



## Infectivity of pestivirus following persistence of acute infection.

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1 **Infectivity of pestivirus following persistence of acute infection.**

2

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15

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19 first authors.

20 **Abstract**

21 Bovine viral diarrhoea virus (BVDV) is an endemic pathogen worldwide and eradication strategies

22 focus on identification and removal of persistently infected (PI) animals arising after *in utero* infection.

23 Despite this, acute infections with BVDV can persist for months or years after the removal of the PI

24 source despite repeated screening for PIs and tight biosecurity measures. Recent evidence for a

25 prolonged duration of viraemia in the testicles of bulls following acute BVDV infection suggests the

1 possibility of a form of chronic persistence that may more closely resemble the persistence strategies of  
2 hepatitis C virus (HCV).

3

4 To investigate the potential for virus transmission from infected and recovered cattle to virus naïve  
5 hosts we established an acute infection of 5 BVDV naïve calves and monitored animals over 129 days.  
6 Infectious BVDV was detected in white blood cells between days 3 and 7 post-challenge. The animals  
7 seroconverted by day 21 post-infection and subsequently were apparently immune and free from  
8 infectious virus and viral antigen.

9

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

12

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

#### 17 **Keywords**

18 BVDV, pestivirus, acute infection, long term persistence, transmission, infectivity.

19

1

## 2 **Introduction**

3

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

11

12 BVDV is endemic in most countries and the consequences of infection in terms of the economic impact  
13 and animal welfare are highly significant (Houe, 2003). The observed range of clinical outcomes  
14 largely depends upon the characteristics of the infecting virus (Bolin and Ridpath, 1992), (Brownlie,  
15 1991) and consequences of infection include decreased milk yield, poor reproductive performance, loss  
16 of condition and high levels of recurrent infection due to the immunosuppressive effects of  
17 BVDV-associated leucopenia. Haemorrhagic strains of BVDV, which are endemic throughout North  
18 America and Canada and increasingly apparent in mainland Europe (Vilcek et al., 2005) also cause  
19 high mortality and severe loss of condition during the acute infection (Brownlie, 1991), (Pellerin et al.,  
20 1994), (Ridpath et al., 2000). As the animal welfare and economic implications of unchecked infection  
21 become apparent, many countries (including Austria, Denmark, Finland, Germany, Norway, Sweden)  
22 are undertaking the national eradication of BVDV.

23

1 The typical course of acute infection of postnatal animals (cattle or sheep) has been well defined  
2 experimentally (Howard, 1990) identifying a period of viraemia between 4 and 10 days post infection  
3 during which infectious BVDV can be detected in serum, blood, buffy coat cells and nasal secretions.  
4  
5 A transient pyrexia and a significant leucopenia is seen between days 3 and 10 post infection.  
6 Seroconversion can be detected, rarely at day 7 but more usually by day 14. As active immunity  
7 develops virus spread is eliminated and infectious virus is rarely detected in buffy coat cells or nasal  
8 secretions beyond 14 days. In this respect, acute BVDV infection seemed to be resolved much more  
9 readily than HCV infection which is known to persist in a significant number of infected individuals  
10 (Tester et al., 2005; Thimme et al., 2001). The use of more sensitive detection techniques such as  
11 RT-PCR can extend the period when BVDV RNA can be detected but isolation of infectious virus is  
12 generally not possible beyond 14 to 21 days post infection as the animal develops significant levels of  
13 anti-viral neutralising antibodies which continue to increase in titre beyond 40 to 50 days post  
14 infection.  
15  
16 A further consequence of acute infection with BVDV occurs when the period of active viraemia occurs  
17 during the first trimester of pregnancy. In these circumstances, virus can cross the placenta and  
18 establish extensive viraemia within the immuno-incompetent foetus. Many such infections result in  
19 abortion but up to 60% will survive to produce a calf which is persistently infected (PI),  
20 immunotolerant to the virus and will actively shed virus throughout its life (Brownlie, 1990).  
21  
22 To date, the persistently infected, immunotolerant animal has been considered to be the major source of  
23 BVDV spread and eradication schemes have focussed on the identification and culling of such animals  
24 (Houe, 1999). However, many units have experienced great difficulty in eliminating the circulation of  
25 BVDV even in the absence of PI animals and the presence of tight biosecurity measures (Houe, 1999).

