Reproductive performance in experimentally BVDV infected ewes and seroconversion rates in sheep co-mingled with BVDV PI calves. *Small Ruminant Research* 2015;123:314-319.
4. Broaddus CC, Holyoak GR, Dawson L et al. Transmission of bovine viral diarrhea virus to adult goats from persistently infected cattle. *Journal of Veterinary Diagnostic Investigation* 2007;19:545-548.
5. Nettleton PF. Pestivirus infections in ruminants other than cattle. *Revue Scientifique et Technique* 1990;9:131-150.
6. Goyal SM, Boujihad M, Haugerud S, Ridpath JF. Isolation of bovine viral diarrhea virus from an alpaca. *Journal of Veterinary Diagnostic Investigation* 2002;14:523-525.
7. Kim SG, Anderson RR, Yu JZ et al. Genotyping and phylogenetic analysis of bovine viral diarrhea virus isolates from BVDV infected alpacas in North America. *Veterinary Microbiology* 2009;136:209-216.
8. Foster AP, Houlihan M, Higgins RJ et al. BVD virus in a British alpaca. *The Veterinary Record* 2005;156:718-719.
9. Cockcroft PD, Dornin LE, Lambert RC, Perry J, Reichel MP. Serological survey for antibodies against bovine viral diarrhoea virus and Neospora caninum in a population of South Australian alpacas (*Vicugna pacos*). *Australian Veterinary Journal* 2015;93:476-478.

10. Danuser R, Vogt HR, Kaufmann T, Peterhans E, Zanoni R. Seroprevalence and characterization of pestivirus infections in small ruminants and new world camelids in Switzerland. *Schweizer Archiv Fur Tierheilkunde* 2009;151:109-117.

11. Mattson DE, Baker RJ, Catania JE et al. Persistent infection with bovine viral diarrhea virus in an alpaca. *Journal of American Veterinary Medicine Association* 2006;228:1762-1765.

12. Carman S, Carr N, DeLay J et al. Bovine viral diarrhea virus in alpaca: abortion and persistent infection. *Journal of Veterinary Diagnostic Investigation* 2005;17:589-593.

13. van Amstel S, Kennedy M. Bovine viral diarrhea infections in new world camelids-A review. *Small Ruminant Research* 2010;91:121-126.

14. Wentz PA, Belknap EB, Brock KV, Collins JK, Pugh DG. Evaluation of bovine viral diarrhea virus in New World camelids. *Journal of American Veterinary Medicine Association* 2003;223:223-228.

15. Byers SR, Snekvik KR, Righter DJ et al. Disseminated Bovine viral diarrhea virus in a persistently infected alpaca (*Vicugna pacos*) cria. *Journal of Veterinary Diagnostic Investigation* 2009;21:145-148.

16. Ridpath JF, Fulton RW, Kirkland PD, Neill JD. Prevalence and antigenic differences observed between Bovine viral diarrhea virus subgenotypes isolated from cattle in Australia and feedlots in the southwestern United States. *Journal of Veterinary Diagnostic Investigation* 2010;22:184-191.

17. Lanyon SR, Sims SK, Cockcroft PD, Reichel MP. Comparison of serum, ear notches, and nasal and saliva swabs for Bovine viral diarrhea virus antigen detection in colostrum-fed persistently infected (PI) calves and non-PI calves. *Journal of Veterinary Diagnostic Investigation* 2014;26:783-787.

18. Evans, CA, Lanyon, SR, Reichel, MP. Investigation of AGID and two commercial ELISAs for the detection of Bovine viral diarrhea virus-specific antibodies in sheep serum. *Journal of Veterinary Diagnostic Investigation* In Press

19 Kessell A, The practice laboratory. In: Cockcroft PD, editor. Bovine medicine. 3rd edn. Wiley Blackwell, Oxford, 2015

20. Lassen. E. D, Weiser. G, 2011. Laboratory Technology for Veterinary Medicine. In: Thrall. M.E (Ed.), Veterinary Hematology and Clinical Chemistry, Blackwell Publishing, Ames, Iowa, pp. 3-38.

21. Hengrave BI, Tschudi P, Martig J, Liesegang A, Meylan M. South American camelids in Switzerland. II. Reference values for blood parameters. *Schweizer Archiv fur Tierheilkunde* 2005;147:335-343

22. Nettleton PF, Entrican G. Ruminant pestiviruses *British Veterinary Journal* 1995;151:615-642.

23. Walz PH, Bell TG, Wells JL et al. Relationship between degree of viremia and disease manifestation in calves with experimentally induced bovine viral diarrhea virus infection. *American Journal of Veterinary Research* 2001;62:1095-1103.

24. Rondeau M, Hanie EA. Cahpter 7 History and Physical Examination. In: Bassert LM, Thomasas JA, editors. *McCurnins Clinical Text for Veterinary Technicians*. 8th Edition edn. Elsevier, 2014:222-257.

25. Byers SR, Evermann JF, Bradway DS et al. The effects of exposure of susceptible alpacas to alpacas persistently infected with bovine viral diarrhea virus. *Canadian Veterinary Journal* 2011;52:263-271.

26. Cebra C, Anderson DE, Tibary A, Van Saun RJ, Johnson LW. Chapter 30 Physical Examinatina and Conformation. *Llama and Alpaca Care: Medicine, Surgery,*

Reproduction, Nutrition, and Herd Health. 1st Edition edn. Elsevier, Canada, 2014:328-337.

27. Jones M, Miesner M, Grondin T. Outbreak of Strptococcus equi ssp. zooepidemicus Polyserosistis in an Alpaca Herd. *Journal of Veterinary Internal Medicine* 2009;23:220-223.

5.2 Chapter discussion

Results from this study indicated that natural transmission occurred readily between a BVDV-1c PI heifer and BVDV naïve alpacas. All four alpacas tested positive for BVDV-specific antibodies by the end of the sampling period, thus indicating acute infection had occurred.

In cattle, the common clinical signs associated with acute infections with BVDV are: fever, oro-nasal discharge and diarrhoea (van Amstel and Kennedy 2010). Similarly in alpacas clinical signs relating to acute BVDV infections are mild to inapparent and include lethargy, anorexia and a break in the fleece. Despite often being a sign of infection in cattle, diarrhoea has not been reported as a consistent indicator of BVDV infection in alpacas (Carman *et al.* 2005; van Amstel and Kennedy 2010). In the present study, clinical signs in infected alpacas were inapparent with no indication of prolonged pyrexia or oro-nasal discharge, and although the occasional soft or dry pellet was reported, diarrhoea was not observed. There was also no change in behavior or temperament in any animal. These findings are in line with previous reports of acute BVDV infections in alpacas.

Similarly, blood biochemistry and haematology revealed only limited and mild changes in the two alpacas sampled. Blood biochemistry indicated slightly reduced levels of sodium, blood urea, total protein and chloride. However as all alpacas were reported as being clinically normal it is unlikely that these transient changes could be attributed to any pathophysiological processes, such as diarrhoea. It is more likely that these changes are related to nutritional factors and unrelated to the acute BVDV-1c infection. Likewise, haematological parameters tended to remain within normal limits. The lymphocytosis, monocytosis and neutrophilia recorded in these alpacas were mild and cannot be explained.

Overall these results show that acute BVDV-1c infections can occur in alpacas following co-mingling with BVDV PI cattle. Results also showed that acute infections in alpacas are clinically inapparent and would most likely go undetected if infection was to occur. In non-pregnant animals this has little obvious clinical impact, although reproduction may be affected as it is in other species (Grooms 2004; Evans *et al.* 2017). However, if acute BVDV-1c infections were to develop in pregnant alpacas, it is possible that similar reproductive outcomes as those seen in cattle and sheep may also be observed. Previous reports have indicated that BVDV infections in pregnant alpacas have resulted in abortion, stillbirth (Goyal *et al.* 2002; Carman *et al.* 2005) and the birth of persistently BVDV infected cria (Carman *et al.* 2005; Foster *et al.* 2005; Mattson *et al.* 2006), however, it is unknown how these outcomes relate to infection with the predominant Australian strain, BVDV-1c.

Although the current study has demonstrated that acute BVDV-1c infections in are clinically in-apparent, the reproductive effects associated with acute BVDV-1c in pregnant alpacas remains unknown. Before an effective control program for BVDV can be established in Australia, the full effect of the disease on pregnant alpacas needs to be understood. Further work needs to be undertaken to determine what reproductive effects can result from BVDV-1c infections in pregnant alpacas and whether viable and persistently BVDV-1c infected crias may develop. Only then can the real risk alpacas pose to the spread and persistence of BVDV in Australia be fully determined.

Chapter 6: Prevalence of antibodies to Pestiviruses in Australian sheep and other livestock species

Cross-species transmission of both BVDV and BDV has been shown to occur naturally. Antibodies to BVDV have been identified in many non-bovine species including, sheep, goats, alpacas and deer (Nettleton 1990), while antibodies to BDV have been identified in non-ovine species including cattle (McFadden *et al.* 2012), goats (Nettleton *et al.* 1998) and pigs (Vilcek and Belak 1996). In relation to sheep, it has been demonstrated that acute infections with the predominant Australian BVDV strain (BVDV-1c) generally cause mild or inapparent clinical signs of infection, except when infection occurs during pregnancy (Evans *et al.* 2015; Evans *et al.* 2017). Infections during pregnancy have been shown to lead to high foetal losses, low lambing rates, lambs born with developmental abnormalities or persistently infected with BVDV-1c (Evans *et al.* 2015; Evans *et al.* 2017). While it has been reported that both BVDV and BDV infections can naturally develop in sheep (Nettleton *et al.* 1998; Evans *et al.* 2015), it is unknown to what extent *Pestiviruses* are currently circulating in Australian sheep populations.

The most recent seroprevalence study for antibodies to *Pestiviruses* in Australian sheep was undertaken in 1971. This study reported that 13% of Australian sheep flocks and 8% of individual sheep had antibodies to mucosal disease/BVDV (St George 1971). Since this time the only other published study relating to the prevalence of *Pestiviruses* in Australian sheep was via a postal survey of Victorian stud sheep producers in 1984. This study identified animals that presented with clinical signs of Border disease virus (BDV) and reported that 3.5% of individual sheep had clinical signs consistent with BDV (Lim and Carnegie 1984).

Currently it is unknown how prevalent antibodies to *Pestiviruses* are in Australian sheep or even if infection is occurring naturally. Understanding the current prevalence of BVDV and/or BDV-specific antibodies within sheep populations will help to quantify the potential risk of sheep as a reservoir source of BVDV infection in cattle. This will in turn assist in determining if sheep should be included in the development of BVDV control/eradication programs in Australia.

Infections with BVDV have also previously been reported in many farmed and feral ungulate species common to Northern Australia, including camels and water buffalo (Martucciello *et al.* 2009; Gao *et al.* 2013). While camels are widespread across central and northern Australia (Pople and McLeod 2010), large-scale testing of these animals was not possible within the time frame of this project. However, we were given access to a historical data set of serum from Northern Territory water buffalo (*Bubalus bubalis*) thanks to Berrimah Veterinary Laboratory and the Northern Territory Government. Serological testing of these samples was possible and would allow for a better understanding of the host range of BVDV within Australia.

Infections with BVDV have previously been reported in buffalo from Italy (Martucciello *et al.* 2009) and the Philippines (Mingala *et al.* 2009) however it is unknown if BVDV infections are present in Australian water buffalo. In the Northern Territory of Australia farmed and feral water buffalo are often located in areas were beef cattle production is widespread thus allowing for the cross species transmission of BVDV between cattle and water buffalo to occur. Due to the interest in identifying potential reservoir hosts of BVDV infection, understanding the prevalence of BVDV in Australian water buffalo was considered important.

The following two manuscripts report the results of two state-wide serological studies for the presence of antibodies to *Pestiviruses* in two potential reservoir hosts, sheep and water buffalo (B.b).

6.1 Prevalence of antibodies to Pestiviruses in sheep

The manuscript presented in this section reports the prevalence of antibodies to *Pestiviruses,* BVDV and/or BDV, from 29 South Australian sheep farms. This manuscript has been prepared for submission to the Australian Veterinary Journal (submitted).

Short communication: Seroprevalence of antibodies to Pestivirus infections in South Australian sheep flocks

CA Evans, SR Lanyon, R O'Handley, MP Reichel and PD Cockcroft

Seroprevalence of antibodies to Pestivirus infections in South Australian sheep flocks

Australian Veterinary Journal (submitted)

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Seroprevalence of antibodies to *Pestivirus* infections in South Australian sheep flocks

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Abstract

Bovine viral diarrhoea virus (BVDV) and Border Disease Virus (BDV) are of the genus *Pestivirus*. They are known to cause significant reproductive and production losses, with BVDV acknowledged as a major source of economic loss to the Australian cattle industry. Very little is currently known about the prevalence and impact of Pestiviruses in the Australian sheep industry. The present study aimed to examine the seroprevalence and impact of both BVDV and BDV in South Australian sheep flocks. In total, 875 breeding ewes on 29 properties were serologically tested by ELISA, AGID and VNT assays for the presence of *Pestivirus* specific antibodies. Three (0.34%) individual animals returned serological results suggestive of previous BDV infection. All three positive animals were collected from one property, giving a property level seroprevalence of 3.45% and a within flock seroprevalence of 10%. These results suggest that BDV infection is present, albeit at a very low incidence, in the South Australian sheep flock while BVDV infection appears to be absent. Consequently, *Pestiviruses* are unlikely to impair production in South Australian sheep populations.

Keywords – AGID, Pestivirus, Sheep, South Australia, ELISA, VNT

Bovine viral diarrhoea virus (BVDV) in cattle and Border disease virus (BDV) in sheep belong to a group of RNA viruses from the *Pestivirus* genus. Infection with either BVDV or BDV in susceptible animals can cause immunosuppression, growth restriction, the onset of other diseases such as mastitis and respiratory disease while also causing severe reproductive dysfunction in pregnant animals including abortions, congenital and physical defects of offspring and the birth of highly infectious, persistently infected (PI) animals[1-3]. Inter-species transmission of both BVDV and BDV has been shown to occur naturally. Antibodies to BVDV have been identified in a wide range of non-bovine species including sheep, goats and deer[4], while antibodies to BDV have been identified in cattle[5], goats[2] and pigs[6] in addition to the native 'host' species, sheep[2].

There is very little currently known about the prevalence of Pestiviruses in Australian sheep. A 1971 study reported that 13% of Australian sheep flocks and 8% of individual Australian sheep had antibodies to mucosal disease virus/BVDV [7]. Similarly, a postal survey to Victorian stud sheep producers in 1984 reported only 3.5% of sheep presenting with symptoms of BDV[8]. Limited diagnostic testing in that study revealed that eleven of the seventeen (65%) sheep farms experiencing hairy-shaker-like signs had neutralising antibodies against mucosal disease virus (BVDV), with the mean within-flock seroprevalence at 26.4%. Diagnostic testing for BDV specific antibodies was not undertaken. To the authors' knowledge, no further prevalence studies on *Pestiviruses* have been undertaken on sheep in Australia since this time. Therefore, the prevalence of *Pestiviruses* in the modern Australian sheep population is unknown. Furthermore, whether the presence of *Pestiviruses* has a significant impact on the sheep industry is unquantified.

It has recently been identified that the prevalence of BVDV in Australian cattle herds is as high as 80% [9]. Losses to the Australian cattle industry due to BVDV infection are estimated to be at least AU$114.4 million[10]. This has prompted discussion on the feasibility of systematic BVDV

control programmes in Australia[11]. The ability of non-bovine species to maintain BVDV infection and act as a reservoir host has been identified as a possible threat to control efforts[11]. Currently, it is unknown whether sheep populations in Australia are capable of sustaining an active BVDV infection in the absence of continual re-infection from endemically infected cattle populations.

