Infectivity of pestivirus following persistence of acute infection

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ABSTRACT

Bovine viral diarrhoea virus (BVDV) is an endemic pathogen worldwide and eradication strategies focus on the identification and removal of persistently infected (PI) animals arising after *in utero* infection. Despite this, acute infections with BVDV can persist for months or years after the removal of the PI source despite repeated screening for PIs and tight biosecurity measures. Recent evidence for a prolonged duration of viraemia in the testicles of bulls following acute BVDV infection suggests the possibility of a form of chronic persistence that may more closely resemble the persistence strategies of hepatitis C virus (HCV). To investigate the potential for virus transmission from infected and recovered cattle to virus naïve hosts we established an acute infection of 5 BVDV-naïve calves and monitored animals over 129 days. Infectious BVDV was detected in white blood cells between days 3 and 7 post-challenge. The animals seroconverted by day 21 post-infection and subsequently were apparently immune and free from infectious virus and viral antigen.

Animals were further monitored and purified white blood cells were stimulated *in vitro* with phytohaemagglutinin A (PHA) during which time BVDV RNA was detected intermittently.

Ninety-eight days following challenge, blood was transferred from these apparently virus-free and actively immune animals to a further group of 5 BVDV-naïve calves and transmission of infection was achieved. This indicates that BVDV-infected, recovered and immune animals have the potential to remain infectious for BVDV-naïve cohorts for longer than previously demonstrated.

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1. Introduction

As members of the Flaviviridae, pestiviruses are enveloped RNA viruses with a positive sense 12.5 kb genome encoding 11 or 12 proteins (Collett et al., 1988; Elbers et al., 1996) with related viruses infecting cattle, sheep and pigs. The type member of this genus is bovine viral diarrhoea virus (BVDV). The similarities between BVDV and human hepatitis C virus (HCV) are in many ways greater than their relationship to other members of the Flaviviridae as both have similar genome structure and organisation, similarities in their replication strategies and the ability to cause long-term infection of their hosts. These similarities have led to many studies using BVDV as a model for HCV infection.

BVDV is endemic in most countries and the consequences of infection in terms of the economic impact and animal welfare are highly significant (Houe, 2003). The observed range of clinical outcomes largely depends upon the characteristics of the infecting virus (Bolin and Ridpath, 1992; Brownlie, 1991) and consequences of infection include decreased milk yield, poor reproductive performance, loss of condition and high levels of recurrent infection due to the immunosuppressive effects of BVDV-associated leucopenia.

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Haemorrhagic strains of BVDV, which are endemic throughout North America and Canada and increasingly apparent in mainland Europe (Vilcek et al., 2005) also cause high mortality and severe loss of condition during the acute infection (Brownlie, 1991; Pellerin et al., 1994; Ridpath et al., 2000). As the animal welfare and economic implications of unchecked infection become apparent, many countries (including Austria, Denmark, Finland, Germany, Norway, Sweden) are undertaking the national eradication of BVDV.

The typical course of acute infection of postnatal animals (cattle or sheep) has been well defined experimentally (Howard, 1990) identifying a period of viraemia between 4 and 10 days post-infection during which infectious BVDV can be detected in serum, blood, buffy coat cells and nasal secretions.

A transient pyrexia and a significant leucopenia are seen between days 3 and 10 post-infection. Seroconversion can be detected, rarely at day 7 but more usually by day 14. As active immunity develops virus spread is eliminated and infectious virus is rarely detected in buffy coat cells or nasal secretions beyond 14 days. In this respect, acute BVDV infection seemed to be resolved much more readily than HCV infection which is known to persist in a significant number of infected individuals (Tester et al., 2005; Thimme et al., 2001). The use of more sensitive detection techniques such as RT-PCR can extend the period when BVDV RNA can be detected but isolation of infectious virus is generally not possible beyond 14–21 days post-infection as the animal develops significant levels of antiviral neutralising antibodies which continue to increase in titre beyond 40–50 days post-infection.

