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Sheep persistently infected with Border disease readily transmit virus to calves seronegative to BVD virus

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Abstract

Bovine viral diarrhea- and Border disease viruses of sheep belong to the highly diverse genus pestivirus of the Flaviviridae. Ruminant pestiviruses may infect a wide range of domestic and wild cloven-hooved mammals (artiodactyla). Due to its economic importance, programs to eradicate bovine viral diarrhea are a high priority in the cattle industry. By contrast, Border disease is not a target of eradication, although the Border disease virus is known to be capable of also infecting cattle. In this work, we compared single dose experimental inoculation of calves with Border disease virus with co-mingling of calves with sheep persistently infected with this virus. As indicated by seroconversion, infection was achieved only in one out of seven calves with a dose of Border disease virus that was previously shown to be successful in calves inoculated with BVD virus. By contrast, all calves kept together with persistently infected sheep readily became infected with Border disease virus. The ease of viral transmission from sheep to cattle and the antigenic similarity of bovine and ovine pestiviruses may become a problem for demonstrating freedom of BVD by serology in the cattle population.

Keywords

Pestivirus, Border disease virus, Bovine viral diarrhea virus (BVD virus), BVD eradication, sheep, cattle, infection
Introduction

Originally, the pestiviruses of ruminants were differentiated according to the host species from which they were isolated and referred to as bovine viral diarrhea virus (BVD virus) and Border disease virus of sheep (Carlsson 1991, Carlsson and Belak 1994, Campell et al. 1995, Paton et al. 1997; for reviews, see Nettleton and Entrican, 1995; Nettleton et al., 1998; Løken 1995). More recently, nucleotide sequencing documented the wide genetic heterogeneity and taxonomic position of these viruses (Becher et al., 1997; 2003; Vilcek et al., 2005; reviewed by Becher and Thiel, 2011) and confirmed that ruminant pestiviruses may not only jump the species barrier between bovines and sheep, but also infect other ruminants, including wild species. (reviewed by Passler and Walz, 2010). In domestic animals, the interspecies infection appears to occur particularly in the direction of cattle to sheep (Løken 1995; Carlsson and Belak 1994, Krametter-Frötscher et al. 2008; Danuser et al., 2009). In contrast, infections of cattle with the sheep pestivirus have been noted less frequently (Becher et al., 1997; Cranwell et al. 2007; Krametter-Frötscher et al. 2008; Hornberg et al. 2009). It was shown that calves kept together with sheep seroconverted and that the antibodies were directed against Border disease virus (Krametter-Frötscher et al. 2008). Information on the clinical effects of infection with Border disease virus in cattle is scarce. By contrast, Border disease virus was shown to cause severe infections in chamois in the Spanish Pyrenees (Marco et al., 2007, 2011). In this work we compared the effects of co-mingling of calves with sheep persistently infected with Border disease virus vs. inoculation of calves with a single virus dose.
Animals, materials and methods

Sheep persistently infected with Border disease virus (PI sheep) and characterization of viruses.

Two female one-year-old sheep persistently infected with Border disease virus were used in this study. Sheep #1, of the white alpine breed, was initially taken to the clinic for investigation at two days of age with clinical signs typical of border disease, i.e. ataxia, tremor and hairy fleece. Sheep #2, of the black-brown alpine breed, was transferred to the clinic at three months of age with head tremor and ataxia. Interestingly, the clinical signs of infection gradually decreased in both animals and had disappeared to almost undetectable levels when the sheep were brought in contact with the calves. Both animals tested positive for pestivirus antigen by immunohistological investigation of skin biopsies as described previously (Arquint, 2003; Thür et al, 1997). Over a period of one year, leucocytes, and intermittently also nasal swabs, of sheep #1 tested positive for pestiviral RNA by RT-PCR. Leucocytes, epilated hair, urine, muzzle and nasal swabs of sheep #2 were positive for viral RNA. Both animals were negative for pestivirus antibody. Virus was repeatedly isolated from leucocytes of both animals in cultured sheep synovial membrane cells. Sequencing showed that the viruses isolated belong to the species Border disease virus of the pestivirus genus and may form a new genetic cluster (Figure 1, marked by arrow). The virus isolated from sheep # 1 was labeled CH-R4786 and that of sheep # 2 CH-R4785. After passaging twice both viruses we decided to use strain CH-R4786 for single dose inoculation because it grew to a higher titer than strain CH-R4785. Both PI sheep were also tested for ovine herpes virus type 2 (Hüssy et
al., 2001) because this virus may cause malignant catarrhal fever in cattle (reviewed by Russell et al., 2009). Both animals were found to be positive.

All animal experiments were approved by the Veterinary Office of the Canton of Zurich.

**Pestiviral status of the calves before initiation of the experiments.**

The two experiments consisted of a quarantine period followed by the infection phase. For logistic reasons, the quarantine was set at 17 days for the orally inoculated calves and at 30 days for the calves exposed to PI sheep. Before purchase and at the end of the quarantine period all animals tested negative for pestivirus antigen and antibodies in blood samples and for pestivirus antigen in skin biopsies (Thür et al., 1997).