The aims of the present study were: to determine the prevalence of exposure to BVDV or BDV in South Australian sheep flocks; to assess the impact of Pestiviruses in the local sheep industry; and, to examine whether the presence of *Pestivirus* antibodies in sheep are associated with the presence of cattle on farm.

Samples had been previously collected for a study of toxoplasmosis in South Australian sheep flocks (unpublished data). A total of 875 serum samples were collected from breeding ewes on 29 properties throughout South Australia. Properties were selected based on the availability of lambing records and the geographic region (high rainfall Mediterranean (n=8), low rainfall Mediterranean (n=12), South East (n=9)). A mean of 30 randomly selected ewes were sampled on each property (range 28-32) by local veterinarians and blood samples were transported on ice to the University of Adelaide's Roseworthy Campus. On arrival, blood samples were centrifuged at 2,400 x g for 10 mins and serum was decanted into 1ml storage tubes and stored at -80°C.

Thawed serum samples were tested for the presence of *Pestivirus*-specific antibodies using the IDEXX BVDV/MD/BDV p80 Protein Antibody Test Kit (IDEXX Laboratories Inc, Rydalmere, NSW). Samples which returned a sample-to-negative ratio (S/N) of <63.5%[12] were considered to be positive for *Pestivirus* antibodies. Samples which were positive for antibodies by ELISA were submitted to the Elizabeth Macarthur Agricultural Institute (EMAI) and analysed by Agarose gel immunodiffusion assay (AGID) using the C24V reference strain

(BVDV-1a) [13] as antigen. A score of 0 was considered negative and scores of 1–3 considered positive. The ELISA-positive samples were also tested by virus neutralization test (VNT) at EMAI, for BVDV and BDV-specific antibodies. The VNT titre was recorded as the last dilution at which complete protection from cytopathic effect was observed, with a titre of <1:8 being recorded as negative.

All samples from properties which included one or more ELISA-positive antibody samples were also tested for BVDV-specific antigen by IDEXX Bovine Viral Diarrhoea Virus (BVDV) Antigen Test Kit/Serum Plus ELISA (IDEXX Laboratories Inc, Rydalmere, NSW). This ELISA kit has been shown to detect persistent infections with both BVDV and BDV in cattle[5, 14]. In addition, this ELISA is not species specific and has been shown to detect persistently BVDV infected sheep[3]. Samples which returned a corrected OD of >0.3 in the ELISA were considered positive for Pestivirus-specific antigen, as per the manufacturer's instructions. Sero-prevalences with 95% confidence intervals (CI) were calculated using an online calculator[15]. The Wilson score interval was used as the method for 95% CI calculation.

Table 1: Diagnostic results of four samples for BVDV/BDV antibodies by ELISA, AGID and VNT

Sample Number	Property	ELISA titre (Pos ≤63.5 S/N)	AGID score (0-3)	VNT (BDV) Titre	VNT (BVDV) Titre
1	1	19.5	0	Neg at 1:4	Neg at 1:4
2	2	17.0	2	512	256
3	2	49.6	1	256	8
4	2	17.4	2	≥512	16

Four sera (0.46%) returned a positive result in at least one assay. The diagnostic results for ELISA, AGID and VNT for those four samples are given in Table 1. Sample number 1 returned a positive result by ELISA, but was negative by both AGID and VNT. Therefore, this sample

has been considered negative for further analysis. The remaining three samples were positive in all assays and, therefore, considered positive. This resulted in an animal level seroprevalence of 0.34% (3/875; 95% CI: 0.12% – 1.00%). All three positive samples were collected from one property giving a property level seroprevalence of 3.45% (1/29; 95% CI: 0.61% - 17.18%) and a within property seroprevalence of 10% (3/30; 95% CI: 3.46% - 25.62%). This property was located in the South-East of South Australia, near the Victorian border, and ran both sheep and cattle on the property (Figure 1). None of the samples tested returned a positive result for *Pestivirus* antigen.

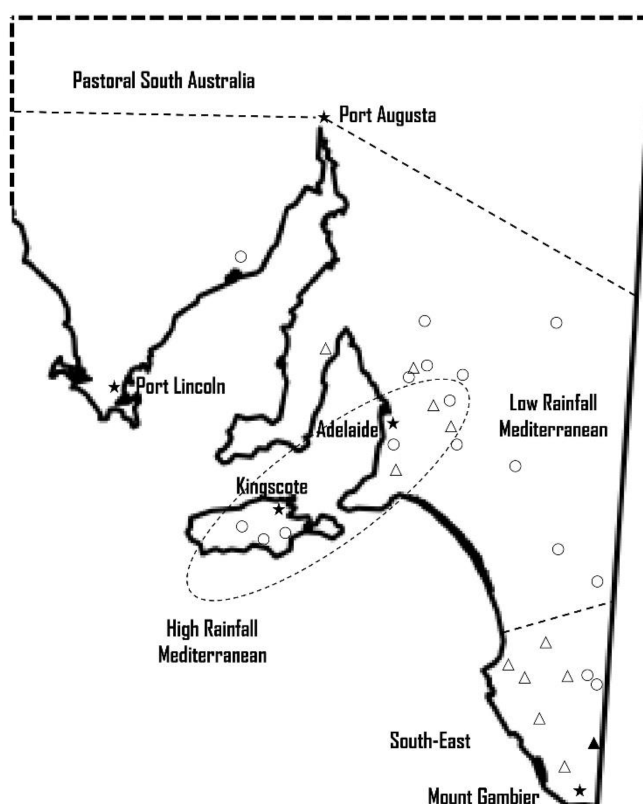

Figure 1: Distribution map of all 29 sampled South Australian sheep properties as shown by sampling region. ○ = properties with only sheep present while Δ= properties with both sheep and cattle present. Solid fill = property with samples positive for BVDV/BDV antibodies.

Virus neutralisation results indicated that the three positive samples were more indicative of an antibody response to BDV infection rather than BVDV infection, as the BDV titres were higher

than those for BVDV (Table 1). The results from this study indicate that only 0.34% of 875 South Australian sheep tested were positive for antibodies to BDV with no antibodies to BVDV detected. This, in turn, suggests that BDV may be present in South Australian sheep but at a very low incidence. Furthermore, the results suggest that infections with BVDV are unlikely to occur in South Australian sheep populations despite high prevalence in South Australian cattle[11]. These results are in line with previous findings, where <10% of Australian sheep were considered infected with Pestiviruses[7, 8]. This suggests that sheep populations in South Australia are unlikely to act as a reservoir of BVDV infection or suffer impaired production due to *Pestivirus* infection and as such are likely to be of little to no consequence in regards to BVDV control programs.

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References

1. Grooms DL. Reproductive consequences of infection with bovine viral diarrhea virus. *Veterinary Clinics Food Animal Practice* 2004;20:5-19.

2. Nettleton PF, Gilray JA, Russo P, Dlissi E. Border disease in sheep and goats. *Veterinary Research* 1998;29:327-340.

3. Evans CA, Reichel MP, Hemmatzadeh F, Cockcroft PD. Clinical responses and reproductive outcomes in pregnant ewes experimentally infected with bovine viral diarrhoea virus (Type-1c) between days 59 and 69 of gestation. *Small Ruminant Res* 2017.

4. Nettleton PF. Pestivirus infections in ruminants other than cattle. *Revue scientifique et technique* 1990;9:131-150.

5. McFadden AMJ, Tisdall DJ, Hill FI et al. The first case of a bull persistently infected with Border disease virus in New Zealand. *New Zealand Veterinary Journal* 2012;60:290-296.

6. Vilcek S, Belak S. Genetic identification of pestivirus strain Frijters as a border disease virus from pigs. *Journal of Virological Methods* 1996;60:103-108.

7. St George TD. A survey of sheep throughout Australia for antibody to parainfluenza type 3 virus and to mucosal disease virus. *Australian veterinary journal* 1971;47:370-374.

8. Lim CF, Carnegie PR. A survey of hairy shaker disease (border disease, hypomyelinogenesis congenita) in sheep. *Australian veterinary journal* 1984;61:174-177.

9. Taylor L. Findings of an Australia wide serological survey of beef and dairy herds for Bovine Viral Diarrhoea Virus conducted between 2007 and 2009. *The Australian Cattle Veterinarian*, 2010:14-19.

10. MLA. Priority list of endemic diseases for the red meat industries. 2015.

11. Lanyon S, Reichel M. Bovine viral diarrhoea virus ('pestivirus') in Australia: to control or not to control? *Australian veterinary journal* 2014;92:277-282.

12. Evans CA, Lanyon SR, Reichel MP. Investigation of AGID and two commercial ELISAs for the detection of Bovine viral diarrhea virus-specific antibodies in sheep serum. *Journal of Veterinary Diagnostic Investigation* 2017;29:181-185.

13. Ridpath JF, Lovell G, Neill JD et al. Change in predominance of Bovine viral diarrhea virus subgenotypes among samples submitted to a diagnostic laboratory over a 20-year time span. *J Vet Diagn Invest* 2011;23:185-193.

14. Lanyon SR, Sims SK, Cockcroft PD, Reichel MP. Comparison of serum, ear notches, and nasal and saliva swabs for Bovine viral diarrhea virus antigen detection in colostrum-fed persistently infected (PI) calves and non-PI calves. *Journal of Veterinary Diagnostic Investigation* 2014;26:783-787.

15. Sergeant. E. S. G. Epitools epidemiological calculators. http://epitools.ausvet.com.au. 2017. Retrieved 01/05/2017.

6.2 Prevalence of antibodies to Pestiviruses in other livestock species

The manuscript presented in this section reports the prevalence of antibodies to BVDV in cattle and water buffalo (*Bubalus bubalis)* from several geographical regions of the Northern Territory of Australia. This manuscript has been published by the *Australian Veterinary Journal.* Vol 94, Pp. 423- 426.

Original article: Antibodies to bovine viral diarrhoea virus (BVDV) in water buffalo (Bubalus bubalis) and cattle from the Northern Territory of Australia

CA Evans, PD Cockcroft and MP Reichel (2016)

Antibodies to bovine viral diarrhoea virus (BVDV) in water buffalo (*Bubalus bubalis*) and cattle from the Northern Territory of Australia

Australian Veterinary Journal Vol 94, Pp. 423- 426

Statement of Authorship

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Publication Details	CA Evans, PD Cockcroft and MP Reichel (2016) Antibodies to bovine viral diarrhoea virus (BVDV) in water buffalo (Bubalus bubalis) and cattle from the Northern Territory of Australia Australian Veterinary Journal Vol 94, Pp. 423- 426

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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution

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Antibodies to bovine viral diarrhoea virus (BVDV) in water buffalo (*Bubalus bubalis*) and cattle from the Northern Territory of Australia

CA Evans,* PD Cockcroft and MP Reichel

Background Farmed and feral water buffaloes (*Bubalus bubalis*) populations often coexist with cattle in the Northern Territory of Australia, but their level of exposure to bovine viral diarrhoea virus (BVDV) is unknown.

Methods Water buffalo (n = 245) and cattle (n = 184) serum samples were collected by the NT Government as part of an ongoing disease surveillance scheme at varying intervals between 1993 and 2001. All samples were frozen and stored at −80°C until testing. Water buffalo samples from farming properties were identified as 'farmed' animals and the remaining samples as 'feral' populations. Serum samples were analysed using commercially available ELISAs to test for the presence of BVDV antibodies.

Results Testing of historical water buffalo sera for BVDV antibodies revealed a low level of exposure, with 4.5% (95% CI ± 2.6%) being sero-positive; cattle from the same geographical area and time period had higher levels of exposure at 74.5% (95% CI ± 6.3%).

Discussion This survey showed that water buffalo are susceptible to infection with BVDV. No persistently infected water buffalo were identified in this study.

Keywords antibodies; bovine viral diarrhoea virus; cattle; Northern Territory; water buffalo

Abbreviation BDV, border disease virus; BVDV, bovine viral diarrhoea virus; CSFV, classical swine fever virus; NT, Northern Territory; S/N, sample to negative; S/P, sample to positive

Aust Vet J 2016;94:423–426 doi: 10.1111/avj.12517

Bovine viral diarrhoea virus (BVDV) is one of the most widely distributed and economically important pathogens of cattle.[1] Depending on the type and isolate present in a population, acute infections often produce unapparent or mild clinical signs and remain largely undetected. However, immunosuppression and reproductive losses from abortions, stillbirths and early calf deaths, as well as the birth of persistently infected and highly viraemic animals can also occur following infection.[1,2] Border disease virus (BDV) in sheep, classical swine fever virus (CSFV), in pigs and BVDV belong to the genus *Pestivirus* from the Flaviviridae family; however, unlike BDV and CSFV, there are two distinct BVDV: types 1 and 2.[3]

Although principally a disease of cattle, antibodies to BVDV have been isolated from other species, including sheep,[4] pigs,[5] goats,[6] deer, alpaca[7] and a variety of wild, predominantly ruminant species.[8] Although there is a limited understanding of the pathophysiological effects following BVDV infection in water buffalo (*Bubalus bubalis*), mild clinical signs appear in experimentally infected water buffalo calves[9] and increases in abortion rates in water buffalo could be linked to maternal infection with BVDV.[10]

Water buffalo were first introduced to Australia in the late 1820s, as a source of meat for settlers living in the Northern Territory (NT). In 2014 an aerial survey estimated at least 100,000 water buffalo in Arnhem Land in the NT.[11] Both feral and farmed water buffalo often coexist with extensively farmed domestic beef cattle in the NT.

In Australia, BVDV can be found in the majority of dairy and beef cattle herds and one study showed 91.5% of all Australian cattle herds have evidence of past exposure to the virus, with one or more animals in each herd being positive for BVDV antibodies.[12] As a result there is increasing interest in developing an Australia-wide BVDV control program,[13] despite the risk posed by other susceptible species as potential reservoir hosts.

A pooled buffalo fetal serum sample from the NT collected in 1994 was identified positive for a BVDV-1 genotype,[14] but it remains unknown whether BVDV infection is present in Australian water buffalo. It is possible that interactions between cattle and water buffalo in the NT are allowing for cross-species transmission.

The aim of this study was to assess whether antibodies to BVDV were present in historical water buffalo serum samples from the NT of Australia and to compare with seropositive cattle from the same region between 1993 and 2001. This information will help develop a better understanding of which Australian cattle species are at risk of BVDV infection and provide further information for the formulation of effective control programs.

Materials and methods

A total of 245 water buffalo serum samples were obtained through the Berrimah Veterinary Laboratory, Darwin, from 18 different survey sites in the NT, north of Tennant Creek. The samples were collected by the NT Government as part of an ongoing disease surveillance scheme at varying intervals between 1993 and 2001. The year and location of each sample was recorded; no information was available regarding the age or sex of the water buffalo and only limited information on whether they were wild-caught or domestically managed. Water buffalo samples identified as being from farming properties were classified as 'farmed' animals and the remaining samples were believed to be from feral populations. Samples were frozen and stored at −80°C until testing.