A further consequence of acute infection with BVDV occurs when the period of active viraemia occurs during the first trimester of pregnancy. In these circumstances, virus can cross the placenta and establish extensive viraemia within the immuno-incompetent foetus. Many such infections result in abortion but up to 60% will survive to produce a calf which is persistently infected (PI), immunotolerant to the virus and will actively shed virus throughout its life (Brownlie, 1990).

To date, the persistently infected, immunotolerant animal has been considered to be the major source of BVDV spread and eradication schemes have focussed on the identification and culling of such animals (Houe, 1999). However, many units have experienced great difficulty in eliminating the circulation of BVDV even in the absence of PI animals and the presence of tight biosecurity measures (Houe, 1999).

These observations have prompted attempts to identify a previously unrecognised form of viral persistence in acutely infected and antibody positive animals. A number of authors (including Ssentongo et al., 1980; Grooms et al., 1998; Niskanen et al., 2002; Givens et al., 2003) have reported detection of BVDV in reproductive tissues (ovary, testis or semen) of cattle at prolonged times following initial acute infection: in ovarian tissue 60 days following challenge (Grooms et al., 1998) and in semen up to 7 months after initial infection with a live BVDV vaccine. These authors (Givens et al., 2003) were unable to culture virus directly from semen but could detect viral RNA by nested RT-PCR and did transmit virus to one calf of three following intravenous injection of semen collected at 5 months post-infection.

With the exclusion of the known incidents of BVDV transmission in virus-infected semen, it was not clear whether these animals with long-term persistence of viral antigen in their ovaries or testes were infectious for BVDV-naïve herd mates. To our knowledge, there is no published report of BVDV immune animals transmitting infectious virus except through semen. The reports of long-term persistence of viral antigen in ovaries or detection or viral RNA in white blood cells (unpublished observations) cannot yet be equated with infectivity and the status of these animals is unresolved.

Similar questions are raised for HCV. Recent publications of Pham et al. (2005, 2004) which confirm that for HCV, *ex vivo* stimulation of PBMCs with mitogens and cytokines will stimulate the previously undetectable replication of viral RNA in both lymphocytes and dendritic cells of infected patients. Questions have been raised as to whether such cases represent the detection of "occult RNA" or whether there is the real potential for virus infectivity and transmission in these samples (Quiroga and Carreno, 2005; Quiroga et al., 2006). A recent paper by Gogorza et al. (2005) has also indicated that *ex vivo* stimulation of PBMCs from cattle in a number of BVDV antibody positive herds was successful in producing antigen-positive samples and infectious virus from a small number of individual cattle but in this field situation it was not possible to comment on the infection history of these individuals within the herds. Although antibody levels could be measured, it was impossible to know when the individuals had been exposed to virus or the duration of persistence and again, the potential for spread of infectious virus was not assessed.

In the light of these observations a review of the pathogenesis of acute BVDV infections is essential as the virus-free status of antibody positive, immune animals can no longer be assumed. Important questions remain:

- does infectious virus persist more widely in recovered, immune and apparently virus-free cattle?
- can this virus be transmitted to BVDV-naïve herdmates?

To this end, we undertook an experimental study of the longer term detection of BVDV following acute infection and the potential for virus transmission from immune animals.

In the following paper we describe the infection of BVDV-naïve calves with a noncytopathic strain of BVDV and attempts to transfer infection from recovered animals to further BVDV-naïve cattle. The hypothesis underlying this study is that BVDV can persist, long-term, following acute infection of the postnatal immunocompetent animal and is transmissible to BVDV-naïve herdmates.