1  
2 These observations have prompted attempts to identify a previously unrecognised form of viral  
3 persistence in acutely infected and antibody positive animals. A number of authors (including  
4 (Ssentongo et al., 1980), (Grooms et al., 1998), (Niskanen et al., 2002), (Givens et al., 2003)) have  
5 reported detection of BVDV in reproductive tissues (ovary, testis or semen) of cattle at prolonged times  
6 following initial acute infection: in ovarian tissue 60 days following challenge (Grooms et al., 1998)  
7 and in semen up to 7 months after initial infection with a live BVDV vaccine. These authors (Givens et  
8 al., 2003) were unable to culture virus directly from semen but could detect viral RNA by nested  
9 RT-PCR and did transmit virus to one calf of three following intravenous injection of semen collected  
10 at 5 months post infection.  
11  
12 With the exclusion of the known incidents of BVDV transmission in virus-infected semen, it was not  
13 clear whether these animals with long term persistence of viral antigen in their ovaries or testes were  
14 infectious for BVDV naïve herd mates. To our knowledge, there is no published report of BVDV  
15 immune animals transmitting infectious virus except through semen. The reports of long term  
16 persistence of viral antigen in ovaries or detection of viral RNA in white blood cells (unpublished  
17 observations) cannot yet be equated with infectivity and the status of these animals is unresolved.  
18  
19 Similar questions are raised for HCV. Recent publications of Pham *et al.* (Pham et al., 2005), (Pham et  
20 al., 2004) which confirm that for HCV, *ex vivo* stimulation of PBMCs with mitogens and cytokines will  
21 stimulate the previously undetectable replication of viral RNA in both lymphocytes and dendritic cells  
22 of infected patients. Questions have been raised as to whether such cases represent the detection of  
23 "occult RNA" or whether there is the real potential for virus infectivity and transmission in these  
24 samples (Quiroga and Carreno, 2005; Quiroga et al., 2006). A recent paper by Gogorza *et al.* (Gogorza  
25 et al., 2005) has also indicated that *ex vivo* stimulation of PBMCs from cattle in a number of BVDV

1 antibody positive herds was successful in producing antigen-positive samples and infectious virus from  
2 a small number of individual cattle but in this field situation it was not possible to comment on the  
3 infection history of these individuals within the herds. Although antibody levels could be measured, it  
4 was impossible to know when the individuals had been exposed to virus or the duration of persistence  
5 and again, the potential for spread of infectious virus was not assessed.

6

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

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

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

14

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

19

20



1

## 2 **Materials & Methods**

3

4 **Study design:** Five BVDV antibody- and antigen-free calves (numbers 321, 330, 339, 345 and 360:  
 5 study group A), of approximately three months of age sourced from a BVDV-free herd, were  
 6 challenged intranasally with 10 ml of isolate \$456497 ( $5 \times 10^6$  TCID<sub>50</sub>), a type 1a BVDVnc identified  
 7 as a more clinically virulent field isolate (T. Drew, VLA, personal communication). This infectious  
 8 dose has been shown to be effective in previous studies (Brownlie et al., 1995). See Fig 1 for summary  
 9 of experimental outline. Blood samples (plain clotted and EDTA) were taken by jugular venepuncture  
 10 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,  
 11 105, 112, 119, 126, 133, 140, 147 and 154 where day 0 is the day of virus challenge).

12

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

16

17 On study day 98 a further 3 BVDV naïve calves were introduced to the group (467, 469 and 473). At  
 18 this stage, 20 ml blood from the original group of 5 calves (study group A: numbers 321, 330, 339, 345  
 19 and 360) was injected, 10 ml intravenously and 10 ml subcutaneously, into the younger animals (399,  
 20 408, 467, 469 and 473: study group B). Sampling of all calves continued on study days 112, 119 and  
 21 126 after which time the older, study group A calves were removed from the study. The remaining  
 22 study group B calves were sampled again on study days 133, 140, 147 and 154 when the study ended.

23

24 **Virus isolation:** Following purification of the buffy coat cells from EDTA blood by ammonium  
 25 chloride lysis, virus was isolated by co-culture of white blood cells (WBCs) with uninfected foetal

1 bovine lung (FBL) cells followed by immunodetection of the virus as described previously (Nobiron et  
2 al., 2003).

3

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

7 **NS3 indirect antibody ELISA:** The NS3 antigen used in this assay (Young et al., 2005) was captured  
8 from a lysate obtained from NADL infected FBL cells using the monoclonal antibody WB112  
9 (Veterinary Laboratories Agency) diluted 1:100 in carbonate coating buffer (Sigma). Uninfected cells  
10 were also prepared as a negative background controlThe ELISA plate was blocked with 5% NPS  
11 before positive and negative antigen was added. Test sera were added, diluted 1:50 and binding  
12 detected using biotinylated monoclonal anti-bovine IgG clone BG18 (Sigma) followed by incubation  
13 with Streptavidin-horseradish peroxidase conjugate (Amersham Biosciences). The ELISA was  
14 developed with OPD substrate (Sigma) and optical density measured using an Optimax microplate  
15 reader (Molecular Diagnostics) at 490nm using SOFTmax®PRO version 5.0.