Cattle serum samples (n = 184) were collected by the NT Government from 9 of the same locations as for the water buffalo and

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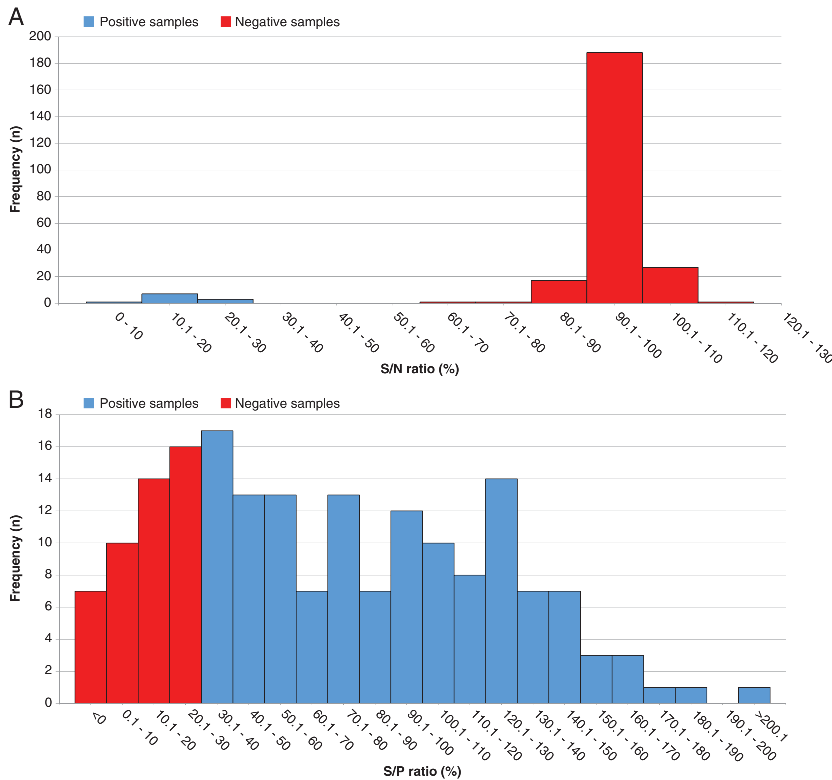

Figure 1. Histogram of the frequencies of bovine viral diarrhoea virus (BVDV) antibody titre levels detected in sera samples from water buffalo (n = 254) and cattle (n = 184) collected across 19 farms in the Northern Territory between1993 and 2001. (A) Antibody titre levels in water buffalo sera. Red columns are samples that returned negative results (S/N ratio >40%), and blue columns are samples that returned positive results (S/N ratio ≤40%). (B) Antibody titre levels in cattle sera. Red columns are samples that returned negative results (S/P ratio <30%), and blue columns are samples that returned positive results (S/P ratio >30%). S/N, sample to negative; S/P, sample to positive.

during a comparable timeframe. All samples were frozen and stored at −80°C until testing.

Serum samples were analysed using commercially available ELISAs to test for the presence of BVDV antibodies. The indirect ELISA used for the water buffalo sera was the IDEXX BVDV/MD/BDV p80 Protein Antibody Test Kit (IDEXX Laboratories, NSW, Aust; specificity 97.6%, sensitivity 97.3%[15]), according to the manufacturer's instructions for bovine serum. This assay uses a non-species-specific conjugate and has been validated for use on cattle and sheep serum samples. Samples that returned a sample to negative (S/N) ratio ≤40% were considered positive as per the manufacturer's instructions.

The direct ELISA used for the cattle sera was the IDEXX BVDV Antibody ELISA (IDEXX Laboratories), which uses a species-specific conjugate. Performance characteristics for the use of this assay on cattle serum have been previously established, with sensitivity and specificity of 96.7% and 97.1%, respectively.[16] Samples that returned a sample to positive (S/P) ratio <30% were considered negative and ≥30% were considered positive, as per the manufacturer's instructions.

All cattle samples that returned negative results on the antibody ELISA were tested for BVDV antigen. Water buffalo samples that returned negative antibody ELISA results, and were from locations where positive antibody results were obtained, were also tested for the presence of BVDV antigen. These samples were analysed using the IDEXX BVDV antigen ELISA (IDEXX Laboratories). For both cattle and water buffalo, samples that returned S/N ratio ≤30% in the antigen-capture ELISA were considered negative and those >30% were considered positive, as per the manufacturer's instructions.

The mean and 95% confidence intervals (95% CI) were calculated.

Results

Antibody S/N ratios for BVDV in water buffalo sera ranged from 5.0% to 119.0% and, using the manufacturer's cut-off of 40%, 11/245 samples were positive for antibodies to BVDV (Figure 1a) (4.5%; 95% CI ±2.6%) (Table 1). Of the 156 samples tested for BVDV antigen, none returned positive results. The 11 seropositive water buffalo samples were all found during 1 year at one location (farm A), where cattle and water buffalo were both farmed. No additional antibody-positive water buffalo were detected before or after this period.

Antibody S/P ratios for BVDV in cattle sera ranged from −5.7% to 206.8% and, using the manufacturer's cut-off of 30%, 137/184 samples were positive (Figure 1b), giving cattle a final proportion of 74.5% seropositive animals (95% CI ±6.3%) (Table 1). The remaining 53 cattle samples testing negative for BVDV antibodies were tested for BVDV antigen, with none returning an S/N ratio considered positive on antigen ELISA.

Discussion

The results of the survey of water buffalo and cattle sera showed disparate results for exposure to BVDV in these two bovid species. The

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Table 1. **Proportion of water buffalo (n = 245) and cattle sera (n = 184) from the Northern Territory (NT) classified as positive for bovine viral diarrhoea virus antibodies**

Location	Water buffalo				Cattle			
	Total (n)	Positive (n)	Proportion (%)	95% CI (%)	Total (n)	Positive (n)	Proportion (%)	95% CI (%)
Beatrice Hill*	63	11	17.5	8.1–26.9	58	45	77.6	66.9–87.7
Berrimah*	3	0	0	0	23	14	60.9	40.9–80.9
Cape Arnhem	1	0	0	0				
Cobourg Peninsula	14	0	0	0				
Daly River Port Keats	5	0	0	0	23	18	78.3	61.4–95.2
Darwin rural area*	4	0	0	0	15	7	46.7	21.4–72
Koolatong	2	0	0	0				
Maningrida	4	0	0	0				
Marrakai	1	0	0	0				
Milingimbi	1	0	0	0	34	25	73.5	58.7–73.5
Murgenella	6	0	0	0				
Nathan River	1	0	0	0				
North-east Arnhem	50	0	0	0				
North-west Arnhem	20	0	0	0				
Numbulwar	8	0	0	0				
South-east Arnhem	14	0	0	0				
Tiwi	41	0	0	0				
Walker River	7	0	0	0				
Other NT					31	28	90.3	79.9–100
Total	245	11	4.5	1.9–7.1	184	137	74.5	68.2–80.8

* Farmed.
CI, confidence interval.

proportion of cattle positive for BVDV antibodies (74.5%) in our survey is similar to that in previous studies outlining the prevalence of BVD virus in cattle populations around Australia, with one study finding 54% of individual Australian cattle were positive for BVDV antibodies.[12]

In contrast, only 4.5% of water buffalo samples were positive for BVDV antibodies and all were located on the same property (farm A), one of five locations where both cattle and water buffalo sera were sampled. On this farm, 77.6% of cattle sera tested positive for BVDV antibodies, which was the highest antibody numbers on any of the properties sampled, and this may reflect cross-species transmission between cattle and water buffalo or vice versa. The current potential of water buffalo to infect cattle in northern Australia is unknown, but is likely to be low, as evidenced by the low level of antibodies observed in this survey and the absence of persistently infected buffalo.

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References

1. Nettleton PF, Entrican G. Ruminant pestiviruses. *Br Vet J* 1995;151:615–642.
2. Grooms DL. Reproductive consequences of infection with bovine viral diarrhea virus. *Vet Clin Food Anim Pract* 2004;20:5–19.
3. Ridpath JF. Bovine viral diarrhea virus: global status. *Vet Clin Food Anim Pract* 2010;26:105–121.
4. Scherer CFC, Flores EF, Weiblen R et al. Experimental infection of pregnant ewes with bovine viral diarrhoea virus type-2 (BVDV-2): effects on the pregnancy and fetus. *Vet Microbiol* 2001;79:285–299.
5. Tao J, Liao JH, Wang Y et al. Bovine viral diarrhea virus (BVDV) infections in pigs. *Vet Microbiol* 2013;165:185–189.
6. Bachofen C, Vogt HR, Stalder H et al. Persisitent infections after natural transmission of bovine viral diarrhoea virus from cattle to goats among goats. *Vet Res* 2013;44:32–42.
7. Goyal SM, Boujihad M, Haugerud S et al. Isolation of bovine viral diarrhea virus from an alpaca. *J Vet Diagn Invest* 2002;14:523–525.
8. Nettleton PF. Pestivirus infections in ruminants other than cattle. *Rev Sci Tech* 1990;9:131–150.
9. Martucciello A, De Mia GM, Giammarioli M et al. Detection of bovine viral diarrhea virus from three water buffalo fetuses (*Bubalus bubalis*) in southern Italy. *J Vet Diagn Invest* 2009;21:137–140.
10. Mingala CN, Konnai S, Tajima M et al. Classification of new BVDV isolates from Philippine water buffalo using the viral E2 region. *J Basic Microbiol* 2009;49:495–500.
11. Department of Land Resource Management. *Feral buffalo (*Bubalus bubalis*): distribution and abundance in Arnhem Land, Northern Territory*. The Department, 2014. http://www.lrm.nt.gov.au/__data/assets/pdf_file/0005/355802/2014-Arnhem-Buffalo-Survey-report.pdf. Accessed September 2015.

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12. Taylor L. Findings of an Australia wide serological survey of beef and dairy herds for Bovine Viral Diarrhoea Virus conducted between 2007 and 2009. *Aust Cattle Vet* 2010;57:14–19.
13. Lanyon S, Reichel M. Bovine viral diarrhoea virus ('pestivirus') in Australia: to control or not to control? *Aust Vet J* 2014;92:277–282.
14. Becher P, Orlich M, Shannon AD et al. Phylogenetic analysis of pestiviruses from domestic and wild ruminants. *J Gen Virol* 1997;78:1357–1366.
15. Cuttance WG, Cuttance EL. Analysis of individual farm investigations into bovine viral diarrhoea in beef herds in the North Island of New Zealand. *NZ Vet J* 2014;62:338–342.
16. Lanyon SR, Anderson ML, Bergman E et al. Validation and evaluation of a commercially available ELISA for the detection of antibodies specific to bovine viral diarrhoea virus (bovine pestivirus). *Aust Vet J*2013;91:52–56.
17. Pfeiffer DU. *Veterinary epidemiology: an introduction*. Wiley-Blackwell, West Sussex, 2010:54–59.

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OBITUARY

Hugh Malcolm Deakin

(1928–2016)

Hugh Malcolm Deakin, a Sydney graduate of 1949, died at home at Central McDonald, near Wisemans Ferry, NSW, on 10 July 2016, just short of his 88th birthday.

Hugh grew up in Epsom Rd, Rosebery, when his family operated a big suburban dairy, opposite the old Victoria Park racecourse. However, the family also owned properties near Picton, where he spent considerable time, giving him also a rural background.

On graduation he was associated at Randwick with VEH Davis, one of the few equine veterinarians at the time, before setting up his own mixed practice in Gardener's Rd, Rosebery, which he conducted for some years. Seeking a change, he purchased a property at Appin, which he worked as well as conducting a part-time large animal practice, mainly for his neighbours.

On selling the Appin property, he moved to Moss Vale, where he was part of a group practice that catered for the booming dairy industry of the 1960s. Eventually, he set up a mixed practice of his own in Moss Vale. Following the death of his wife, Regina, from cancer, Hugh sold the practice and moved back to Sydney, from where he did some locums in country practices, mainly to help out old friends, but included one locum in an equine practice in WA, during the breeding season.

After marrying Cynthia in 1987, he settled down in the Macdonald Valley, where they had a Lowline cattle stud and bred Maremma dogs. Again, his neighbours prevailed upon him to enter veterinary practice and he didn't need a great deal of persuasion to do so. A few years ago, he suffered severe injuries in an accident with a motor vehicle, which, together with failing eyesight, greatly restricted his activities.

Outside of veterinary work his interests focused on many aspects of horse sports. As a young man, he rode trackwork at the old Rosebery race course and, as an amateur rider at picnic meetings in NSW, over jumps at horse shows and in hunt club point-to-points. Turning his attention to fox-hunting, he had his own pack of hounds when at Appin. At Moss Vale he played polo for Goulburn, raced horses on country tracks and was associated with the Bong Bong Race Club, of which he was a life member. He was also a long-time member of the Australian Jockey Club (now the Australian Turf Club) and a foundation member of the Sydney Turf Club.

Hugh was very generous of his time in officiating at equestrian events, frequently in an honorary capacity, whether it be horse shows, endurance rides, hunt clubs, polo tournaments or race meetings.

For a long period he was head equine veterinarian at the RAS Show at Sydney and worked with several Olympic equestrian teams training at Bowral.

He is survived by his wife, Cynthia, and four children from his previous marriage to Regina.

Hugh will be remembered in the profession for the help and encouragement that he frequently gave to students and young graduates.

AJ Chittick

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6.3 Chapter discussion

The final step in clarifying the full effect of BVDV on sheep populations and the subsequent role sheep play in the spread and persistence of BVDV throughout Australia is to understand the prevalence of antibodies to ruminant Pestiviruses in sheep. While an Australia-wide seroprevalence study could not be undertaken, we were able to test samples obtained for a prevalence study of toxoplasmosis in South Australian sheep. A total of 875 serum samples from breeding ewes, collected from 29 South Australian properties were tested for antibodies to both BVDV and BDV. Border disease virus is the ovine *Pestivirus* and as such correct identification of the two viruses was considered essential. Furthermore, natural infection of cattle with BDV has previously been associated with high abortion rates (Krametter-Froetscher *et al.* 2010b), low pregnancy rates (McFadden *et al.* 2012) and persistent infections in cattle (Braun *et al.* 2015). Therefore testing for BDV-and BVDV-specific antibodies was considered appropriate when testing sheep.

Results from this study suggested, that there was a low prevalence of antibodies to *Pestiviruses* in South Australian sheep flocks. Only three samples, of the 875 tested, were positive for antibodies to BVDV/BDV, with all three samples originating from the same property. This resulted in a prevalence at the individual animal level of 0.34 % (95%CI: 0.12%-1.00%) and 3.45% (95%CI: 0.16%-17.18%) at the property level. These findings are similar to historical studies relating to *Pestivirus* infections in Australian sheep, which reported prevalences of between 3.5% and 8% (St George 1971; Lim and Carnegie 1984) at the individual animal level and 13% at the flock level (St George 1971).

The property from which all three positive samples were collected was recorded as being a mixed sheep and cattle farming property. As such, it was originally thought that cross-species transmission of BVDV must have been occurring from cattle and sheep. However, testing by

VNT strongly suggested that the three positive samples were positive for antibodies to BDV not BVDV. This serological survey demonstrated that Pestiviruses are present in South Australian sheep at a relatively low level, and that serological results were suggestive of BDV rather than BVDV.

The second study in this section focused on the presence of BVDV antibodies in 184 cattle and 245 water buffalo from the Northern Territory of Australia. This study reported that 74.5% (95%CI: ±6.3%) of the cattle tested were positive for BVDV antibodies, which is in line with previous reports in Australian cattle (Taylor 2010). In comparison only 4.5% (95%CI: ±2.6%) of the water buffalo tested were positive for BVDV antibodies. Results suggested that all 11 positive water buffalo were from a single property, which was also shown to farm cattle. While the proportion of water buffalo positive for BVDV antibodies on this farm was 17.5%, the proportion of BVDV antibody positive cattle was 77.6%. These findings suggest that cattle are the true reservoir of BVDV infection, however cross-species transmission of BVDV may occur between cattle and water buffalo in Australia.

Although natural infections with BVDV have been shown to occur in both sheep (Pratelli *et al.* 2001; Evans *et al.* 2015) and water buffalo (Mingala *et al.* 2009), the current studies indicate that it is unlikely that these species are acting as a reservoir source of BVDV infection for cattle in Australia. It appears that cattle are the true reservoir of BVDV in Australia and although cross-species transmission can occasionally occur on mixed farming properties, infections with BVDV do not appear to be actively circulating in these non-bovine species.