2. Materials and methods

2.1. Study design

Five BVDV antibody- and antigen-free calves (numbers 321, 330, 339, 345 and 360: study group A), of approximately 3 months of age sourced from a BVDV-free herd,
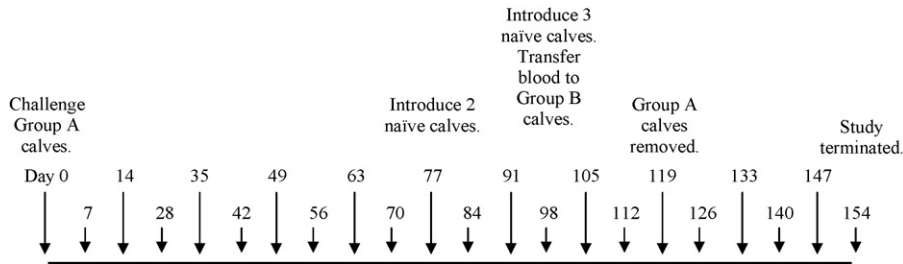
Fig. 1. Summary of the sampling schedules for the study.

were challenged intranasally with 10 ml of isolate $456497 ($5 \times 10^6$ TCID$_{50}$), a type 1a BVDVnc identified as a more clinically virulent field isolate (T. Drew, VLA, personal communication). This infectious dose has been shown to be effective in the previous studies (Brownlie et al., 1995). See Fig. 1 for summary of experimental outline. Blood samples (plain clotted and EDTA) were taken by jugular venepuncture at regular intervals (on days −2, −1, 0, 4, 5, 6, 7, 8, 11, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91, 98, 105, 112, 119, 126, 133, 140, 147 and 154 where day 0 is the day of virus challenge).

At day 77 two additional BVDV-naïve calves (399 and 408) were introduced to the group to ascertain the potential for horizontal transmission of viral infection and were also sampled on study days 84, 91 and 98 together with the original calves.

On study day 98 a further 3 BVDV-naïve calves were introduced to the group (467, 469 and 473). At this stage, 20 ml blood from the original group of 5 calves (study group A: numbers 321, 330, 339, 345 and 360) was injected, 10 ml intravenously and 10 ml subcutaneously, into the younger animals (399, 408, 467, 469 and 473: study group B). Sampling of all calves continued on study days 112, 119 and 126 after which time the older, study group A calves were removed from the study. The remaining study group B calves were sampled again on study days 133, 140, 147 and 154 when the study ended.

2.2. Virus isolation

Following purification of the buffy coat cells from EDTA blood by ammonium chloride lysis, virus was isolated by co-culture of white blood cells (WBCs) with uninfected foetal bovine lung (FBL) cells followed by immunodetection of the virus as described previously (Nobiron et al., 2003).

2.3. BVDV antibody ELISA

A BVDV antibody ELISA was performed using prepared antigen from cells infected with the cytopathic BVDV strain NADL (also using mock-infected cells as a negative control) as previously described (Howard et al., 1985).

2.4. NS3 indirect antibody ELISA

The NS3 antigen used in this assay (Young et al., 2005) was captured from a lysate obtained from NADL infected FBL cells using the monoclonal antibody WB112 (Veter-

inary Laboratories Agency) diluted 1:100 in carbonate coating buffer (Sigma). Uninfected cells were also prepared as a negative background control. The ELISA plate was blocked with 5% NPS before positive and negative antigen was added. Test sera were added, diluted 1:50 and binding detected using biotinylated monoclonal anti-bovine IgG clone BG18 (Sigma) followed by incubation with Streptavidin–horseradish peroxidase conjugate (Amersham Biosciences). The ELISA was developed with OPD substrate (Sigma) and optical density measured using an Optimax microplate reader (Molecular Diagnostics) at 490 nm using SOFTmax®PRO version 5.0.

2.5. E2 antibody ELISA

The E2 antigen for the detection of anti-E2 antibodies was recombinant his-tagged protein expressed from a baculovirus system (Nobiron et al., 2003) and diluted in carbonate buffer. Plates were washed and blocked with 5% NPS. Test sera were added and binding of bovine IgG was detected as above.