16

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

21

22 **Ex vivo stimulation of PBMCs:** PBMCs were isolated from EDTA blood (5 ml) by density gradient  
23 centrifugation using Histopaque1083. Leucocytes were removed from the top of the Histopaque  
24 cushion and washed in 20 ml of ice cold PBS. Any remaining red blood cells were lysed by briefly  
25 resuspending the pellet in 2 ml of sterile ultrapure water before adding 20 ml of PBS and washing twice

1 in PBS. The cells were finally resuspended in 10 ml of pre-warmed RPMI-1640 medium (Sigma)  
2 containing 10% FCS, glutamine 200 mM, penicillin 100 IU/ml and streptomycin 100 µg/ml with  
3 phytohaemagglutinin (PHA, Sigma L8754, 5 µg/ml) for 72-hours. After stimulation, PBMCs were  
4 pelleted by centrifugation at 400 x g, 4 °C for 10 minutes before RNA extraction.

5  
6 **RNA extraction, RT-PCR and sequencing:** Total RNA was extracted from potentially virus infected  
7 cells using 0.8 ml of RNA Stat-60 (AMS Biotechnology Ltd, UK) according to the manufacturer's  
8 instructions. RNA was precipitated from the aqueous phase with 0.5 ml of isopropanol and 1 µl of  
9 glycogen (20 mg/ml, Roche Diagnostics, USA) at 4 °C for 30 min and then collected by centrifugation.  
10 The RNA pellet was washed in 1 ml of 75% ethanol and resuspended in 50 µl of diethylpyrocarbonate  
11 (DEPC)-treated ultrapure water.

12  
13 RNA was reverse transcribed (RT) to cDNA with random hexamer primers, (50 ng/µl, Amersham  
14 Biosciences, UK) and Superscript II, reverse transcriptase (200 units/µl, Invitrogen) according to the  
15 manufacturer's instructions. The reactions were held at 42 °C for 50 min and at 70 °C for a final 15  
16 min.

17  
18 cDNA (5 µl) generated by RT was amplified by PCR (0.5 µl of *Pfu* polymerase (5 units/µl, Promega),  
19 2.5 µl of 10X buffer (Promega), 1 µl 10 mM dNTPs, using 2.5 µl primer 324 and 2.5 µl primer 326  
20 (Vilcek, 1994), both at 10 pmol/ml, in a final volume of 20 µl with sterile ultrapure water. These  
21 primer sequences are highly conserved in different BVDV isolates and amplify a 288-bp fragment of  
22 the 5' untranslated region of the BVDV genome. This fragment was sequenced using Cy-5 labelled  
23 primers and a Thermosequenase kit on an Amersham Biosciences ALF Express DNA analyser.

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

4

5

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1

## 2 Results

3

4 Virus challenge: Back titration of the type 1a BVDVnc isolate \$456497 challenge inoculum confirmed  
5 that all study group A calves (numbers 321, 330, 339, 345 and 360) received a high infectious dose of  
6 approximately  $5 \times 10^6$  TCID<sub>50</sub>.

7

8 Clinical responses to virus challenge were recorded from day –1 to day 12. All calves experienced  
9 some degree of pyrexia, peaking around day 8 or 9 post infection and leucopenia between days 3 and  
10 14 (data not shown). Both of these are typical signs of BVDV infection. Additionally, the calves were  
11 observed for clinical signs of disease. Occasional coughing was noted, but calves appeared healthy in  
12 all other respects.

13

14 Seroconversion: A total anti-BVDV antibody ELISA was performed to detect BVDV-specific (total  
15 IgG) antibodies in the serum. The results (Fig 2) indicate that by day 21 post challenge all calves had  
16 seroconverted to BVDV, showing a positive ELISA result. Antibody titres continued to rise from day  
17 21, and for 4 animals was still increasing in titre up to day 105 post challenge. The group A calves were  
18 removed from the study at day 129 post challenge at which time all five showed antibody titres that  
19 would indicate a high degree of protection to infection with BVDV.

20

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

1 the onset of seroconversion by day 14. No infectious virus was isolated directly from purified PBMCs  
2 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  
5 98 post challenge PBMCs purified from the five, study group A calves were stimulated by incubation  
6 with the mitogen PHA before cells were used for conventional virus isolation or for RNA extraction  
7 followed by RT-PCR.

8  
9 Between days 28 and 98 post infection viral RNA was detected in PBMCs on a number of occasions in  
10 each animal (table 2). This was evident in unstimulated PBMCs in 16 separate samples and following  
11 stimulation of PBMCs with PHA for 72 hr, viral RNA was detected in 26 different samples indicating  
12 that stimulation did increase the frequency of RNA detection. No infectious virus was recovered from  
13 any of these samples following immunostaining using standard protocols (data not shown).

14  
15 Virus transmission: To assess the potential for horizontal transmission of BVDV, on day 77, BVDV  
16 naïve calves 399 and 408 were introduced into the existing group and monitored weekly for any signs  
17 of virus transmission. During a three week period the new animals were consistently virus negative by  
18 virus isolation and RT-PCR and did not seroconvert to BVDV (data not shown).