Chapter 7: Discussion and Conclusions

While BVDV is predominantly considered a disease of cattle, numerous reports have indicated that many non-bovine species including sheep, goats and alpaca are also susceptible to infection. For a country such as Australia, cattle are frequently farmed in close proximity to sheep and other non-bovine species. Therefore, understanding the clinical and economic significance BVDV has on these non-bovine species and in turn the role these non-bovine species play in the epidemiology, including the spread and persistence, of BVDV in cattle populations is important, if effective control programs are to be developed. As such, the objective of this thesis was to investigate important aspects of the epidemiological and clinical impacts of BVDV in sheep, and other commonly farmed Australian livestock species.

Evidence from the studies in this thesis support the assertions that sheep populations pose a low risk to the spread and persistence of BVDV-1c in Australia and that sheep are likely to be a dead-end host. The seroprevalence study (Chapter 6.1) identified no BVDV-specific antibodies in any of the 875 ewes tested and indicates that acute BVDV infections are rare in South Australian sheep. This study did identify three sheep which were positive (seroprevalence of 0.34%) for BDV-specific antibodies indicating that BDV is present in Australia, although at a low prevalence. This is, as far as the authors are aware, the first published report of BDV in Australian sheep in 30+ years.

While Chapter 6.1 reported that *Pestivirus* infections are uncommon in South Australian sheep, this was a geographically limited cross-sectional study and the prevalence in other states/territories of Australia are unquantified and need to be determined. Future cross-sectional surveys could collect serum samples from properties where cattle and sheep co-graze or from properties where higher than usual reproductive losses have recently been reported. While this wouldn't give an accurate representation of the prevalence of BVDV infections in Australian

sheep populations, it would more accurately demonstrate if cross-species transmission is naturally occurring from cattle to sheep, or not. Once this is known, State or National seroprevalence studies could be undertaken. This would then determine the prevalence of both BDV and BVDV-specific antibodies in sheep throughout other sheep farming regions of Australia, as it has been more than 30 years since any prevalence work has been undertaken for Australian sheep. However, results from Chapter 6.1 indicate that there is a low prevalence of *Pestivirus* antibodies, and thus acute infections, in South Australian sheep.

The low risk sheep populations appear to pose to the spread and persistence of BVDV within Australia is further supported by the papers presented in Chapter 4. These papers demonstrated that there is low risk of BVDV-1c being transmitted from acutely BVDV-1c infected sheep or from pre-weaned BVDV PI lambs which have had successful passive transfer of maternal antibodies. Transmission from BVDV infected sheep was only shown to occur on one occasion and was as a result of co-paddocking a neonatal, PI lamb with susceptible, naïve ewes and lambs. One lamb from this study was reported to develop BVDV-specific antibodies at 7 weeks of age and was considered to be acutely infected with BVDV-1c (Chapter 4.1). In addition, transmission was not shown to occur from interactions with the same PI lamb and naïve cattle (Chapter 4.1) or from sheep undergoing acute BVDV-1c infections (Chapter 4.2). This low rate of transmission from BVDV infected sheep suggests that if cross-species transmission was to occur from cattle to sheep, the likelihood that acutely infected sheep or pre-weaned BVDV PI lambs (with successful passive transfer of maternal antibodies) would infect other susceptible animals is low.

While these two papers suggest that the risk posed by BVDV infected sheep to the spread and persistence of BVDV is low, there were a number of limitations that need addressing. Firstly, only a single BVDV-1c PI lamb was produced in Chapter 3.1 and although its survival was longer than that of BVDV-1c PI lambs reported by Evans *et al.* (2015), it was of a small and

weak stature. As such the studies relating to transmission of BVDV from a PI lamb were carried out earlier than planned, in case that PI lamb did not survive. Because of this, the PI lamb was of a young age (0-8 weeks of age) and still affected by high levels of maternal antibodies directed against BVDV, through ingested colostrum. It appears from an analysis of the present study that colostral antibodies may not only inhibit the accurate detection but also the infective nature of a young PI animal. Results from antigen ELISA testing of serum samples support the conclusion that the lamb was persistently infected with BVDV, and indicate that the amount of antigen in the serum dropped significantly after colostral ingestion, before increasing at 3 weeks of age. Serum viral load when tested by RT-PCR was also shown to increase 10-fold at 7 weeks of age. While the relationship between the concentration of virus present in serum and the amount of virus shed in nasal or saliva secretions is not known, the results (lack of transmission) do suggest that colostral antibodies have an influence on the amount of virus present in young PI animals. Unfortunately saliva swabs were not collected from the PI lamb to determine the amount of virus actually being shed during the study period.

In addition, the ideal method to study transmission of BVDV from a PI lamb to susceptible cattle might be to co-house them for a period of time. Unfortunately, due to the young age of the PI lamb there were welfare concerns that in a co-housed situation the steers might kill the lamb, whether intentionally or accidentally. Thus the two trials described (adjacent fence line and 5 mins of contact/ambient air) were used. In retrospect, the amount of contact a lamb and steer would undergo naturally would be limited, as such it was believed these two trial designs were adequate to represent an on farm encounter for studying the ability of a neonatal, PI lamb to infect susceptible cattle. While the papers from Chapter 4 indicate that the risk a neonatal BVDV PI lamb poses to the spread of BVDV to susceptible animals is low, there is an opportunity for further work to be undertaken in determining the risk posed by older BVDV PI sheep, including co-housing them with naïve cattle. However from the results presented previously by Evans *et al.* (2015), and in Chapter 3, BVDV-1c PI lambs have a poor survival

rate and the chance of a PI lamb surviving long enough to pose a significant risk to the spread of the virus is limited.

The absence of BVDV-specific antibodies in South Australian sheep, despite them being highly susceptible under experimental conditions, combined with the low rate of transmission from BVDV-1c infected sheep suggest that sheep are likely a dead-end host for BVDV. However, work from this thesis has also demonstrated that if acute infection with BVDV-1c develops in a pregnant ewe flock then severe reproductive losses are possible. Low lambing rates, poor lamb survival, the birth of PI lambs and severe physical malformations in lambs were observed following maternal infection of ewes with BVDV-1c during early-mid gestation (Chapter 3). These findings are similar to previous reports associated with other BVDV species/strains (Parsonson *et al.* 1979; Pratelli *et al.* 2001; Scherer *et al.* 2001) and suggests that although the current body of work focused on the pre-dominant Australian strain of BVDV (BVDV-1c), severe reproductive losses are likely to occur following acute BVDV infections in pregnant ewes, irrespective of the species or strain present.

Chapter 3 also reports that a single viable and persistently BVDV-1c infected lamb was produced following maternal infection with BVDV between 59 and 69 days of gestation. While this lamb survived until euthanasia at 8 weeks of age, the lamb was significantly smaller and weaker than non PI lambs of the same age. It is unknown to what age the surviving PI lamb would have lived had it not been euthanased. There was also a high foetal death rate and low survival rate for lambs suspected of being persistently infected with BVDV (positive for BVDV antigen and negative for BVDV antibodies) in this study. The poor survival of lambs suspected of and confirmed to be PI with BVDV-1c not only supports the findings of previous studies (Evans *et al.* 2015) but also suggests that viability of BVDV PI sheep is poor. The poor survival of BVDV-1c PI lambs further supports the low risk sheep populations pose to the spread and

persistence of BVDV within Australia, as BVDV PI lambs appear to die long before having any real chance of passing on the infection to susceptible animals.

This thesis also investigated BVDV infections on non-bovine species, other than sheep. Chapter 5 reported that acute BVDV infections in alpacas can develop, following the co-housing of a PI heifer and susceptible alpacas. This paper also determined that the clinical signs associated with acute BVDV-1c infections in alpacas are inapparent, a finding which is consistent with previous reports in cattle and sheep (Nettleton and Entrican 1995). While the effects associated with acute BVDV-1c infection in pregnant alpacas were not investigated in this thesis it is likely that similar reproductive losses to those seen in acutely infected cattle and sheep would occur as has also been reported in BVDV infected alpacas elsewhere (Carman *et al.* 2005; Mattson *et al.* 2006). However to fully comprehend the role alpacas play in the spread and persistence of BVDV further work should be undertaken in relation to the reproductive effects associated with BVDV-1c infections in pregnant alpacas, particularly in relation to the development and survival of BVDV-1c PI crias.

Finally, this thesis reported on the presence of BVDV-specific antibodies in water buffalo (*Bubalus bubalis*) from the Northern Territory of Australia (Chapter 6.2). Results from this study determined that while there is a high prevalence (74.5%) of BVDV-specific antibodies in cattle, there is only a low prevalence (4.5%) in water buffalo from the same geographical regions. These findings would suggest that water buffalo (*B. b.*) are susceptible to acute infections with BVDV however BVDV-specific antibodies in these populations appears to be uncommon.

In conclusion, the research presented in this thesis contributes to a better understanding of the role that non-bovine species play in the spread and persistence of BVDV-1c in Australia (and possibly around the world). It is evident that infection of sheep with BVDV-1c can lead to

severe reproductive losses and indicates a need to eliminate close contact of cattle, which may be infected with BVDV, and pregnant sheep. However, results have also shown that sheep are unlikely to play a pivotal role in the spread and persistence of the virus in Australia and the inclusion of sheep in BVDV control and eradication programs appears to be unnecessary. Furthermore, results from this study have indicated that alpacas are susceptible to acute BVDV-1c infections following co-mingling with PI cattle, although infections are clinically inapparent. Finally, antibodies to BVDV and BDV were identified, at low prevalences, in Northern Territory water buffalo and South Australian sheep, respectively. While it is clear that many non-bovine species are susceptible to *Pestivirus* infections in Australia, further work needs to be undertaken, in species other than sheep, before a suitable decision can be made regarding their inclusion in BVDV control and eradication programs.

Appendix 1: Supporting Publications – Published papers

The following manuscript supports the main body of work presented in this thesis. Although the manuscript was published during the course of the PhD it is from work undertaken prior to the commencement of the PhD and was submitted for the award of an Honours degree. It is included here as a supporting publication.

Original article: Reproductive performance in experimentally BVDV infected ewes and seroconversion rates in sheep co-mingled with BVDV PI calves

CA Evans, SR Lanyon, SK Sims and MP Reichel (2015)

Reproductive performance in experimentally BVDV infected ewes and seroconversion rates in sheep co-mingled with BVDV PI calves

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Reproductive performance in experimentally BVDV infected ewes and seroconversion rates in sheep co-mingled with BVDV PI calves

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ABSTRACT

Bovine viral diarrhoea virus (BVDV) is an economically important disease of cattle, causing severe reproductive losses and immunosuppressive effects in cattle populations worldwide. Before a national BVDV control programme can be developed in Australia, it is important to explore the possibility of sources of transmission from and through alternative susceptible populations, such as sheep.

Transmission of BVDV to species other than cattle has been shown to occur following both experimental and natural infection of other ruminants. The aims of this study were to investigate the impact of an experimental infection with a local Australian strain of BVDV (Type 1c) on the reproductive performance of pregnant ewes and the potential for natural transmission of BVDV from persistently infected (PI) cattle to naive sheep.

Twenty-three BVDV naïve, pregnant ewes were experimentally infected with BVDV by subcutaneous inoculation with 2 mL freeze-thawed PI cow serum. Severe reproductive losses including low lambing rates and a high number of young lamb deaths were seen in these ewes. One PI lamb was also produced in this trial, but died at 15 days of age.

Nine BVDV naïve sheep wethers were co-mingled with three BVDV PI calves in a 1500 sqm paddock for four weeks. Transmission of infection, as indicated by seroconversion, occurred in four of nine wethers during a four-week period of co-mingling.

The trials suggest that the potential for infection with BVDV derived from cattle for sheep exists and needs to be taken into account when controlling BVDV infection in cattle.
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1. Introduction

Bovine viral diarrhoea virus (BVDV) is an important pathogen of cattle and the causative agent of bovine viral diarrhoea (BVD) and mucosal disease (MD) (Nettleton and Entrican, 1995). Together with border disease virus (BDV) in sheep and classical swine fever virus (CSFV) in pigs, BVDV belongs to the genus *Pestivirus* in the family *Flaviviridae* (Collett et al., 1988). BVDV is a highly prevalent pathogen, endemic to most cattle-producing countries (including Australia) (Ridpath, 2010). A study undertaken in 2010, sampling 406 Australian cattle herds, showed that 86% of herds had experienced some level of exposure to BVDV (Taylor, 2010) and indicated a need for the development of a national BVD control programme.

In 2012, Australia had a total of 6526 mixed sheep and cattle farms, with a further 11,944 and 46,255 farms specialising solely in sheep and cattle (beef and dairy) production, respectively (Australian Bureau of Statistics, 2013). This high number of mixed farming practices in

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Australia may present a risk for cross-species disease transmission and the subsequent spread of diseases such as Johne's disease, ruminant pestiviruses and leptospirosis.

BVDV infection has been reported to occur in multiple other species, including goats (Bachofen et al., 2013), deer (Nettleton, 1990), camels (Gao et al., 2013), eland (Vilcek et al., 2000), pigs (Tao et al., 2013) and (of particular relevance to the Australian cattle industries) sheep (Scherer et al., 2001).

Previous studies have demonstrated that sheep can be both, experimentally and naturally infected with BVDV (Vilcek et al., 1997; Passler and Walz, 2010; Braun et al., 2013). The lambing rates of ewes infected with BVDV during pregnancy are highly variable, with reported lamb losses ranging from 0% to 85% (Snowdon et al., 1975; Parsonson et al., 1979; Scherer et al., 2001). Studies by Snowdon et al. (1975) and Scherer et al. (2001) have observed the reproductive consequences in sheep following experimental infection with different strains of BVDV and BVDV type 2, respectively. These studies found that reproductive losses were dependent on both the type and the strain of BVDV used, as well as the stage of gestation at which infection occurred. In Australia, there is an overwhelming predominance (>96%) of one BVDV subgenotype, BVDV type 1c (Ridpath, 2010), and although previous studies have described the effects of BVDV infection on sheep, these studies primarily used types or strains of the virus not commonly found in Australia. As such, these results may not be indicative of the implications and losses associated with BVDV infection of sheep in Australia.

The aims of this study were as follows: to assess the impact of infection with an Australian strain of BVDV on the reproductive performance of group of experimentally infected, pregnant ewes and to identify the efficiency of natural transmission of BVDV from PI cattle to sheep under simulated farming conditions.

2. Materials and methods

2.1. Animals

All sheep used in this study were sourced from the University of Adelaide's commercial flock, resident at its Roseworthy Campus. Prior to use in this trial, the flock was tested for BDV and BVDV antibodies by Agarose Gel Immunodiffusion (AGID) and found to be naïve for pestiviruses.

All animal experiments were approved by the University of Adelaide's Animal Ethics Committee prior to this project (S-2012-248) being undertaken.

2.2. Reproductive consequences of BVDV infection in pregnant ewes

Twenty-four ewes, confirmed pregnant by ultrasound examination, were subcutaneously inoculated with 2 mL freeze-thawed BVDV-type 1c PI cow serum at between 60 and 120 days of gestation. Serum samples were collected from each ewe one week prior to inoculation, on the day of inoculation, and weekly for six weeks post-inoculation. Serum samples were tested for BVDV-specific antibodies using the Priocheck BVDV Ab P80 ELISA (Prionics, Martinsried, Germany).

Ewes were observed for clinical signs of BVDV infection daily throughout the trial and were pregnancy scanned by ultrasound on Days 28, 48 and 68 post-inoculation. Pregnancy losses were recorded when, either, a pre-term abortion was observed, or a ewe was found not pregnant at scanning. Day of gestation at inoculation was calculated back from the day of lambing, assuming an average gestation length of 147 days (Forbes, 1967).