2.6. Ex vivo stimulation of PBMCs

PBMCs were isolated from EDTA blood (5 ml) by density gradient centrifugation using Histopaque1083. Leucocytes were removed from the top of the Histopaque cushion and washed in 20 ml of ice cold PBS. Any remaining red blood cells were lysed by briefly resuspending the pellet in 2 ml of sterile ultrapure water before adding 20 ml of PBS and washing twice in PBS. The cells were finally resuspended in 10 ml of pre-warmed RPMI-1640 medium (Sigma) containing 10% FCS, glutamine 200 mM, penicillin 100 IU/ml and streptomycin 100 μg/ml with phytohaemagglutinin (PHA, Sigma L8754, 5 μg/ml) for 72 h. After stimulation, PBMCs were pelleted by centrifugation at $400 \times g$, 4 °C for 10 min before RNA extraction.

2.7. RNA extraction, RT-PCR and sequencing

Total RNA was extracted from potentially virus-infected cells using 0.8 ml of RNA Stat-60 (AMS Biotechnology Ltd., UK) according to the manufacturer's instructions. RNA was precipitated from the aqueous phase with 0.5 ml of isopropanol and 1 μl of glycogen (20 mg/ml, Roche Diagnostics, USA) at 4 °C for 30 min and then collected by centrifugation. The RNA pellet was washed in 1 ml of 75% ethanol and resuspended in 50 μl of diethylpyrocarbonate (DEPC)-treated ultrapure water.

RNA was reverse transcribed (RT) to cDNA with random hexamer primers (50 ng/µl, Amersham Biosciences, UK) and Superscript II, reverse transcriptase (200 units/µl, Invitrogen) according to the manufacturer's instructions. The reactions were held at 42 °C for 50 min and at 70 °C for a final 15 min.

cDNA (5 µl) generated by RT was amplified by PCR 0.5 µl of *Pfu* polymerase (5 units/µl, Promega), 2.5 µl of 10× buffer (Promega), 1 µl 10 mM dNTPs, using 2.5 µl primer 324 and 2.5 µl primer 326 (Vilcek, 1994), both at 10 pmol/ml, in a final volume of 20 µl with sterile ultrapure water. These primer sequences are highly conserved in different BVDV isolates and amplify a 288-bp fragment of the 5′ untranslated region of the BVDV genome. This fragment was sequenced using Cy-5 labelled primers and a Thermosequenase kit on an Amersham Biosciences ALF Express DNA analyser.

A negative control was provided by a 'no template' reaction, with sterile ultrapure water added. For positive controls cDNA generated from foetal bovine lung (FBL) cells infected with BVDV isolate Ky1203nc was used.

3. Results

3.1. Virus challenge

Back titration of the type 1a BVDVnc isolate \$456497 challenge inoculum confirmed that all study group A calves (numbers 321, 330, 339, 345 and 360) received a high infectious dose of approximately 5×10^6 TCID$_{50}$.

Clinical responses to virus challenge were recorded from days −1 to 12. All calves experienced some degree of pyrexia, peaking around day 8 or 9 post-infection and leucopenia between days 3 and 14 (data not shown). Both of these are typical signs of BVDV infection. Additionally, the calves were observed for clinical signs of disease. Occasional coughing was noted, but calves appeared healthy in all other respects.

3.2. Seroconversion

A total anti-BVDV antibody ELISA was performed to detect BVDV-specific (total IgG) antibodies in the serum. The results (Fig. 2) indicate that by day 21 post-challenge all calves had seroconverted to BVDV, showing a positive ELISA result. Antibody titres continued to rise from day 21, and for 4 animals were still increasing in titre up to day 105 post-challenge. The group A calves were removed from the study at day 129 post-challenge at which time all five showed antibody titres that would indicate a high degree of protection to infection with BVDV.