19  
20 On study day 98, a further 3 BVDV-naïve calves (467, 469 and 473), were introduced to the group and  
21 exchange of blood from study group A (numbers 321, 330, 339, 345 and 360) to study group B (399,  
22 408, 467, 469 and 473) was undertaken. Both groups were subjected to continued monitoring for virus  
23 isolation, RT-PCR and seroconversion after sampling at weekly intervals.

24

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

5

6 Animals in study group B were observed to seroconvert to BVDV, beginning at day 14 post blood  
7 transmission (animal 408) and all group B animals were seropositive in the standard BVDV antigen  
8 ELISA by study day 140, 42 days following blood exchange between group A and group B animals  
9 (Fig 4). To ensure that this was a result of active virus replication in group B animals and not the  
10 passive transfer of antigen, seroconversion to recombinant BVDV E2 glycoprotein and to the BVDV  
11 non-structural protein NS3 were measured independently and antibody levels increased in all animals  
12 over the 42 day period of observation (Fig 3).

13

14 Viral RNA was detected in purified PBMCs of animal 469 on day 7 post transmission, with other  
15 group B animals, except 408, being virus RNA positive up to 21 days post infection (table 3). Sequence  
16 analysis of the RT-PCR product (corresponding to the viral 5' untranslated region), indicated that the  
17 recovered virus from 469 was identical to that of the original challenge virus over the range of bases  
18 sequenced (data not shown). Only animal 408 appeared to be virus free by these methods but did show  
19 the strongest seroconversion to BVDV in all three ELISA assays (for total anti-BVDV, anti-E2 and  
20 anti-NS3 antibody).

21

22

1

## 2 Discussion

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

12

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

24



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

5

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

16

17 The current study provided a more extensive time course following BVDV infection.  
18 We were unable to demonstrate horizontal transmission of the virus to BVDV naïve calves housed for  
19 3 weeks with the seropositive, infectious virus-negative but RNA positive study group A animals in  
20 agreement with other studies describing poor transmission from acutely infected animals. Direct  
21 contact with an immunotolerant persistently infected animal has been shown to be a more efficient  
22 means of infection although even then, less so than the consistently high transmission of virus achieved  
23 when subcutaneous injection occurs transferring blood or serum or on needles used with shared BVDV  
24 contaminated vials, even through use of contaminated rectal examination gloves (Cook et al., 1990),  
25 (Lang-Ree et al., 1994; Niskanen and Lindberg, 2003). Similarly in the current study, transfer of 20ml

1 of blood from the apparently virus free and immune group A calves to five BVDV naïve group B  
2 calves resulted in the effective transmission of virus to the new hosts. Seroconversion was, relatively  
3 speaking, a slower and less synchronous event than normal, perhaps reflecting the fact that variable  
4 amounts of virus were transmitted in each inoculum as has been reported previously when different  
5 sources of virus and differing routes of transmission were compared (Cook et al., 1990). While blood  
6 transfer was an "artificial" procedure, it does genuinely represent the possibility of exchange of blood  
7 or fluids through biting flies (Tarry et al., 1991) or other veterinary intervention (Lang-Ree et al.,  
8 1994), (Niskanen and Lindberg, 2003).

9

10 We cannot absolutely determine whether virus spread to all of the group B calves is as a result of  
11 transmission through blood or horizontal transmission within the group. Calves 408 and 469  
12 seroconverted rapidly (day 14 and 21) suggesting that these animals were infected initially. As animal  
13 473 was RT-PCR positive by day 7 post blood transfer (7 days is not usually regarded as sufficient time  
14 for seroconversion following horizontal transmission from calves 408 and 469) this suggests that three  
15 of the five calves were directly infected by transmission via blood transfer. Calves 467 and 473 were  
16 both RNA positive by day 21 post transmission but showed a slower seroconversion, 42 days post  
17 blood transfer and it is impossible to assess whether their infection arose following blood transfer or  
18 horizontal transmission from other calves in the group in the current study.