All ewes that carried their pregnancies to term were allowed to lamb naturally. Serum samples were collected weekly from live lambs until six weeks of age. Aborted foetuses and carcases of lambs that died after birth were submitted to the Veterinary Diagnostic Laboratory of the University of Adelaide for post-mortem examination, where abdominal or peritoneal fluid and ear tissue samples were collected for virus detection. One ewe (#24) died at 24 days post-inoculation. Post-mortem examination of this ewe showed no evidence implicating BVD infection as the cause. Twin foetuses were recovered from this ewe, with abdominal or peritoneal fluid and tissue samples collected for inclusion in the study.

All serum samples from live lambs were tested for BVDV-specific antibodies using the Priocheck BVDV Ab P80 ELISA (Prionics, Martinsried, Germany), a competitive ELISA. Either serum, post-mortem fluid or ear tissue samples from all live, aborted or dead lambs were tested for BVDV viral antigen using IDEXX Serum/Ag PLUS ELISA (IDEXX Laboratories Inc. Rydalmere, NSW), as per manufacturer's instructions.

2.3. Transmission of BVDV from PI calves to naïve wethers

A total of nine mature wethers (confirmed BVDV naïve by AGID and Ab ELISA) were co-mingled with three confirmed BVDV PI calves for four weeks. During the first week, the calves and sheep were separated by a fence but shared feed and water sources. For the following three weeks, the calves and sheep were able to mingle unrestricted in a 1500 sqm paddock under simulated farming conditions. Serum samples were collected weekly from each wether, from the initial day of mixing (Day 0) until three weeks post-separation (7 weeks post-mixing) and stored at −80 °C until testing.

Serum samples were tested for the presence of BVDV-specific antibodies using the Priocheck BVDV Ab P80 ELISA (Prionics, Martinsried, Germany). Any wethers that had not seroconverted within three weeks of separation from the PI calves were considered uninfected.

2.4. BVDV antibody serology

Ewe, wether and lamb samples were tested for the presence of BVDV antibodies by the Priocheck BVDV Ab P80 ELISA (Prionics, Martinsried, Germany). Initial validation was carried out internally, establishing a new positivity threshold for the competitive ELISA of 35% inhibition (Inh) after comparison with AGID test results (performed at the Elizabeth Macarthur Agricultural Institute (EMAI), Camden, NSW).

2.5. RT-PCR and characterisation of the persisting BVD strain

Total viral RNA was extracted from freeze-thawed serum samples from the PI lamb (L14) and PI cow using the QIAmp Viral RNA Mini Kit (Qiagen, Germany). Copy DNA (cDNA) was synthesised using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) with each reaction consisting of 1× reverse transcriptase (RT) buffer, 4 μM dNTP mix, 2.5 U/μL MultiScribe Reverse Transcriptase (Applied Biosystems, California, USA), 1 mM of a single 21-mer reverse primer (CTATCCTTCTCT-GATTCTCTG) that targeted the 5′ UTR of the BVDV genome and 10 μL of RNA extract in a total reaction volume of 20 μL. cDNA was synthesised under the following thermal cycler conditions (per manufacturer's instructions): 10 min at 25 °C, followed by 120 min at 37 °C. The reaction was terminated by inhibition of the reverse transcriptase enzyme for 5 min at 85 °C.

Detection of BVDV was achieved by polymerase chain reaction (PCR) amplification of a 292 base pair fragment that targeted the 5′ untranslated region (UTR) of the BVDV genome (Primers F: CTAGCCATGC-CCTTAGTAGGACTA and R: CAACTCCATGTGCCATGTACAGCA). Reactions were performed in a BioRad T100 Thermal cycler (BioRad USA). Each reaction consisted of 1x reaction buffer (Fisher Biotech, Australia), 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM forward primer, 0.2 mM reverse primer, 0.2 μL Taq DNA polymerase (Fisher Biotech, Australia) and 2 μL cDNA template in a total reaction volume of 50 μL. The reaction was incubated for 2 min at 94 °C for initial denaturation, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s. Positive PCR products were purified using the MinElute PCR Purification Kit (Qiagen, Germany) and sequenced at the Australian Genome Research Facility, Urrbrae, South Australia. Further sequence analysis was carried out using ClustalX 2.1 and BioEdit 7.2.5.

3. Results

3.1. Reproductive consequences of acute BVDV infection in pregnant ewes

3.1.1. Seroconversion

All 23 ewes seroconverted to BVDV antibody positive status (at the threshold of 35%Inh) between two and five weeks post-inoculation. The mean observed time from inoculation (Day 0) to seroconversion for these animals was between 14 and 21 days (Fig. 1) (see Supplementary Table 1 for detailed inhibition results). No clinical signs of infection were observed in any ewes.

Supplementary Table 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.smallrumres.2014.11.018.

3.1.2. Reproductive performance

Of the 23 pregnant ewes inoculated with BVDV, only 13 ewes (56.6%; 95% CI: 36.3–76.8%) carried their pregnancies to term. A further six ewes (26%; 95% CI: 8.1–44.1%) were found to be not pregnant by pregnancy scanning and four ewes (17.4%; 95% CI: 1.9–32.9%) aborted pre-term lambs (Fig. 2).

3.1.3. Lamb survival

The 13 ewes that carried their pregnancies to term delivered a total of 19 lambs. Seven lambs (36.8%; 95% CI: 15.2–58.5%) died between one hour and 15 days after birth. Three died within 24 hours after birth and a further three died within three days of birth (Table 1). One lamb died at two weeks of age. Post-mortem examination showed all full term lambs had been born alive (i.e. had breathed) (Table 1).

A total of 12 lambs survived to marking, that is, until the routine muster for management procedures including castration and vaccination at two to five months of age. This gave a lamb marking percentage $\left(\frac{\text{\# lambs alivs atmarking}}{\text{\# ewes mated}}\right)$ of 52.2% (95% CI: 30.8–72.6%). By comparison, the flock from which the ewes in this study were sourced achieved a lamb marking percentage of 120% (J. Matheson, pers. commun.).

3.1.4. BVDV antigen testing on lambs

Post-mortem abdominal or peritoneal fluids and/or ear tissue obtained from two aborted foetuses (ewes 18 and 23) and two foetuses recovered from the deceased ewe (ewe 24) at post-mortem examination returned positive BVDV antigen ELISA results (Table 1). All lambs (n = 19) born alive returned negative BVDV antigen ELISA results, except for two lambs (born to ewe #1) which died within three days of birth and one lamb (L14), which died at fifteen days of age (Table 1).

This lamb (L14) returned positive BVDV antigen ELISA results on serum collected on the day of birth, at 12 days of age, and on post-mortem peritoneal fluid (Table 2). However, this lamb also tested negative for BVDV antigen on serum collected at 5 days of age, after confirmed colostrum ingestion. PCR identified serum samples from the day of birth and 12 days of age and the post-mortem peritoneal fluid as positive for BVDV-specific RNA indicating that this lamb was PI with BVDV. The serum collected from the PI lamb at 5 days of age returned a negative PCR result.

All samples for this lamb returned positive BVDV antibody ELISA results despite the confirmation, by PCR, that this lamb was persistently infected with BVDV. It was presumed that the Day 0 sample from this lamb was collected prior to colostrum ingestion, however this cannot be completely confirmed. The positive antibody result on Day 0 may suggest that this lamb had consumed a small amount of colostrum prior to blood collection.

Table 1
Lamb survival following experimental BVDV infection of 23 pregnant ewes (with corresponding estimated day of gestation at time of inoculation). Ewes that did not lamb are not included. PI = positive for BVDV antigen on sequential samples, Ag+ on post-mortem = positive, by antigen ELISA, for BVDV virus on single (post-mortem) sample.

Ewe #	Estimated day of gestation at which infection occurred	Lamb #	Outcome
25	63	L15	Healthy
7	67	L13	Healthy
		L14	Death at 15 days, PI
11	80	L12	Healthy
26	87	n/a	Death at <24 h
19	93	L11	Healthy
28	94	L10	Healthy
22	97	L09	Death at 1 day
		n/a	Death at < 24 h
2	99	L08	Healthy
10	100	L06	Healthy
		L07	Healthy
9	106	L03	Healthy
12	106	L04	Healthy
		L05	Death at 2 days
6	109	L01	Healthy
		L02	Healthy
1	117	n/a	Death at <24 h, Ag+ on post-mortem
		n/a	Death at 3 days, Ag+ on post-mortem
23	Aborted	n/a	Ag+ on post-mortem
16	Aborted	n/a	
18	Aborted	n/a	Ag+ on post-mortem
24	Ewe died	Fetus	Ag+ on post-mortem
		Fetus	Ag+ on post-mortem

3.2. Genotype of BVDV strain

Sequencing of the 5′ UTR region confirmed that the BVDV strain persisting in the PI cow from which the inoculating serum was collected showed 98% homology with BVDV type 1c strains deposited with GenBank (GenBank accession number: EU159702) (Xue et al., 2010). There was also 99.6% homology between the sequences of the PI cow and L14, with a single nucleotide polymorphism on the 212th position of the 292 bp UTR region sequenced, where the cytosine was substituted for a thyamine. The high similarity between the sequences from the PI cow and L14 indicate that cross-species transmission of the same BVDV strain (BVDV type 1c) occurred.

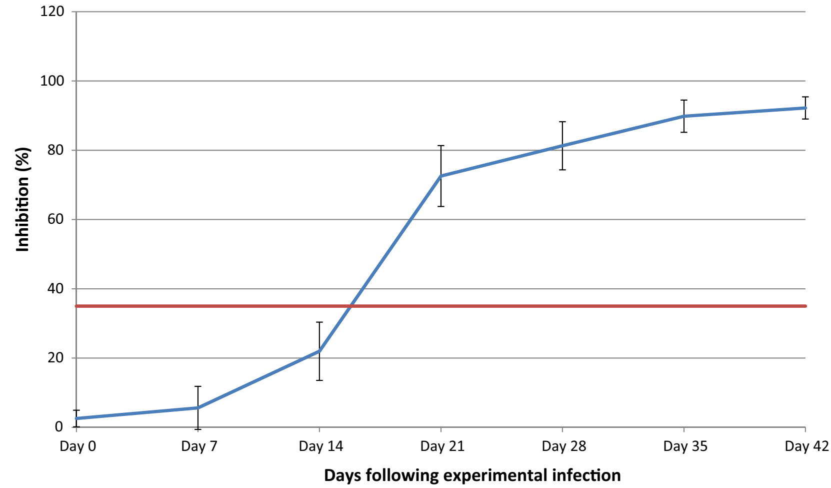

Fig. 1. Mean BVDV antibody results (with 95% confidence intervals) for experimentally infected ewe flock (23 ewes) from day of infection (Day 0) until 42 days post infection. Red line shows the 35%Inh cut off for positive samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

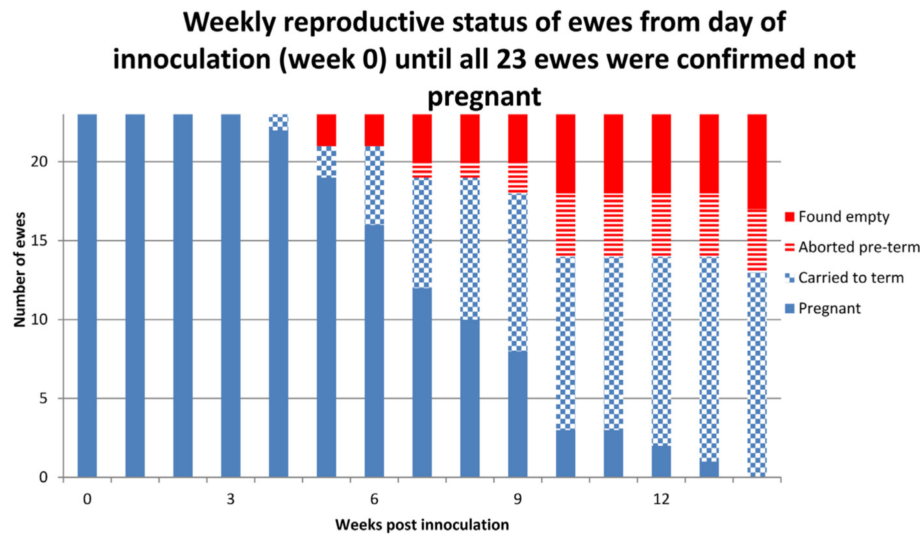

Fig. 2. Weekly reproductive status of ewes from inoculation (week 0) until all 23 ewes were confirmed not pregnant after an experimental inoculation with 2 ml BVDV PI cow serum.

3.3. Transmission of BVDV infection from PI calves to naïve sheep

During the four week mixing period, BVDV infection appeared to be transmitted (as evidenced by seroconversion within three weeks) from the three PI calves to four of the nine (44.4%: 95% CI: 11.9–76.9%), previously pestivirus naïve wethers. The first wether (W04) seroconverted by Day 21, with the final two wethers (W01 and W02) seroconverting by Day 49 (Table 3). No clinical signs of BVDV infection were observed in any wethers.

Table 2
BVDV antibody (Ab) and antigen (Ag) ELISA and PCR results at different ages obtained from a lamb (L14) persistently infected with bovine viral diarrhoea virus (BVDV) following foetal infection at approximately Day 67 of gestation.

Blood sample	Ab ELISA (%INH[a])	Ag ELISA (corrected OD[b])	PCR
Day of birth (0 days of age)	55.122	2.146	Positive
Week 1 (5 days of age)	99.512	0.022	Negative
Week 2 (12 days of age)	97.236	0.323	Positive
Post-mortem (15 days of age)	82.358	0.626	Positive

[a] Percentage inhibition.
[b] Optical density (450 nm).

Table 3
BVDV antibody ELISA results for each of the four wethers showing positive infection to BVDV following a four-week co-mingling period with three BVDV PI calves. Days are from when the calves were initially housed with wethers (Day 0) until three weeks post-separation of animals (Day 49). Values are expressed as percent inhibition (Inh) with red values indicating positive BVDV antibody results.

ID number	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49
W01	22.79	19.49	16.26	13.58	26.26	23.98	27.15	43.41
W02	24.97	20.97	7.24	7.64	6.50	10.89	26.26	48.78
W04	28.36	20.79	29.59	62.76	86.99	70.24	80.24	77.56
W09	19.05	17.31	17.80	16.50	27.80	9.92	71.30	81.46

4. Discussion

Seroconversion in cattle following exposure to BVDV can take anywhere between 13 and 28 days (Kirkland et al., 1997; Niskanen and Lindberg, 2003; Lindberg et al., 2004), while in experimental infection of ewes with BVDV type-2, seroconversion took between 15 and 30 days (Scherer et al., 2001). The present study found that seven ewes (of 23) returned positive BVDV antibody results within 14 days after inoculation with BVDV, while the remaining 16 ewes seroconverted over the following three weeks (and at most by 35 days post-inoculation). Mean Inhibition percentages crossed the positive threshold after 14 and before 21 days. By comparison, a study of heifers naturally infected with the same BVDV strain used in the present study seroconverted between 14 and 28 days following exposure to a PI cow (Lanyon et al., 2014b). Time to seroconversion for pestivirus species thus appears to be similar, in both sheep and cattle.

In the present study, 23 ewes experimentally inoculated with BVDV had a marking percentage of just 52.2% (95% CI: 30.8–72.6%), with a total of 12 lambs surviving to marking. This percentage is considerably lower (less than half) than the 120% marking percentage achieved in the flock of origin (also pestivirus naïve) over the same time period, suggesting that the experimental introduction of BVDV into a pestivirus naïve, pregnant ewe flock can have the potential to negatively impact reproductive performance. The authors do acknowledge that these flocks were managed under different conditions, particularly during the time of lambing, and differences in marking percentages may not be exclusively attributed to BVDV infection. However, such a drastic difference is suggesting that maternal infection of BVDV accounted for a substantial percentage of the losses seen in this trial.