3.3. Virus isolation

Infectious virus was isolated from PBMCs by co-culture with FBL cells and detected by immunostaining. The results (Table 1) indicate that infectious BVDV was isolated from every calf between days 3 and 7, with some individual variation in the timing of viraemia. All animals were apparently virus-free by standard virus isolation techniques by day 10 post-challenge and evidence for the onset
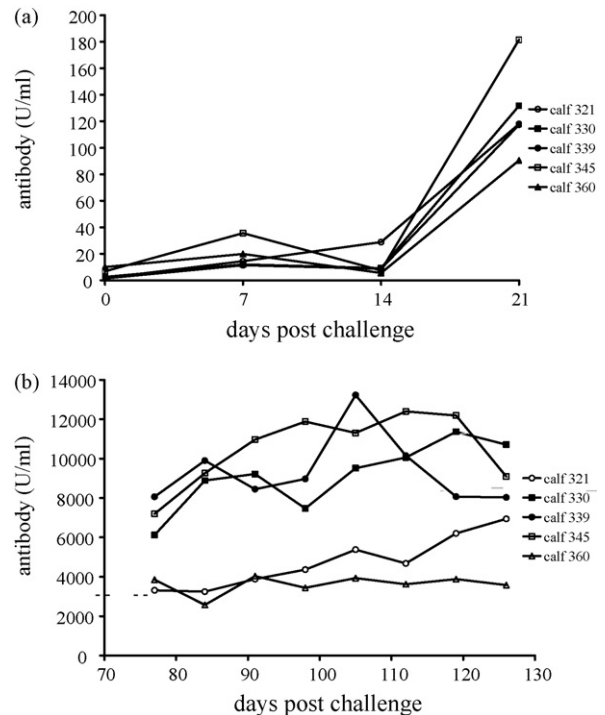

Fig. 2. Seroconversion following challenge of group A calves. Serum antibody titres to total BVDV antigen were measured in all 5 calves using an antibody capture ELISA following challenge with BVDV isolate \$456497. A standard curve using a reference BVDV hyperimmune serum allowed the results to be expressed as units/ml (Howard et al., 1985). Clear evidence of seroconversion (90–180 antibody units) was seen by day 21 post-infection (panel a) and titres continued to rise in all animals (4500–44,000 antibody units) at least up to day 105 post-challenge (panel b).

of seroconversion by day 14. No infectious virus was isolated directly from purified PBMCs after day 10 post-infection.

3.4. Stimulation of PBMCs using PHA and viral RNA detection

At days 28, 42, 56, 63, 70, 77, 84, 91 and 98 post-challenge PBMCs purified from the five, study group A calves were stimulated by incubation with the mitogen PHA before cells were used for conventional virus isolation or for RNA extraction followed by RT-PCR.

Between days 28 and 98 post-infection viral RNA was detected in PBMCs on a number of occasions in each animal (Table 2). This was evident in unstimulated PBMCs in 16 separate samples and following stimulation of PBMCs with PHA for 72 h, viral RNA was detected in 26 different samples indicating that stimulation did increase the frequency of RNA detection. No infectious virus was recovered from any of these samples following immunostaining using standard protocols (data not shown).

3.5. Virus transmission

To assess the potential for horizontal transmission of BVDV, on day 77, BVDV-naïve calves 399 and 408 were introduced into the existing group and monitored weekly

Table 1

BVDV virus isolation from white blood cells between study days 0 and 21. The buffy coat cell population from all calves challenged with BVDV isolate $456497 was purified from whole blood as described and the cell lysate co-cultured with foetal bovine lung cells for two passages before virus antigen was detected using a standard immunofluorescence assay. Unambiguous staining for viral antigen was seen as early as day 3 post-challenge in calves 339 and 360 and all 5 calves were virus positive on day 5. By day 10 post-infection all calves were definitely virus negative in this assay.

Study day	Calf number				
	321	330	339	345	360
0	–	–	–	–	–
3	–	–	+	–	+
5	+	+	+	+	+
6	+	+	+	–	+
7	–	+	+	+	+
10	–	–	–	–	–
14	–	–	–	–	–
21	–	–	–	–	–

for any signs of virus transmission. During a 3-week period the new animals were consistently virus negative by virus isolation and RT-PCR and did not seroconvert to BVDV (data not shown).

On study day 98, a further 3 BVDV-naïve calves (467, 469 and 473), were introduced to the group and exchange of blood from study group A (numbers 321, 330, 339, 345 and 360) to study group B (399, 408, 467, 469 and 473) was undertaken. Both groups were subjected to continued monitoring for virus isolation, RT-PCR and seroconversion after sampling at weekly intervals.