19

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

25

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

15

16

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

21

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

1

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Accepted Manuscript

- 1 Bolin, S.R., Ridpath, J.F., 1992, Differences in virulence between two noncytopathic bovine viral diarrhea viruses in calves.  
2 Am J Vet Res 53, 2157-2163.
- 3 Bramley, A.M., Vitalis, T.Z., Wiggs, B.R., Hegele, R.G., 1999, Effects of respiratory syncytial virus persistence on airway  
4 responsiveness and inflammation in guinea-pigs. Eur Respir J 14, 1061-1067.
- 5 Brownlie, J., 1990, Pathogenesis of mucosal disease and molecular aspects of bovine virus diarrhoea virus. Vet Microbiol  
6 23, 371-382.
- 7 Brownlie, J., 1991, The pathways for bovine virus diarrhoea virus biotypes in the pathogenesis of disease. Arch Virol Suppl  
8 3, 79-96.
- 9 Brownlie, J., Clarke, M.C., Hooper, L.B., Bell, G.D., 1995, Protection of the bovine fetus from bovine viral diarrhoea virus  
10 by means of a new inactivated vaccine. Vet Rec 137, 58-62.
- 11 Collett, M.S., Larson, R., Belzer, S.K., Retzel, E., 1988, Proteins encoded by bovine viral diarrhea virus: the genomic  
12 organization of a pestivirus. Virology 165, 200-208.
- 13 Cook, L.G., Littlejohns, I.R., Jessep, T.M., 1990, Induced sero-conversion in heifers with a field strain of bovine pestivirus-  
14 -a comparison of methods and doses. Aust Vet J 67, 393-395.
- 15 Elbers, K., Tautz, N., Becher, P., Stoll, D., Rumenapf, T., Thiel, H.J., 1996, Processing in the pestivirus E2-NS2 region:  
16 identification of proteins p7 and E2p7. J Virol 70, 4131-4135.
- 17 Fulton, R.W., Purdy, C.W., Confer, A.W., Saliki, J.T., Loan, R.W., Briggs, R.E., Burge, L.J., 2000, Bovine viral diarrhea  
18 viral infections in feeder calves with respiratory disease: interactions with Pasteurella spp., parainfluenza-3 virus,  
19 and bovine respiratory syncytial virus. Can J Vet Res 64, 151-159.
- 20 Givens, M.D., Heath, A.M., Brock, K.V., Brodersen, B.W., Carson, R.L., Stringfellow, D.A., 2003, Detection of bovine  
21 viral diarrhea virus in semen obtained after inoculation of seronegative postpubertal bulls. Am J Vet Res 64, 428-  
22 434.
- 23 Gogorza, L.M., Moran, P.E., Larghi, J.L., Segui, R., Lissarrague, C., Saracco, M., Braun, M., Esteban, E.N., 2005,  
24 Detection of bovine viral diarrhea virus (BVDV) in seropositive cattle. Prev Vet Med 72, 49-54; discussion 215-  
25 219.
- 26 Grooms, D.L., Brock, K.V., Ward, L.A., 1998, Detection of bovine viral diarrhea virus in the ovaries of cattle acutely  
27 infected with bovine viral diarrhea virus. J Vet Diagn Invest 10, 125-129.
- 28 Houe, H., 1999, Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections.  
29 Vet Microbiol 64, 89-107.
- 30 Houe, H., 2003, Economic impact of BVDV infection in dairies. Biologicals 31, 137-143.

- 1 Howard, C.J., 1990, Immunological responses to bovine virus diarrhoea virus infections. *Rev Sci Tech* 9, 95-103.
- 2 Howard CJ, Clarke MC, Brownlie J. An enzyme-linked immune-sorbent assay (ELISA) for the detection of antibodies to  
3 bovine viral diarrhoea virus (BVDV) in cattle sera. *Vet Microbiol* 1985; 10: 359-69.  
4
- 5 Lang-Ree, J.R., Vatn, T., Kommisrud, E., Loken, T., 1994, Transmission of bovine viral diarrhoea virus by rectal  
6 examination. *Vet Rec* 135, 412-413.
- 7 Muchmore, H.G., Parkinson, A.J., Humphries, J.E., Scott, E.N., McIntosh, D.A., Scott, L.V., Cooney, M.K., Miles, J.A.,  
8 1981, Persistent parainfluenza virus shedding during isolation at the South Pole. *Nature* 289, 187-189.
- 9 Niskanen, R., Alenius, S., Belak, K., Baule, C., Belak, S., Voges, H., Gustafsson, H., 2002, Insemination of susceptible  
10 heifers with semen from a non-viraemic bull with persistent bovine virus diarrhoea virus infection localized in the  
11 testes. *Reprod Domest Anim* 37, 171-175.
- 12 Niskanen, R., Lindberg, A., 2003, Transmission of bovine viral diarrhoea virus by unhygienic vaccination procedures,  
13 ambient air, and from contaminated pens. *Vet J* 165, 125-130.
- 14 Nobiron, I., Thompson, I., Brownlie, J., Collins, M.E., 2003, DNA vaccination against bovine viral diarrhoea virus induces  
15 humoral and cellular responses in cattle with evidence for protection against viral challenge. *Vaccine* 21, 2082-  
16 2092.
- 17 Pellerin, C., van den Hurk, J., Lecomte, J., Tussen, P., 1994, Identification of a new group of bovine viral diarrhoea virus  
18 strains associated with severe outbreaks and high mortalities. *Virology* 203, 260-268.
- 19 Pham, T.N., Macparland, S.A., Coffin, C.S., Lee, S.S., Bursey, F.R., Michalak, T.I., 2005, Mitogen-induced upregulation of  
20 hepatitis C virus expression in human lymphoid cells. *J Gen Virol* 86, 657-666.
- 21 Pham, T.N., MacParland, S.A., Mulrooney, P.M., Cooksley, H., Naoumov, N.V., Michalak, T.I., 2004, Hepatitis C virus  
22 persistence after spontaneous or treatment-induced resolution of hepatitis C. *J Virol* 78, 5867-5874.
- 23 Pollock, J.M., Rowan, T.G., Dixon, J.B., Carter, S.D., Kelly, D.F., 1991, Estimation of immunity in the developing calf:  
24 cellular and humoral responses to the keyhole limpet haemocyanin. *Vet Immunol Immunopathol* 29, 105-113.
- 25 Quiroga, J.A., Carreno, V., 2005, HCV-indeterminate blood donors or occult HCV infection? *Lancet* 365, 1540-1541;  
26 author reply 1541.
- 27 Quiroga, J.A., Llorente, S., Castillo, I., Rodriguez-Inigo, E., Lopez-Alcorocho, J.M., Pardo, M., Carreno, V., 2006, Virus-  
28 specific T-cell responses associated with hepatitis C virus (HCV) persistence in the liver after apparent recovery  
29 from HCV infection. *J Med Virol* 78, 1190-1197.
- 30 Ridpath, J.F., Neill, J.D., Frey, M., Landgraf, J.G., 2000, Phylogenetic, antigenic and clinical characterization of type 2  
31 BVDV from North America. *Vet Microbiol* 77, 145-155.