Two Australian studies, undertaken in the late 1970s showed lambing percentages between 20% and 100% following experimental infection with BVDV during pregnancy (Snowdon et al., 1975; Parsonson et al., 1979). More recently, a Brazilian study indicated that ewes inoculated with BVDV type 2 had a final lambing percentage of just 53% (Scherer et al., 2001). Many of these reported reproductive outcomes of ewes following infection with BVDV were reproduced during the present study, including low lambing percentages and a high number of early lamb deaths.

The present study demonstrated that an acute BVDV infection in a pregnant ewe, inoculated at Day 67 of gestation, can result in the birth of a BVDV PI lamb. This is in agreement with a previous study where the development of BDV PI lambs was reported to arise from the infection in the ewe between days 60 and 80 of gestation (Broaddus et al., 2009). BVDV PI lambs have resulted from infection anywhere between days 38 and 78 of gestation (Parsonson et al., 1979; Scherer et al., 2001). In the present study, a further six lambs (recovered foetuses or dead lambs) also returned positive antigen ELISA results following maternal infection between 65 and 117 days gestation (Table 1), indicating that these lambs may too have been persistently infected with BVDV. Although it is suspected that these lambs were also PI, this cannot be confirmed as sequential samples were not possible. Nonetheless, these results suggest that the gestational range for PI lamb production may be much larger than that previously reported.

Strong BVDV antigen responses for both Day 0 and post-mortem serum samples were observed for the confirmed PI lamb in this study. However, false negative results were observed at 5 days of age. Similar results were seen following PCR of the same samples and although limited, these results suggest that colostrum-derived antibodies may interfere with detection of BVDV antigen by both ELISA and PCR. Previous studies have reported that a decrease in a positive antigen signal (ELISA) and a decrease in the number of virus copies present (PCR) have been seen in PI calves due to the presence of maternal antibodies (Zimmer et al., 2004; Fux and Wolf, 2012; Lanyon et al., 2014a). Further study of the detection of BVDV (including the use of ear notches) in young lambs would be valuable to more clearly identify the period during which the detection of PI lambs may be inhibited by interference from colostrum-derived antibodies.

The twin (L13) to the confirmed PI lamb (L14) appeared to be healthy, antibody positive and antigen negative. A study by Schoder et al. (2004) reported a similar scenario in cattle: at three months of age, one dizygotic twin heifer tested BVDV antibody positive and BVD virus free while the other heifer calf was antibody negative but virus positive. These results indicate that the response to infection and development of immunocompetence may vary between foetuses and suggest that the windows for foetal infection resulting in persistent infection are not absolute.

The confirmed BVDV PI lamb in this study survived only to 15 days of age, however, previous studies have shown that lambs persistently infected with BVDV-2 have survived for up to 6 months of age (Scherer et al., 2001). BVDV-2 is not found in Australia (Ridpath et al., 2010), and, as such, the survivability of BVDV PI lambs in Australia and, therefore, the ability of sheep flocks to maintain BVDV infection and act as a reservoir of infection may require further investigation with local strains and isolates.

This study was able to show that cross-species transmission of BVDV from PI calves to sheep can occur under simulated farming conditions. The first of these animals

seroconverted by 21 days, when calves and sheep remained co-mingled, with the remaining three wethers seroconverting after calves and sheep were separated. There is a possibility that due to the extended period of time it took W01 and W02 to seroconvert, that these animals contracted BVDV from sheep to sheep transmission.

The ability of naïve sheep to become infected with BVDV through co-mingling with PI cattle, however, indicates that sheep may be at risk of contracting BVDV from endemically infected cattle populations. For the duration of this study, no clinical signs were observed in any of the sheep acutely infected with BVDV, by either experimental inoculation or natural infection. Thus, if infection with BVDV was to occur in a flock of naive sheep it is quite possible that there would be little to no indication that BVDV was present. However, if that infection occurred in a flock of pestivirus naïve pregnant ewes, abortions and poor lambing rates may occur, resulting in severe economic losses to the producer.

While the presence of PI cattle appears to be the most dominant method by which BVDV is spread and maintained in cattle populations (Niskanen and Lindberg, 2003), it is likely, once these PI cattle have been removed from a population, that less efficient means of transmission, such as cross-species transmission from reservoir populations, may become more important. Further research in this area would be beneficial. In particular, studies on the viability of PI lambs, and efficiency of transmission from these animals to naïve sheep and cattle could reveal the potential of sheep to act as a reservoir of BVDV, while studies on the current prevalence of BVD in Australian sheep populations could further inform on BVDV infection and associated losses in the sheep industry.

Conflict of interest statement

None.

Role of the funding source

The University of Adelaide had no part in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

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References

Australian Bureau of Statistics, 2013. Agricultural Business.

Bachofen, C., Vogt, H.R., Stalder, H., Mathys, T., Zanoni, R., Hilbe, M., Schweizer, M., Peterhans, E., 2013. Persisitent infections after natural transmission of bovine viral diarrhoea virus from cattle to goats among goats. Vet. Res. 44.

Braun, U., Bachofen, C., Schenk, B., Hassig, M., Peterhans, E., 2013. Investigation of border disease and bovine virus diarrhoea in sheep from 76 mixed cattle and sheep farms in eastern Switzerland. Schweiz. Arch. Tierheilkd. 155, 293–298.

Broaddus, C.C., Lamm, C.G., Kapil, S., Dawson, L., Holyoak, G.R., 2009. Bovine viral diarrhea virus abortion in goats housed with persistently infected cattle. Vet. Pathol. 46, 45–53.

Collett, M.S., Anderson, D.K., Retzel, E., 1988. Comparisons of the pestivirus bovine viral diarrhoea viurs with members of the flaviviridae. J. Gen. Virol. 69, 2637–2643.

Forbes, J.M., 1967. Factors affecting gestational length in sheep. J. Agric. Sci. 68, 191–194.

Fux, R., Wolf, G., 2012. Transient elimination of circulating bovine viral diarrhoea virus by colostral antibodies in persistently infected calves: a pitfall for BVDV-eradication programs? Vet. Microbiol. 161, 13–19.

Gao, S.D., Luo, J.H., Du, J.Z., Lang, Y.F., Cong, G.Z., Shao, J.J., Lin, T., Zhao, F.R., Belak, S., Liu, L.H., Chang, H.Y., Yin, H., 2013. Serological and molecular evidence for natural infection of Bactrian camels with multiple subgenotypes of bovine viral diarrhea virus in Western China. Vet. Microbiol. 163, 172–176.

Kirkland, P.D., McGowan, M.R., Mackintosh, S.G., Moyle, A., 1997. Insemination of cattle with semen from a bull transiently infected with pestivirus. Vet. Rec. 140, 124–127.

Lanyon, S.R., Hill, F.I., Reichel, M.P., Brownlie, J., 2014a. Bovine viral diarrhoea: pathogenesis and diagnosis. Vet. J. 199, 201–209.

Lanyon, S.R., McCoy, R., Cockcroft, P.D., Reichel, M.P., 2014. A longitudinal study of the antibody levels in serum from cows with differing gestational outcomes following acute infection with bovine viral diarrhoea virus (BVDV) during early gestation. Vet. Microbiol. (submitted for publication).

Lindberg, A., Stokstad, M., Loken, T., Alenius, S., Niskanen, R., 2004. Indirect transmission of bovine viral diarrhoea virus at calving and during the postparturient period. Vet. Rec. 154, 463–467.

Nettleton, P.F., 1990. Pestivirus infections in ruminants other than cattle. Rev. Sci. Tech. 9, 131–150.

Nettleton, P.F., Entrican, G., 1995. Ruminant pestiviruses. Br. Vet. J. 151, 615–642.

Niskanen, R., Lindberg, A., 2003. Transmission of bovine viral diarrhoea virus by unhygienic vaccination procedures, ambient air, and from contaminated pens. Vet. J. 165, 125–130.

Parsonson, I.M., Ohalloran, M.L., Zee, Y.C., Snowdon, W.A., 1979. Effects of bovine viral diarrhoea-mucosal disease (BVD) virus on the ovine fetus. Vet. Microbiol. 4, 279–292.

Passler, T., Walz, P.H., 2010. Bovine viral diarrhea virus infections in heterologous species. Anim. Health Res. Rev./Conf. Res. Work. Anim. Dis. 11, 191–205.

Ridpath, J.F., 2010. Bovine viral diarrhea virus: global status. Vet. Clin. North Am.: Food Anim. Pract. 26, 105–121.

Ridpath, J.F., Fulton, R.W., Kirkland, P.D., Neill, J.D., 2010. Prevalence and antigenic differences observed between Bovine viral diarrhea virus subgenotypes isolated from cattle in Australia and feedlots in the southwestern United States. J. Vet. Diagn. Invest. 22, 184–191.

Scherer, C.F.C., Flores, E.F., Weiblen, R., Caron, L., Irigoyen, L.F., Neves, J.P., Maciel, M.N., 2001. Experimental infection of pregnant ewes with bovine viral diarrhoea virus type-2 (BVDV-2): effects on the pregnancy and fetus. Vet. Microbiol. 79, 285–299.

Schoder, G., Mostl, K., Benetka, V., Baumgartner, W., 2004. Different outcome of intrauterine infection with bovine viral diarrhoea (BVD) virus in twin calves. Vet. Rec. 154, 52–53.

Snowdon, W.A., Parsonson, I.M., Broun, M.L., 1975. Reaction of pregnant ewes to innoculation with mucosal disease virus of bovine origin. J. Comp. Pathol. 85, 241–251.

Tao, J., Liao, J.H., Wang, Y., Zhang, X.J., Wang, J.Y., Zhu, G.Q., 2013. Bovine viral diarrhea virus (BVDV) infections in pigs. Vet. Microbiol. 165, 185–189.

Taylor, L., 2010. Findings of an Australia wide serological survey of beef and dairy herds for bovine viral diarrhoea virus conducted between 2007 and 2009. Aust. Cattle Vet. 57, 14–19.

Vilcek, S., Nettleton, P.F., Paton, D.J., Belak, S., 1997. Molecular characterisation of ovine pestivirus. J. Gen. Virol. 78, 725–735.

Vilcek, S., Paton, D.J., Rowe, L.W., Anderson, E.C., 2000. Typing of pestiviruses from eland in Zimbabwe. J. Wildl. Dis. 36, 165–168.

Xue, F., Zhu, Y.M., Li, J., Zhu, L.C., Ren, X.G., Feng, J.K., Shi, H.F., Gao, Y.R., 2010. Genotyping of bovine viral diarrhea viruses from cattle in China between 2005 and 2008. Vet. Microbiol. 143, 379–383.

Zimmer, G.M., Van Maanen, C., De Goey, I., Brinkhof, J., Wentink, G.H., 2004. The effect of maternal antibodies on the detection of bovine virus diarrhoea virus in peripheral blood samples. Vet. Microbiol. 100, 145–149.

Appendix 2: Supporting Publications – Conference papers

The following supporting publications are oral and poster contributions presented during the course of the PhD. Presentations are from work undertaken during the PhD or the preceding Honours degree (marked by an asterisk) at both international and domestic scientific conferences. The presenting author is underlined.

Oral Conference Presentations

Reichel MP, Neverauskas C, Evans CA and Nasir A (2015) Co-infections of *Neospora caninum* and BVDV in water buffalo (*Bubalus bubalis*) and cattle in the Northern Territory of Australia 25th WAAVP 2015, Liverpool, United Kingdom

*Evans CA, Lanyon SR, Manning SK and Reichel MP (2014) Reproductive performance in pregnant ewes experimentally infected with BVDV and transmission rates in sheep co-mingled with BVDV PI calves XXVIII World Buiatrics Congress 2014, Cairns, Australia

Poster Conference Presentations

Evans CA, Reichel MP, Hemmatzadeh F and Cockcroft PD (2016) Reproductive outcomes in pregnant ewes experimentally infected with Bovine viral diarrhoea virus between days 59 and 69 of gestation XXIX World Buiatrics Congress 2016, Dublin, Ireland

Evans CA, Reichel MP, Hemmatzadeh F and Cockcroft PD (2016) Transmission of Bovine viral diarrhoea virus from a persistently infected lamb to naïve sheep XXIX World Buiatrics Congress 2016, Dublin, Ireland

References

Acland, HM, Gard, GP, Plant, JW (1972) Infection of sheep with a mucosal disease virus. *Aust Vet J* **48**, 70-70.

Arnal, M, Fernandez-de-Luco, D, Riba, L, Maley, M, Gilray, J, Willoughby, K, Vilcek, S, Nettleton, PF (2004) A novel pestivirus associated with deaths in Pyrenean chamois (Rupicapra pyrenaica pyrenaica). *J Gen Virol* **85**, 3653-7.

Bachofen, C, Vogt, HR, Stalder, H, Mathys, T, Zanoni, R, Hilbe, M, Schweizer, M, Peterhans, E (2013) Persisitent infections after natural transmission of bovine viral diarrhoea virus from cattle to goats among goats. *Veterinary Research* **44**, 32-42.

Baker, JC (1990) Clinical aspects of bovine virus diarrhoea virus infection. *Rev Sci Tech* **9**, 25-41.

Barber, DM, Nettleton, PF (1993) Investigations into bovine viral diarrhoea virus in a dairy herd. *Vet Rec* **133**, 549-50.

Barnett, J, Twomey, DF, Millar, MF, Bell, S, Bradshaw, J, Higgins, RJ, Scholes, SF, Errington, J, Bromage, GG, Oxenham, GJ (2008) BVDV in British alpacas. *Vet Rec* **162**, 795.

Becher, P, Avalos Ramirez, R, Orlich, M, Cedillo Rosales, S, Konig, M, Schweizer, M, Stalder, H, Schirrmeier, H, Thiel, HJ (2003) Genetic and antigenic characterization of novel pestivirus genotypes: implications for classification. *Virology* **311**, 96-104.

Becher, P, Fischer, N, Grundhoff, A, Stalder, H, Schweizer, M, Postel, A (2014) Complete genome sequence of bovine pestivirus strain PG-2, a second member of the tentative pestivirus species giraffe. *Genome Announcements* **2**, e00376-14.

Becher, P, Shannon, AD, Tautz, N, Thiel, HJ (1994) Molecular characterization of border disease virus, a pestivirus from sheep. *Virology* **198**, 542-51.

Bhudevi, B, Weinstock, D (2003) Detection of bovine viral diarrhea virus in formalin fixed paraffin embedded tissue sections by real time RT-PCR (Taqman). *Journal of Virological Methods* **109**, 25-30.

Blood, DC, Hutchins., DR, Jubb, KV, Whittem, JH, Littlejohns, I (1957) "Mucosal Disease" of cattle in Australia. *Aust Vet J* **33**, 75-76.

Boardman, W (2015) Biosecurity. In 'Bovine Medicine.' (Ed. PD Cockcroft.) pp. 189-197. (Wiley Blackwell: West Sussex, UK)

Braun, U, Bachofen, C, Buchi, R, Hassig, M, Peterhans, E (2013) Infection of cattle with Border disease virus by sheep on communal alpine pastures. *Schweizer Archiv Fur Tierheilkunde* **155**, 123-128.

Braun, U, Hilbe, M, Janett, F, Hassig, M, Zanoni, R, Frei, S, Schweizer, M (2015) Transmission of border disease virus from a persistently infected calf to seronegative heifers in early pregnancy. *BMC Vet Res* **11**, 43.