During the next 28 days, animals in study group A were consistently negative for virus isolation and continued to demonstrate high levels of circulating anti-BVDV antibodies (Fig. 2b). Study group A animals were removed from the study on day 129, 4 weeks after the transmission of blood to study group B animals.

Animals in study group B were observed to seroconvert to BVDV, beginning at day 14 post-blood transmission (animal 408) and all group B animals were seropositive in the standard BVDV antigen ELISA by study day 140, 42 days following blood exchange between group A and group B animals (Fig. 3). To ensure that this was a result of active

Table 2

Detection of virus specific RNA by RT-PCR in PBMCs of study group A animals.

Days post-infection	Animal number									
	321		330		339		345		360	
	NS	S	NS	S	NS	S	NS	S	NS	S
28	+	+	+	+	+	+	–	+	–	+
42	–	–	–	–	–	+	–	+	–	+
56	+	+	+	+	+	+	+	+	+	+
63	+	+	–	+	+	+	+	+	+	+
70	–	–	–	–	–	–	–	–	–	–
77	–	–	–	–	–	–	–	–	–	+
84	–	–	–	–	–	–	–	–	–	–
91	+	+	–	+	–	+	+	+	+	+
98	–	–	–	+	–	–	–	+	+	–

NS = nonstimulated PBMCs; S = PHA stimulated PBMCs; + = RNA detected by RT-PCR; − = RNA not detected by RT-PCR.
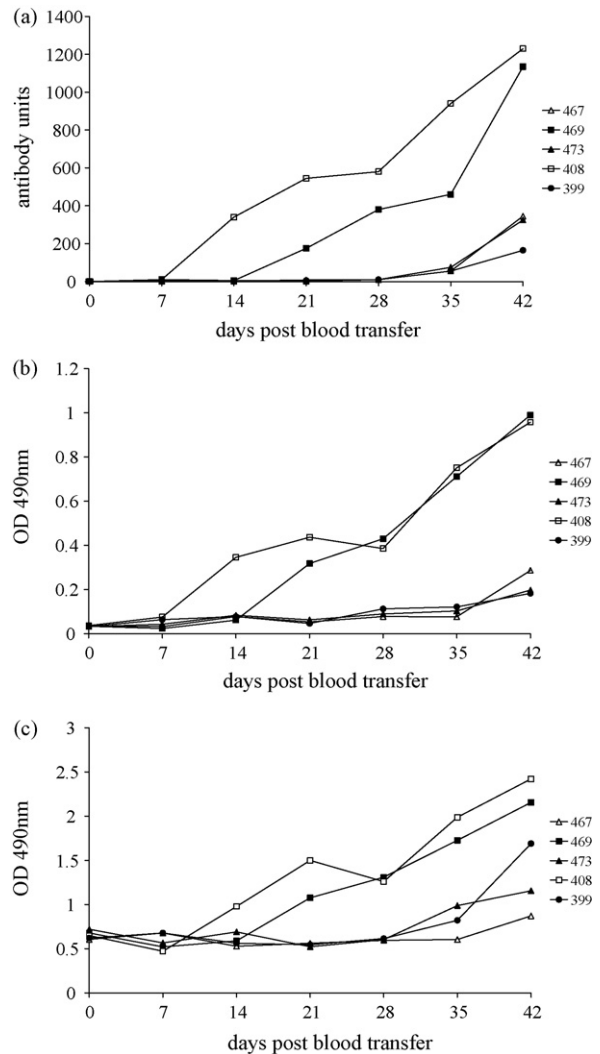
Fig. 3. Seroconversion of study group B animals following transfer of blood.

(a) Total anti-BVDV antibody. Early seroconversion is noted in calf 408 by day 14 post-transfer with all animals becoming seropositive (over 100 units) by day 42.

(b) Anti-E2 antibody. Using an ELISA to purified recombinant E2, the major structural glycoprotein, again animal 408 becomes seropositive by day 14 post-transfer with other animals seroconverting later.