- 1 Ssentongo, Y.K., Johnson, R.H., Smith, J.R., 1980, Association of bovine viral diarrhoea-mucosal disease virus with  
2 ovaritis in cattle. *Aust Vet J* 56, 272-273.
- 3 Tarry, D.W., Bernal, L., Edwards, S., 1991, Transmission of bovine virus diarrhoea virus by blood feeding flies. *Vet Rec*  
4 128, 82-84.
- 5 Tester, I., Smyk-Pearson, S., Wang, P., Wertheimer, A., Yao, E., Lewinsohn, D.M., Tavis, J.E., Rosen, H.R., 2005, Immune  
6 evasion versus recovery after acute hepatitis C virus infection from a shared source. *J Exp Med* 201, 1725-1731.
- 7 Thimme, R., Oldach, D., Chang, K.M., Steiger, C., Ray, S.C., Chisari, F.V., 2001, Determinants of viral clearance and  
8 persistence during acute hepatitis C virus infection. *J Exp Med* 194, 1395-1406.
- 9 Vilcek, S., 1994, [Development of PCR tests for the detection of bovine herpesvirus-1, bovine respiratory syncytial viruses  
10 and pestiviruses]. *Vet Med (Praha)* 39, 687-700.
- 11 Vilcek, S., Durkovic, B., Kolesarova, M., Paton, D.J., 2005, Genetic diversity of BVDV: consequences for classification  
12 and molecular epidemiology. *Prev Vet Med* 72, 31-35; discussion 215-219.
- 13 Young, N.J., Thomas, C.J., Thompson, I., Collins, M.E., Brownlie, J., 2005, Immune responses to non-structural protein 3  
14 (NS3) of bovine viral diarrhoea virus (BVDV) in NS3 DNA vaccinated and naturally infected cattle. *Prev Vet Med*  
15 72, 115-120; discussion 215-119.
- 16  
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18

1 Figure legends:

2 Figure 1. Summary of the sampling schedules for the study.

3

4 Figure 2. Seroconversion following challenge of Group A calves

5 Serum antibody titres to total BVDV antigen were measured in all five calves using an antibody

6 capture ELISA following challenge with BVDV isolate \$456497. A standard curve using a reference

7 BVDV hyperimmune serum allowed the results to be expressed as units/ml (Howard et al., 1985).

8 Clear evidence of seroconversion (90 to 180 antibody units) was seen by day 21 post infection (panel a)

9 and titres continued to rise in all animals (4,500 to 44,000 antibody units) at least up to day 105 post  
10 challenge (panel b).