**Inoculation of calves with Border disease virus and co-mingling of calves with PI sheep.**

Seven calves (4 male/3 female) were orally inoculated with Border disease virus (see below). The animals were of various breeds (Simmental, Holstein Friesian, Charolais x Braunvieh, Charolais x Holstein Friesian) and were between 87 and 176 days old at the beginning of the quarantine period (145.9 [32.65] (mean [SD]). The protocol and virus dose for oral inoculation were the same as previously used for experimental infection of calves with BVD virus (Hilbe et al., 2007). Each animal received a total of 4.8ml of Border disease virus BD4 isolated from PI sheep #1, dispensed in the oropharyngeal cavity by four strokes using a Foxy Plus™ hand sprayer (Birchmeier Corporation, Stetten, Switzerland, www.birchmeier.com). The
total dose per animal of $7 \times 10^5$ TCID$_{50}$ was verified by backtitration. In the co-mingling experiment, nine male calves of various breeds (Braunvieh, Simmental, Holstein Friesian) that were between 99 and 156 days old (131 ± 18.8 days) were kept together with sheep # 1 and # 2 for 72 days.

Clinical investigations and laboratory tests

To avoid contamination strict quarantine rules were observed throughout the experiments, involving separation of the animal groups, changing clothes and disinfection between attending different groups of animals.

The animals were subjected to standardized clinical investigation by two of the authors (CR: oral inoculation, SR: co-mingling of calves and PI sheep). The investigations included recording of body temperature, heart beat and respiratory frequencies, lung and digestive tract auscultation, inspection for signs of diarrhea, nasal and ocular discharge and inspection of mucosae. In the oral inoculation experiment, clinical investigations were done on days -9, -6, -3, thereafter daily until day 21, followed by days 25, 28, 32, and 35. Nasal swabs were collected daily on the first 21 days post-infection. Blood samples for virus detection and hematological investigations followed the same schedule as the clinical investigation but continued until the termination of the experiment on day 70. The schedule of antibody determination is seen in Figure 3A and B. Hematological investigations included erythrocyte and differential leucocyte counts, hemoglobin, packed cell volume, mean corpuscular hemoglobin concentration, mean corpuscular hemoglobin, and mean corpuscular volume. In the co-mingling experiment, the calves were subjected to standardized clinical investigation at the beginning and end of the quarantine period. The schedule of clinical and laboratory investigations was as follows: hematology:
days 0, 3,6,7,8,9,10,11,12,13,14, days 16 through 72: every second day. BVD virus
and – serology: 0,2,4,6, thereafter daily until day 20, followed by every second day
until the termination of the experiment; nasal swabs: up to day 41 for all calves as for
virus and serology, thereafter every second day for calves # 2,5,6, and continued
until day 72 for calves # 5 and # 6.

Detection of pestiviral RNA
Quantitative (q) RT-PCR was used to detect viral RNA in the EDTA blood and in the
nasal swab medium. RNA was extracted from 150μl of anti-coagulated whole blood
using the BioRobot Universal System and the QIAamp virus BioRobot MDx Kit
(Qiagen, CH-8694 Hombrechtikon, Switzerland). Viral RNA was quantitated using
the Qiagen Cador BVDV RT-PCR kit. Volumes were chosen to give a final dilution
factor of 12.5. The test recognizes a genome sequence conserved in pestiviruses
and can therefore also be used for the detection of border disease virus (Stalder et
al., 2008). Following the evaluation of the raw data the amount of the viral DNA
present in the sample was expressed in CT values, whereby CT values of >45 were
considered to be negative and those with a CT value of 30-45 were considered to be
weakly positive. The RNA isolations and RT-PCR preparations were done in
separate laboratories. The testing for viral RNA in the nasal swab medium was done
analogously to the method described for the viral RNA testing in the blood.

Sequencing
A genome fragment of 504 nucleotides spanning from the 5′ untranslated region to
the core protein including the Npro was amplified as described previously (Bachofen
et al., 2008) and the DNA fragments of the correct length were sequenced by
Microsynth GmbH (CH-9436 Balgach). The sequence data were analysed by means of the SeqMan (DNASTAR Inc., Madison, USA) and the Clone Manager (Scientific & Educational Software, Cary, USA) softwares. The sequences are deposited in Genbank (CH-R4785: GU244490; CH-R4786: GU244489)

Serological investigations

ELISA

A biphasic in-house ELISA was used for antibody screening as previously described (Rüfenacht et al., 2000). The antigen of this ELISA contains a high concentration of the conserved immunogenic NS3 protein and only little of the more strain-specific E2 protein (Wageck Canal et al., 1998). ELISA microtiter plates (Maxisorp, A/S Nunc, Kamstrup, Denmark) were coated with antigen derived from bovine turbinate cells infected with the cytopathic BVD viral strain R1935/72 (Oregon C24V, subgenotype 1a). A horse radish peroxidase labelled goat anti-bovine IgG antibody (light and heavy chain, Kirkegaard and Perry Labs., Gaithersburg, USA) was used to detect bound antibody. To visualize bound labelled antibody, the substrate ABTS (2,2'-azino-di-(3-ethyl benzthiazoline-6- sulphonic acid), Roche Diagnostics) was added and color measured in an ELISA reader at 405nm.