(c) Anti-NS3 antibody. Using an ELISA to purified recombinant NS3, four animals are clearly seropositive by day 42 post-transfer while anti-NS3 titres in animal 467 are still increasing.

virus replication in group B animals and not the passive transfer of antigen, seroconversion to recombinant BVDV E2 glycoprotein and to the BVDV non-structural protein NS3 were measured independently and antibody levels increased in all animals over the 42-day period of observation (Fig. 3).

Viral RNA was detected in purified PBMCs of animal 469 on day 7 post-transmission, with other group B animals, except 408, being virus RNA positive up to 21 days post-infection (Table 3). Sequence analysis of the RT-PCR product (corresponding to the viral 5′ untranslated region), indicated that the recovered virus from 469 was

Table 3

Detection of virus specific RNA by RT-PCR in PBMCs of study group B animals.

Days post-blood transmission	Animal number									
	399		408		467		469		473	
	NS	S	NS	S	NS	S	NS	S	NS	S
7	−	−	−	−	−	−	−	−	+	−
14	−	−	−	−	−	−	−	−	−	−
21	+	−	−	−	+	−	−	+	+	+

NS = nonstimulated PBMCs; S = PHA stimulated PBMCs; + = RNA detected by RT-PCR; − = RNA not detected by RT-PCR.

identical to that of the original challenge virus over the range of bases sequenced (data not shown). Only animal 408 appeared to be virus-free by these methods but did show the strongest seroconversion to BVDV in all three ELISA assays (for total anti-BVDV, anti-E2 and anti-NS3 antibody).

4. Discussion

The hypothesis underlying this study was that BVDV can persist, long-term, following acute infection of the immunocompetent animal and be transferred to BVDV-naïve herdmates. That BVDV has the ability to cause persistent infection, a lifelong viraemia following *in utero* infection of the developing foetus, is well-established. In contrast, BVDV infection of the postnatal animal has been regarded as an acute event, being cleared from the blood within just 14 days. This clearance of viraemia has been assumed to represent clearance of infection, with seroconversion indicating long-term protection from disease. However, the results from this study have satisfied the hypothesis, showing for the first time that BVDV can be transmitted to BVDV-naïve hosts by transfer of blood 98 days after the initial challenge event.

Relatively few published studies of acute BVDV infection have followed the time course of infection beyond days 21–28 post-challenge as all the available evidence suggested that virus had been eliminated before this time. However, it has now been shown that the RNA of BVDV can be detected in PBMCs for prolonged periods when infectious virus cannot be recovered. Although unexpected, this parallels the situation where HCV RNA can be carried in the PBMCs for at least 5 years after spontaneous or treatment-induced resolution of disease (Pham et al., 2004) and again highlights similarities between these two viruses. Another study was able to detect BVDV antigen in PBMCs of seropositive cattle and to recover infectious virus *in vitro* following mitogenic stimulation *ex vivo* (Gogorza et al., 2005). As cattle in this study were in a field situation it was impossible to estimate the original time of exposure of the cattle to live virus or draw conclusions about the duration of persistence of BVDV infection in these animals.

These observations support reports of the detection of persistent viral antigen in reproductive tissues following acute BVDV infection (Ssentongo et al., 1980; Grooms et al., 1998; Givens et al., 2003; Niskanen et al., 2002). The unanswered question remains as to whether such animals are infectious for herdmates by other methods than transfer of semen.

In other systems, longer term persistence of respiratory syncytial virus antigen has been associated with hyper-responsiveness and inflammation (Bramley et al., 1999) which exacerbates the disease state. In contrast most cases of acute BVDV infection seem to be resolved with few serious clinical consequences for the individual although the consequences for both the herd and for the business may often be underestimated. It is many years since long-term circulation of parainfluenza virus infection was first described in completely enclosed communities at the South Pole (Muchmore et al., 1981). This persistence of acute infection in the absence of re-introduction of additional sources of infection provides precedent for our investigation. The defects in protective immunity and the pathogenesis strategies of viruses for immuno-evasion and immuno-modulation which allow virus persistence are still only partly described.