11

12 Figure 3. Seroconversion of study Group B animals following transfer of blood.

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

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

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

20

21

22



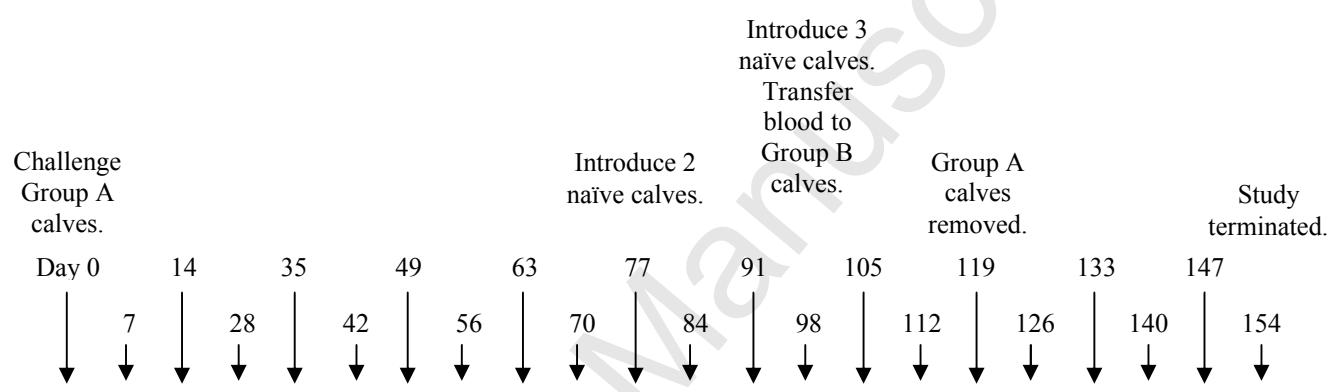
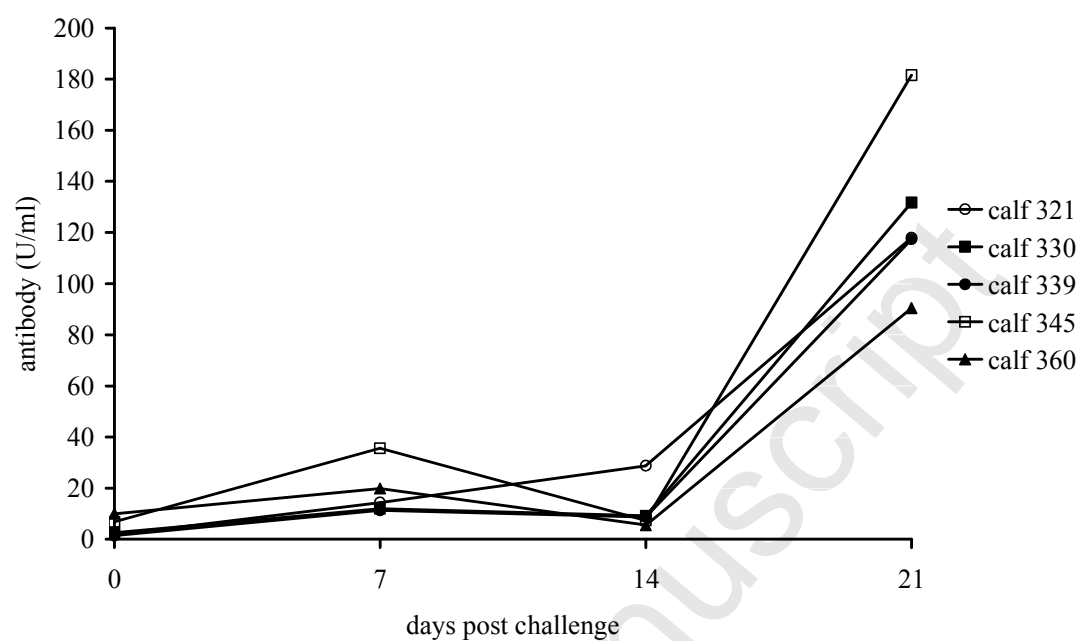


Figure 1.

a)



b)