Braun, U, Reichle, SF, Reichert, C, Hassig, M, Stalder, HP, Bachofen, C, Peterhans, E (2014) Sheep persistently infected with Border disease readily transmit virus to calves seronegative to BVD virus. *Veterinary Microbiology* **168**, 98-104.

Brinkhof, J, Zimmer, G, Westenbrink, F (1996) Comparative study on four enzyme-linked immunosorbent assays and a cocultivation assay for the detection of antigens associated with the bovine viral diarrhoea virus in persistently infected cattle. *Vet Microbiol* **50**, 1-6.

Broaddus, CC, Holyoak, GR, Dawson, L, Step, DL, Funk, RA, Kapil, S (2007) Transmission of bovine viral diarrhea virus to adult goats from persistently infected cattle. *Journal of Veterinary Diagnostic Investigation* **19**, 545-548.

Broaddus, CC, Lamm, CG, Kapil, S, Dawson, L, Holyoak, GR (2009) Bovine viral diarrhea virus abortion in goats housed with persistently infected cattle. *Vet Pathol* **46**, 45-53.

Brock, KV (2004) Strategies for the control and prevention of bovine viral diarrhea virus. *Vet Clin North Am Food Anim Pract* **20**, 171-80.

Brock, KV, Grooms, DL, Ridpath, J, Bolin, SR (1998) Changes in levels of viremia in cattle persistently infected with bovine viral diarrhea virus. *J Vet Diagn Invest* **10**, 22-6.

Brock, KV, Lapin, DR, Skrade, DR (1997) Embryo transfer from donor cattle persistently infected with bovine viral diarrhea virus. *Theriogenology* **47**, 837-44.

Brownlie, J, Clarke, MC, Howard, CJ, Pocock, DH (1987) Pathogenesis and epidemiology of bovine virus diarrhoea virus infection of cattle. *Ann Rech Vet* **18**, 157-66.

Byers, SR, Evermann, JF, Bradway, DS, Grimm, AL, Ridpath, JF, Parish, SM, Tibary, A, Barrington, GM (2011) The effects of exposure of susceptible alpacas to alpacas persistently infected with bovine viral diarrhea virus. *Can Vet J* **52**, 263-71.

Byers, SR, Snekvik, KR, Righter, DJ, Evermann, JF, Bradway, DS, Parish, SM, Barrington, GM (2009) Disseminated Bovine viral diarrhea virus in a persistently infected alpaca (Vicugna pacos) cria. *J Vet Diagn Invest* **21**, 145-8.

Carman, S, Carr, N, DeLay, J, Baxi, M, Deregt, D, Hazlett, M (2005) Bovine viral diarrhea virus in alpaca: abortion and persistent infection. *J Vet Diagn Invest* **17**, 589-93.

Cho, HJ, Masri, SA, Deregt, D, Yeo, SG, Thomas, EJ (1991) Sensitivity and specificity of an enzyme-linked immunosorbent assay for the detection of bovine viral diarrhea virus antibody in cattle. *Can J Vet Res* **55**, 56-9.

Cleveland, SM, Salman, MD, Van Campen, H (2006) Assessment of a bovine viral diarrhea virus antigen capture ELISA and a microtiter virus isolation ELISA using pooled ear notch and serum samples. *Journal of Veterinary Diagnostic Investigation* **18**, 395-398.

Cockcroft, PD, Dornin, LE, Lambert, RC, Perry, J, Reichel, MP (2015) Serological survey for antibodies against bovine viral diarrhoea virus and Neospora caninum in a population of South Australian alpacas (Vicugna pacos). *Aust Vet J* **93**, 476-478.

Collins, ME, Heaney, J, Thomas, CJ, Brownlie, J (2009) Infectivity of pestivirus following persistence of acute infection. *Vet Microbiol* **138**, 289-96.

Cornish, TE, van Olphen, AL, Cavender, JL, Edwards, JM, Jaeger, PT, Vieyra, LL, Woodard, LF, Miller, DR, O'Toole, D (2005) Comparison of ear notch immunohistochemistry, ear notch antigen-capture ELISA, and buffy coat virus isolation for detection of calves persistently infected with bovine viral diarrhea virus. *J Vet Diagn Invest* **17**, 110-7.

Danuser, R, Vogt, HR, Kaufmann, T, Peterhans, E, Zanoni, R (2009) Seroprevalence and characterization of pestivirus infections in small ruminants and new world camelids in Switzerland. *Schweizer Archiv Fur Tierheilkunde* **151**, 109-117.

Driskell, EA, Ridpath, JF (2006) A survey of bovine viral diarrhea virus testing in diagnostic laboratories in the United States from 2004 to 2005. *J Vet Diagn Invest* **18**, 600-5.

Dubois, E, Russo, P, Prigent, M, Thiery, R (2008) Genetic characterization of ovine pestiviruses isolated in France, between 1985 and 2006. *Vet Microbiol* **130**, 69-79.

Durham, PJK, Paine, GD (1997) Serological survey for antibodies to infectious agents in beef cattle in northern South Australia. *Aust Vet J* **75**, 139-140.

Edwards, S, Roehe, PM, Ibata, G (1995) Comparative studies of border disease and closely related virus infections in experimental pigs and sheep. *British Veterinary Journal* **151**, 181-187.

Evans, CA, Lanyon, SR, Sims, SK, Reichel, MP (2015) Reproductive performance in experimentally BVDV infected ewes and seroconversion rates in sheep co-mingled with BVDV PI calves. *Small Ruminant Research* **123**, 314-319.

Evans, CA, Reichel, MP, Hemmatzadeh, F, Cockcroft, PD (2017) Clinical responses and reproductive outcomes in pregnant ewes experimentally infected with bovine viral diarrhoea virus (Type-1c) between days 59 and 69 of gestation. *Small Ruminant Research*

Foster, AP, Houlihan, M, Higgins, RJ, Errington, J, Ibata, G, Wakeley, PR (2005) BVD virus in a British alpaca. *Vet Rec* **156**, 718-9.

French, EL (1962) Mucosal disease-virus diarrhoea complex of cattle. *Aust Vet J* **38**, 477-478.

French, EL, Snowdon, WA (1964) Mucosal disease in Australian cattle. *Aust Vet J* **40**, 99-105.

Fulton, RW, Ridpath, JF, Saliki, JT, Briggs, RE, Confer, AW, Burge, LJ, Purdy, CW, Loan, RW, Duff, GC, Payton, ME (2002) Bovine viral diarrhea virus (BVDV) 1b: predominant BVDV subtype in calves with respiratory disease. *Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire* **66**, 181-190.

Fulton, RW, Step, DL, Ridpath, JF, Saliki, JT, Confer, AW, Johnson, BJ, Briggs, RE, Hawley, RV, Burge, LI, Payton, ME (2003) Response of calves persistently infected with noncytopathic bovine viral diarrhea virus (BVDV) subtype 1b after vaccination with heterologous BVDV strains in modified live virus vaccines and Mannheimia haemolytica bacterin-toxoid. *Vaccine* **21**, 2980-2985

Fux, R, Wolf, G (2012) Transient elimination of circulating bovine viral diarrhoea virus by colostral antibodies in persistently infected calves: a pitfall for BVDV-eradication programs? *Vet Microbiol* **161**, 13-9.

Gao, SD, Luo, JH, Du, JZ, Lang, YF, Cong, GZ, Shao, JJ, Lin, T, Zhao, FR, Belak, S, Liu, LH, Chang, HY, Yin, H (2013) Serological and molecular evidence for natural infection of Bactrian camels with multiple subgenotypes of bovine viral diarrhea virus in Western China. *Veterinary Microbiology* **163**, 172-176.

Garcia-Perez, AL, Minguijon, E, Estevez, L, Barandika, JF, Aduriz, G, Juste, RA, Hurtado, A (2009) Clinical and laboratorial findings in pregnant ewes and their progeny infected with Border disease virus (BDV-4 genotype). *Research in Veterinary Science* **86**, 345-352.

Geoscience Australia (2004) Land areas of States and Territories. Available at http://www.ga.gov.au/scientific-topics/national-location-information/dimensions/area-of-australia-states-and-territories (accessed 20/06/2017

Giammarioli, M, La Rocca, SA, Steinbach, F, Casciari, C, De Mia, GM (2011) Genetic and antigenic typing of border disease virus (BDV) isolates from Italy reveals the existence of a novel BDV group. *Vet Microbiol* **147**, 231-6.

Givens, MD, Heath, AM, Brock, KV, Brodersen, BW, Carson, RL, Stringfellow, DA (2003) Detection of bovine viral diarrhea virus in semen obtained after inoculation of seronegative postpubertal bulls. *Am J Vet Res* **64**, 428-34.

Givens, MD, Riddell, KP, Edmondson, MA, Walz, PH, Gard, JA, Zhang, Y, Galik, PK, Brodersen, BW, Carson, RL, Stringfellow, DA (2009) Epidemiology of prolonged testicular infections with bovine viral diarrhea virus. *Vet Microbiol* **139**, 42-51.

Goyal, SM, Boujihad, M, Haugerud, S, Ridpath, JF (2002) Isolation of bovine viral diarrhea virus from an alpaca. *Journal of Veterinary Diagnostic Investigation* **14**, 523-525.

Grooms, DL (2004) Reproductive consequences of infection with bovine viral diarrhea virus. *Veterinary Clinics Food Animal Practice* **20**, 5-19.

Grubb, P (2005) Order Artiodactyla. In 'Mammal Species of the World.' (Ed. DER Wilson, D.M.) pp. 637-722. (The John Hopkins University Press: Baltimore)

Gunn, GJ, Stott, AW, Humphry, RW (2004) Modelling and costing BVD outbreaks in beef herds. *Vet J* **167**, 143-9.

Harasawa, R, Giangaspero, M, Ibata, G, Paton, DJ (2000) Giraffe strain of pestivirus: its taxonomic status based on the 5'-untranslated region. *Microbiol Immunol* **44**, 915-21.

Hewicker-Trautwein, M, Liess, B, Frey, HR, Trautwein, G (1994) Virological and pathological findings in sheep fetuses following experimental infection of pregnant ewes with cytopathogenic-bovine-virus diarrhoea virus. *Zentralbl Veterinarmed B* **41**, 264-76.

Hewicker-Trautwein, M, Trautwein, G, Frey, HR, Liess, B (1995) Variation in neuropathogenicity in sheep fetuses transplacentally infected with non-cytopathogenic and cytopathogenic biotypes of bovine-virus diarrhoea virus. *Zentralbl Veterinarmed B* **42**, 557-67.

Horner, GW, Orr, DM (1993) An enzyme-linked immunosorbent assay for detection of antibodies against bovine pestivirus. *N Z Vet J* **41**, 123-5.

Horner, GW, Tham, KM, Orr, D, Ralston, J, Rowe, S, Houghton, T (1995) Comparison of an antigen capture enzyme-linked assay with reverse transcription-polymerase chain reaction and cell-culture immunoperoxidase tests for the diagnosis of ruminant pestivirus infections. *Veterinary Microbiology* **43**, 75-84.

Houe, H (1993) Survivorship of animals persistently infected with bovine virus diarrhoea virus (BVDV). *Prev Vet Med* **15**, 275-283.

Houe, H (2003) Economic impact of BVDV infection in dairies. *Biologicals* **31**, 137-43.

Houe, H, Lindberg, A, Moennig, V (2006) Test strategies in bovine viral diarrhea virus control and eradication campaigns in Europe. *Journal of Veterinary Diagnostic Investigation* **18**, 427-436.

Hughes, LE, Kershaw, GF (1959) "B" of Border Disease. *Vet Rec* **71**, 313-317.

IDEXX (2010) IDEXX Laboratories Introduces the SNAP BVD Test for Bovine Viral Diarrhea Virus. Available at www.idexx.com (accessed 7/4/13 2013).

International Committee on Taxonomy of Viruses (2014) Virus Taxonomy: 2014 Release. Available at http://ictvonline.org/virustaxonomy.asp (accessed 8/4/2015

Jenvey, CJ, Weir, AM, Reichel, MP, Cockcroft, PD (2015) Performance characteristics of ELISA to detect bovine viral diarrhoea virus (BVDV) antibodies in colostrum. *Open Journal of Veterinary Medicine* **5**, 35-41.

Kahrs, R, K. (2001) 'Viral Diseaes of Cattle.' (Iowa State University Press: Ames, Iowa)

Kim, SG, Anderson, RR, Yu, JZ, Zylich, NC, Kinde, H, Carman, S, Bedenice, D, Dubovi, EJ (2009) Genotyping and phylogenetic analysis of bovine viral diarrhea virus isolates from BVDV infected alpacas in North America. *Veterinary Microbiology* **136**, 209-216.

Kim, SG, Dubovi, EJ (2003) A novel simple one-step single-tube RT-duplex PCR method with an internal control for detection of bovine viral diarrhoea virus in bulk milk, blood, and follicular fluid samples. *Biologicals* **31**, 103-6.

Kirkland, PD, Frost, MJ, Finlaison, DS, King, KR, Ridpath, JF, Gu, X (2007) Identification of a novel virus in pigs--Bungowannah virus: a possible new species of pestivirus. *Virus Res* **129**, 26-34.

Kirkland, PD, Mackintosh, SG (2006) Ruminant Pestivirus Infections. Australia and New Zealand Standard Diagnostic Procedures April 2006. Elizabeth Macarthur Agricultural Institute, Camden, New South Wales) 1-30.

Kirkland, PD, McGowan, MR, Mackintosh, SG, Moyle, A (1997) Insemination of cattle with semen from a bull transiently infected with pestivirus. *Veterinary Record* **140**, 124-127.

Kosinova, E, Psikal, I, Robesova, B, Kovarcik, K (2007) Reat-time PCR for quantification of bovine viral diarrhea virus RNA using SYBR Green I fluorimetry. *Veterinarni Medicina* **52**, 253-261.

Krametter-Froetscher, R, Benetka, V, Moestl, K, Baumgartner, W (2008) Transmission of Border Disease Virus from sheep to calves - a possible risk factor for the Austrian BVD eradication programme in cattle? *Wiener Tierarztliche Monatsschrift* **95**, 200-203.

Krametter-Froetscher, R, Duenser, M, Preyler, B, Theiner, A, Benetka, V, Moestl, K, Baumgartner, W (2010a) Pestivirus infection in sheep and goats in West Austria. *Vet J* **186**, 342-6.

Krametter-Froetscher, R, Mason, N, Roetzel, J, Benetka, V, Bago, Z, Moestl, K, Baumgartner, W (2010b) Effects of Border disease virus (genotype 3) naturally transmitted by persistently infected sheep to pregnant heifers and their progeny. *Veterinarni Medicina* **55**, 145-153.

Kramps, JA, van Maanen, C, van de Wetering, G, Stienstra, G, Quak, S, Brinkhof, J, Ronsholt, L, Nylin, B (1999) A simple, rapid and reliable enzyme-linked immunosorbent assay for the detection of bovine virus diarrhoea virus (BVDV) specific antibodies in cattle serum, plasma and bulk milk. *Vet Microbiol* **64**, 135-44.

Lanyon, SR, Anderson, ML, Bergman, E, Reichel, MP (2013) Validation and evaluation of a commercially available ELISA for the detection of antibodies specific to bovine viral diarrhoea virus (bovine pestivirus). *Aust Vet J* **91**, 52-6.

Lanyon, SR, Hill, FI, Reichel, MP, Brownlie, J (2014a) Bovine viral diarrhoea: Pathogensis and diagnosis. *The Veterinary Journal* **199**, 201-209.

Lanyon, SR, Sims, SK, Cockcroft, PD, Reichel, MP (2014b) Comparison of serum, ear notches, and nasal and saliva swabs for Bovine viral diarrhea virus antigen detection in colostrum-fed persistently infected (PI) calves and non-PI calves. *Journal of Veterinary Diagnostic Investigation* **26**, 783-787.