The current study provided a more extensive time course following BVDV infection.

We were unable to demonstrate horizontal transmission of the virus to BVDV-naïve calves housed for 3 weeks with the seropositive, infectious virus negative but RNA positive study group A animals in agreement with other studies describing poor transmission from acutely infected animals. Direct contact with an immunotolerant persistently infected animal has been shown to be a more efficient means of infection although even then, less so than the consistently high transmission of virus achieved when subcutaneous injection occurs transferring blood or serum or on needles used with shared BVDV contaminated vials, even through use of contaminated rectal examination gloves (Cook et al., 1990; Lang-Ree et al., 1994; Niskanen and Lindberg, 2003). Similarly in the current study, transfer of 20 ml of blood from the apparently virus-free and immune group A calves to 5 BVDV-naïve group B calves resulted in the effective transmission of virus to the new hosts. Seroconversion was, relatively speaking, a slower and less synchronous event than normal, perhaps reflecting the fact that variable amounts of virus were transmitted in each inoculum as has been reported previously when different sources of virus and differing routes of transmission were compared (Cook et al., 1990). While blood transfer was an "artificial" procedure, it does genuinely represent the possibility of exchange of blood or fluids through biting flies (Tarry et al., 1991) or other veterinary intervention (Lang-Ree et al., 1994; Niskanen and Lindberg, 2003).

We cannot absolutely determine whether virus spread to all of the group B calves is as a result of transmission through blood or horizontal transmission within the group. Calves 408 and 469 seroconverted rapidly (days 14 and 21) suggesting that these animals were infected initially. As animal 473 was RT-PCR positive by day 7 post-blood transfer (7 days is not usually regarded as sufficient time for seroconversion following horizontal transmission from calves 408 and 469) this suggests that three of the 5 calves were directly infected by transmission via blood transfer. Calves 467 and 473 were both RNA positive by day 21 post-transmission but showed a

slower seroconversion, 42 days post-blood transfer and it is impossible to assess whether their infection arose following blood transfer or horizontal transmission from other calves in the group in the current study.

It is certain that transmission from BVDV-infected, seropositive and apparently virus-free cows did occur, probably 98 days after the original infection. It is also possible that in the natural farm environment, stress or concurrent infection may act as a stimulus for virus recrudescence and increase the levels of viraemia in seropositive, recovered cattle making transmission to BVDV-naïve herdmates more likely, a possibility which is worthy of future study.

The significance of these results is that long-term persistence of infectious BVDV following acute viraemia has the potential to hinder BVDV control efforts worldwide and would constitute a mechanism whereby BVDV is maintained within a population over a long period of time, despite the removal of all PI animals. Animals known to have seroconverted to the virus following natural infection, and hence assumed protected could instead act as long-term virus carriers. Replication during this period may be continuous, spontaneous, or it may require a stimulus, such as stress caused by crowding, intercurrent disease or transportation perhaps explaining the role of BVDV in shipping fever (Fulton et al., 2000). In the current study, young calves were challenged with BVDV. A possibility for the prolonged residence of the virus in the young/neonate animal is that the immune response may not be sufficiently mature to remove all virus (Pollock et al., 1991). Against this proposal is the observation that foetuses in late gestation (the last trimester) are able to clear the virus and make an active immune response; such calves have never, to our knowledge, been examined for 'occult' virus. If young calves, however, were found to be more liable to a persistent acute infection, it could have implications for the use of modified–live virus vaccines.

As the group A animals had high titres of circulating antibody at the time of blood exchange it remains a possibility that vaccination against BVDV may not be sufficient to overcome pre-existing persistence of the virus in animals that had already been naturally infected. This observation highlights the importance of being able to readily discriminate between naturally infected and vaccinated cattle.

Further studies are required to elucidate the frequency and duration of long-term persistence in naturally infected cattle, the sites and mechanisms of virus persistence and possible stimuli for recrudescence. It will be of the utmost importance to determine the potential for infectious virus dissemination from virus positive, seropositive cattle to BVDV-naïve animals in natural environments.

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