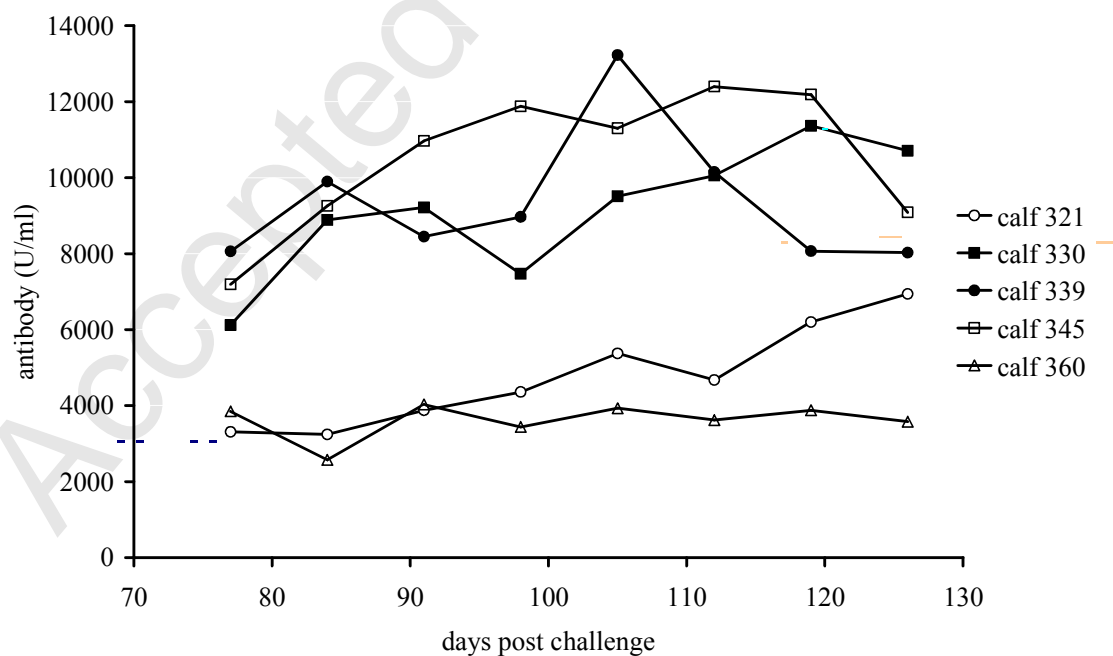


Figure2

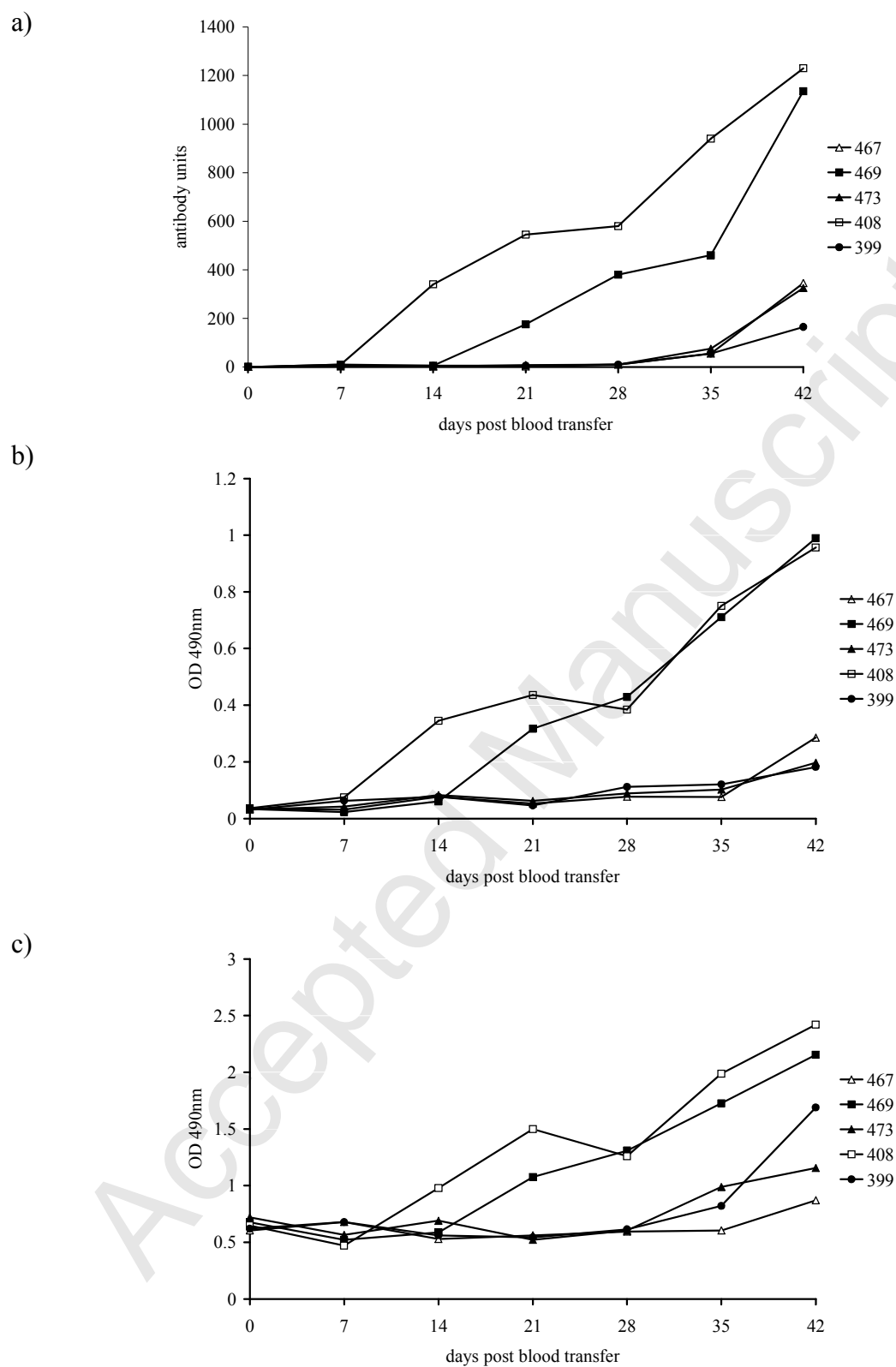


Figure3.

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

**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 three post challenge in calves 339 and 360 and all five calves were virus positive on day 5. By day 10 post infection all calves were definitely virus negative in this assay.

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

Table 2. Detection of virus specific RNA by RT-PCR in PBMCs of study group A animals.

NS = nonstimulated PBMCs

S = PHA stimulated PBMCs

+= RNA detected by RT-PCR

- = RNA not detected by RT-PCR

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

Table 3. Detection of virus specific RNA by RT-PCR in PBMCs of study group B animals.

NS = nonstimulated PBMCs

S = PHA stimulated PBMCs

+ = RNA detected by RT-PCR

- = RNA not detected by RT-PCR