Lanyon.S.R., Rogers.J., Kessell.A., Reichel, MP (2012) Economic analysis of an acute outbreak of bovine viral diarrhoea virus (BVDV) in a South Australian dairy herd - a case study. *The Australian Cattle Veterinarian* **63**, 13-17.

Laureyns, J, Ribbens, S, de Kruif, A (2010) Control of bovine virus diarrhoea at the herd level: reducing the risk of false negatives in the detection of persistently infected cattle. *Vet J* **184**, 21-6.

Letellier, C, Kerkhofs, P, Wellemans, G, Vanopdenbosch, E (1999) Detection and genotyping of bovine diarrhea virus by reverse transcription-polymerase chain amplification of the 5' untranslated region. *Vet Microbiol* **64**, 155-67.

Li, W, Mao, L, Zhao, Y, Sun, Y, He, K, Jiang, J (2013) Detection of border disease virus (BDV) in goat herds suffering diarrhea in eastern China. *Virol J* **10**, 80.

Lim, CF, Carnegie, PR (1984) A survey of hairy shaker disease (border disease, hypomyelinogenesis congenita) in sheep. *Aust Vet J* **61**, 174-7.

Lindberg, A, Alenius, S (1999) Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. *Veterinary Microbiology* **64**,

Lindberg, A, Brownlie, J, Gunn, GJ, Houe, H, Moennig, V, Saatkamp, HW, Sandvik, T, Valle, PS (2006) The control of bovine viral diarrhoea virus in Europe: today and in the future. **25**, 961-979.

Lindberg, A, Houe, H (2005) Characteristics in the epidemiology of bovine viral diarrhea virus (BVDV) of relevance to control. *Prev Vet Med* **72**, 55-73; discussion 215-9.

Loken, T (1995) BORDER DISEASE IN SHEEP. *Veterinary Clinics of North America-Food Animal Practice* **11**, 579-&.

Loken, T, Bjerkas, I, Larsen, HJ (1991a) Experimental pestivirus infections in newborn goat kids. *Journal of Comparative Pathology* **103**, 277-88.

Loken, T, Krogsrud, J, Bjerkas, I (1991b) OUTBREAKS OF BORDER DISEASE IN GOATS INDUCED BY A PESTIVIRUS-CONTAMINATED ORF VACCINE, WITH VIRUS TRANSMISSION TO SHEEP AND CATTLE. *Journal of Comparative Pathology* **104**, 195-209.

Manktelow, BW, Porter, WL, Lewis, KH (1969) Hairy shaker disease of lambs. *N Z Vet J* **17**, 245-8.

Marco, I, Cabezon, O, Rosell, R, Fernandez-Sirera, L, Allepuz, A, Lavin, S (2011) Retrospective study of pestivirus infection in Pyrenean chamois (Rupicapra pyrenaica) and other ungulates in the Pyrenees (NE Spain). *Veterinary Microbiology* **149**, 17-22.

Martucciello, A, De Mia, GM, Giammarioli, M, De Donato, I, Iovane, G, Galiero, G (2009) Detection of Bovine viral diarrhea virus from three water buffalo fetuses (Bubalus bubalis) in southern Italy. *J Vet Diagn Invest* **21**, 137-40.

Mattson, DE, Baker, RJ, Catania, JE, Imbur, SR, Wellejus, KM, Bell, RB (2006) Persistent infection with bovine viral diarrhea virus in an alpaca. *Journal of the American Veterinary Medical Association* **228**, 1762-5.

McCormack, PE, St George-Grambauer, TD, Pulsford, MF (1959) Mucosal type disease of cattle in South Australia. *Aust Vet J* **35**, 482-488.

McFadden, AMJ, Tisdall, DJ, Hill, FI, Otterson, P, Pulford, D, Peake, J, Finnegan, CJ, La Rocca, SA, Kok-Mun, T, Weir, AM (2012) The first case of a bull persistently infected with Border disease virus in New Zealand. *New Zealand Veterinary Journal* **60**, 290-296.

Meyling, A, Houe, H, Jensen, AM (1990) Epidemiology of bovine virus diarrhoea virus. *Rev Sci Tech* **9**, 75-93.

Mignon, B, Waxweiler, S, Thiry, E, Boulanger, D, Dubuisson, J, Pastoret, PP (1992) Epidemiological evaluation of a monoclonal ELISA detecting bovine viral diarrhoea pestivirus antigens in field blood samples of persistently infected cattle. *J Virol Methods* **40**, 85-93.

Mingala, CN, Konnai, S, Tajima, M, Onuma, M, Ohashi, K (2009) Classification of new BVDV isolates from Philippine water buffalo using the viral E2 region. *J Basic Microbiol* **49**, 495-500.

MLA (2016) Fast Facts. Available at https://www.mla.com.au/prices-markets/Trends-analysis/fast-facts/ (accessed 21/05/17

Moerman, A, Straver, PJ, de Jong, MC, Quak, J, Baanvinger, T, van Oirschot, JT (1993) A long term epidemiological study of bovine viral diarrhoea infections in a large herd of dairy cattle. *Vet Rec* **132**, 622-6.

Nettleton, PF (1990) Pestivirus infections in ruminants other than cattle. *Rev Sci Tech* **9**, 131-50.

Nettleton, PF, Entrican, G (1995) Ruminant pestiviruses *British Veterinary Journal* **151**, 615-642.

Nettleton, PF, Gilmour, JS, Herring, JA, Sinclair, JA (1992) The production and survival of lambs persistently infected with border disease virus. *Comparative Immunology Microbiology and Infectious Diseases* **15**, 179-188.

Nettleton, PF, Gilray, JA, Russo, P, Dlissi, E (1998) Border disease in sheep and goats. *Veterinary Research* **29**, 327-340.

Niskanen, R, Alenius, S, Belak, K, Baule, C, Belak, S, Voges, H, Gustafsson, H (2002) Insemination of susceptible heifers with semen from a non-viraemic bull with persistent bovine virus diarrhoea virus infection localized in the testes. *Reprod Domest Anim* **37**, 171-5.

Niskanen, R, Emanuelson, U, Sundberg, J, Larsson, B, Alenius, S (1995) Effects of infection with bovine viral diarrhoea virus on health and reproductive-performance in 213 dairy herds in one county in Sweden. *Prev Vet Med* **23**, 229-237.

Niskanen, R, Lindberg, A (2003) Transmission of Bovine Viral Diarrhoea Virus by Unhygienic Vaccination Procedures, Ambient Air, and from Contaminated Pens. *The Veterinary Journal* **165**, 125-130.

Oguzoglu, TC (2012) A Review of Border Disease Virus Infection in Ruminants: Molecular Characterization, Pathogenesis, Diagnosis and Control. *Animal Health, Production and Hygiene* **1**, 1-9.

Olafson, P, Mac, CA, Fox, FH (1946) An apparently new transmissible disease of cattle. *Cornell Vet* **36**, 205-13.

Parsonson, IM, Ohalloran, ML, Zee, YC, Snowdon, WA (1979) Effects of bovine viral diarrhoea-mucosal disease (BVD) virus on the ovine fetus. *Veterinary Microbiology* **4**, 279-292.

Pople, AR, McLeod, SR (2010) Demography of feral camels in central Australia and its relevance to population control. *Rangeland Journal* **32**, 11-19.

Pratelli, A, Martella, V, Cirone, F, Buonavoglia, D, Elia, G, Tempesta, M, Buonavoglia, C (2001) Genomic characterisation of pestiviruses isolated from lambs and kids in southern Italy. *Journal of Virological Methods* **94**, 81-85.

Presi, P, Struchen, R, Knight-Jones, T, Scholl, S, Heim, D (2011) Bovine viral diarrhea (BVD) eradication in Switzerland-Experiences of the first two years. *Prev Vet Med* **99**, 112-121.

Reichel, MP, Hill, FI, Voges, H (2008) Does control of bovine viral diarrhoea infection make economic sense? *New Zealand Veterinary Journal* **56**, 60-66.

Ridpath, JF (2010) Bovine viral diarrhea virus: global status. *Vet Clin North Am Food Anim Pract* **26**, 105-21.

Ridpath, JF, Bolin, SR, Dubovi, EJ (1994) Segregation of bovine viral diarrhea virus into genotypes. *Virology* **205**, 66-74.

Ridpath, JF, Fulton, RW, Kirkland, PD, Neill, JD (2010) Prevalence and antigenic differences observed between Bovine viral diarrhea virus subgenotypes isolated from cattle in Australia and feedlots in the southwestern United States. *Journal of Veterinary Diagnostic Investigation* **22**, 184-191.

Ridpath, JF, Neill, JD, Frey, M, Landgraf, JG (2000) Phylogenetic, antigenic and clinical characterization of type 2 BVDV from North America. *Vet Microbiol* **77**, 145-55.

Saah, AJ, Hoover, DR (1997) "Sensitivity" and "specificity" reconsidered: the meaning of these terms in analytical and diagnostic settings. *Ann Intern Med* **126**, 91-4.

Saliki, JT, Dubovi, EJ (2004) Laboratory diagnosis of bovine viral diarrhea virus infections. *Vet Clin North Am Food Anim Pract* **20**, 69-83.

Sandvik, T, Krogsrud, J (1995) Evaluation of an antigen-capture ELISA for detection of bovine viral diarrhea virus in cattle blood samples. *J Vet Diagn Invest* **7**, 65-71.

Scherer, CFC, Flores, EF, Weiblen, R, Caron.L., Irigoyen.L.F., Neves.J.P., Maciel.M.N. (2001) Experimental infection of pregnant ewes with bovine viral diarrhoea virus type-2 (BVDV-2): effects on the pregnancy and fetus. *Veterinary Microbiology* **79**, 285-299.

Schweizer, M, Peterhans, E (2014) Pestiviruses. *Annual Review of Animial Biosciences* **2**, 141-163.

Shannon, AD, Richards, SG, Kirkland, PD, Moyle, A (1991) An antigen-capture ELISA detects pestivirus antigens in blood and tissues of immunotolerant carrier cattle *Journal of Virological Methods* **34**, 1-12.

Sharp, MW, Rawson, BC (1986) The cost of Border disease infection in a commercial flock. *Vet Rec* **119**, 128-30.

Snowdon, WA, Parsonson, IM, Broun, ML (1975) Reaction of pregnant ewes to innoculation with mucosal disease virus of bovine origin. *Journal of Comparative Pathology* **85**, 241-251.

St George, TD (1971) A survey of sheep throughout Australia for antibody to parainfluenza type 3 virus and to mucosal disease virus. *Aust Vet J* **47**, 370-4.

St George, TD, Snowdon, WA, Parsonson, IM, French, EL (1967a) A serological survey of mucosal desease and infectious bovine rhinotracheitis in cattle in Australia and New Guinea. *Aust Vet J* **43**, 549-557.

St George, TD, Snowdon, WA, Parsonson, IM, French, EL (1967b) A serological survey of mucosal disease and infectious bovine rhinotracheitis in cattle in Australia and New Guinea. *Aust Vet J* **43**, 549-57.

Stahl, K, Alenius, S (2012) BVDV control and eradication in Europe--an update. *Jpn J Vet Res* **60 Suppl**, S31-9.

Stahl, K, Beer, M, Schirrmeier, H, Hoffmann, B, Belak, S, Alenius, S (2010) Atypical 'HoBi'-like pestiviruses--recent findings and implications thereof. *Vet Microbiol* **142**, 90-3.

Stalder, HP, Meier, P, Pfaffen, G, Wageck-Canal, C, Rufenacht, J, Schaller, P, Bachofen, C, Marti, S, Vogt, HR, Peterhans, E (2005) Genetic heterogeneity of pestiviruses of ruminants in Switzerland. *Prev Vet Med* **72**, 37-41; discussion 215-9.

Strong, R, La Rocca, SA, Ibata, G, Sandvik, T (2010) Antigenic and genetic characterisation of border disease viruses isolated from UK cattle. *Vet Microbiol* **141**, 208-15.

Taylor, L (2010) Findings of an Australia wide serological survey of beef and dairy herds for Bovine Viral Diarrhoea Virus conducted between 2007 and 2009. The Australian Cattle Veterinarian. 57: 14-19.

Taylor, LF, Black, PF, Pitt, DJ, Mackenzie, AR, Johnson, SJ, Rodwell, BJ (2006) A serological study of bovine pestivirus in Queensland beef and dairy herds conducted in 1994/95. *Aust Vet J* **84**, 163-168.

Thabti, F, Letellier, C, Hammami, S, Pepin, M, Ribiere, M, Mesplede, A, Kerkhofs, P, Russo, P (2005) Detection of a novel border disease virus subgroup in Tunisian sheep. *Arch Virol* **150**, 215-29.

Toplu, N, Oguzoglu, TC, Epikmen, ET, Aydogan, A (2011) Neuropathologic study of border disease virus in naturally infected fetal and neonatal small ruminants and its association with apoptosis. *Vet Pathol* **48**, 576-83.

Uttenthal, A, Grondahl, C, Hoyer, MJ, Houe, H, van Maanen, C, Rasmussen, TB, Larsen, LE (2005) Persistent BVDV infection in mousedeer infects calves. Do we know the reservoirs for BVDV? *Prev Vet Med* **72**, 87-91; discussion 215-9.

Valdazo-Gonzalez, B, Alvarez-Martinez, M, Greiser-Wilke, I (2006) Genetic typing and prevalence of Border disease virus (BDV) in small ruminant flocks in Spain. *Veterinary Microbiology* **117**, 141-153.

van Amstel, S, Kennedy, M (2010) Bovine viral diarrhea infections in new world camelids-A review. *Small Ruminant Research* **91**, 121-126.

Van Campen, H, Vorpahl, P, Huzurbazar, S, Edwards, J, Cavender, J (2000) A case report: evidence for type 2 bovine viral diarrhea virus (BVDV)-associated disease in beef herds vaccinated with a modified-live type 1 BVDV vaccine. *J Vet Diagn Invest* **12**, 263-5.

Vilcek, S, Belak, S (1996) Genetic identification of pestivirus strain Frijters as a border disease virus from pigs. *Journal of Virological Methods* **60**, 103-108.

Vilcek, S, Ridpath, JF, Van Campen, H, Cavender, JL, Warg, J (2005) Characterization of a novel pestivirus originating from a pronghorn antelope. *Virus Res* **108**, 187-93.

Waage, S (2000) Influence of new infection with bovine virus diarrhoea virus on udder health in Norwegian dairy cows. *Prev Vet Med* **43**, 123-135.

Wellenberg, GJ, van der Poel, WHM, Van Oirschot, JT (2002) Viral infections and bovine mastitis: a review. *Veterinary Microbiology* **88**, 27-45.

Wentz, PA, Belknap, EB, Brock, KV, Collins, JK, Pugh, DG (2003a) Evaluation of bovine viral diarrhea virus in New World camelids. *Journal of the American Veterinary Medical Association* **223**, 223-228.

Wentz, PA, Belknap, EB, Brock, KV, Collins, JK, Pugh, DG (2003b) Evaluation of bovine viral diarrhea virus in New World camelids. *Journal of the American Veterinary Medical Association* **223**, 223-8.

Young, NJ, Thomas, CJ, Collins, ME, Brownlie, J (2006) Real-time RT-PCR detection of Bovine Viral Diarrhoea virus in whole blood using an external RNA reference. *J Virol Methods* **138**, 218-22.

Zimmer, GM, Van Maanen, C, De Goey, I, Brinkhof, J, Wentink, GH (2004) The effect of maternal antibodies on the detection of bovine virus diarrhoea virus in peripheral blood samples. *Veterinary Microbiology* **100**, 145-149.