Serum neutralization test

To confirm the specificity of the antibodies we carried out serum neutralization assays using Border disease virus CH-R4786 and BVD virus CH-04-01b. The latter is a BVD-1h virus strain, which is the most frequent subgenogroup found in the Swiss cattle population (Bachofen et al., 2008).
Procedure at the onset of erosive changes at the muzzle and the muzzle cavity

Specimens were taken at the onset of erosive changes at the muzzle and the muzzle cavity and investigated for the presence of Border disease virus.

Statistics

The data were collected and evaluated by means of the StatView 5.1 programme (SAS Institute, CHJ-8602 Wangen). The data were descriptively analysed (mean values, standard deviations, frequency distributions). A generalized linear model was also used to check for possible significant changes in the variables during the course of the investigations (programme Stata statistical software, Stata Corp., 2009; Release 101.; College Station, TX, USA: StataCorp LP). P-values of < 0.05 were considered to be significant.
Results

Clinical findings

Four out of the seven orally inoculated calves had a reduced appetite on day 2 post-infection whereas this was observed in three out of nine calves on day six after the introduction of PI sheep. Transient increases >39.5°C in body temperature were noted in individual animals of the two groups both during the quarantine and the infection periods. These changes occurred scattered over time. All calves exposed to PI sheep showed erosions of the muzzle mucosa between days 3 and 17, whereas this was the case with four out of seven orally inoculated animals (animal #1: days 17-22 and days 29/30; animal #2: days 24-30; animal #5: days 4-29; animal #6: days 5-22; animal #7: days 7-15). The erosions were located in the areas of the rostral palate (see Fig. 2A, orally inoculated calf), the gingival area of the incisors and the mucosa of the upper and lower lips (Fig. 3B, calf exposed to PI sheep). The lesions showed a roundish or oblong slightly reddened area and were associated with a loss of integrity of the surface epithelium. The animals often salivated and the edges of the gingiva were inflamed.

Serology

Serum samples obtained at various time points were tested for antibodies using ELISA. In addition, serum samples obtained at the termination of the experiment were assayed by SNT. Sera of all orally inoculated animals remained close (0±2%) to the zero value of positive control until day 28 post-infection and thereafter the sera of individual animals varied between -8% and +9% (Fig 3A). Of the serum samples obtained on day 70 post oral inoculation, only the sample of animal #6 showed a
neutralizing antibody titer of 422 to Border disease virus CH-R4786, whereas the samples of all other animals were negative (<8). The samples of animals exposed to PI sheep remained negative until day 22. Thereafter, with the exception of calf #4, we noted an increase in the ELISA antibody titers. At the termination of the experiment, six out of nine animals were in the ELISA positive range and three (calves #4, 5 and 6) were in the negative range (Fig 3B). To determine the pestiviral specificity of the antibodies, we compared the SNT titers against Border disease virus CH-R4786 and a BVD 1h virus. Using CH-R4786 as the challenge virus, the titers ranged from 450 in the serum of calf #4 to 3,500 in calf #2. These titers were 10-30 times higher than those directed against BVD virus as the challenge, which indicated that the antibodies were indeed directed against Border disease virus (Figure 3C).

**Hematology**
During the entire experiment, i.e. during quarantine and infection period, none of the calves showed leukopenia or significant changes in the parameters of red blood cells (erythrocyte number, PCV, hemoglobin concentration, MCH, MCHC, MCV).

**Virological investigations**
Viral RNA was not detected in any of the nasal swabs and blood samples taken from the calves exposed to PI sheep, and the same was true for the orally inoculated calves.
Discussion

BVD and Border disease viruses may be viewed as “partial generalists”, infecting not only cattle and sheep, but also a wide array of wildlife species including deer, antelopes, camelids and wildebeest (reviewed in Passler and Walz, 2010). The spread of Border disease virus to cattle is likely to gain in importance because existing BVD eradication programs are run without concomitant efforts to control Border disease (Krametter-Frötscher et al., 2010). Therefore, the focus of this work was on the transmission of Border disease virus from sheep to cattle. In a herd of susceptible bovines exposed to sheep PI with Border disease virus, the time point of transmission of virus may differ between individual animals. Consequently, seroconversion as well as possible clinical signs of infection with Border disease virus may occur scattered over time. To control for this, we compared single dose experimental inoculation with contact exposure to PI sheep. The oral route for inoculation of the calves with Border disease virus and dose of infection were chosen to allow a direct comparison with a previous experiment in which we used BVD virus. As indicated by seroconversion in only one out of seven calves orally inoculated with Border disease virus vs. 6/6 calves inoculated with BVD virus (Hilbe et al., 2007), Border disease virus was less efficient than BVD virus at infecting cattle. The relative inefficiency of Border disease virus correlates with a poor growth of this virus in cultured bovine turbinate cells (unpublished observation). The virus concentrations of the strain of Border disease virus shed by the PI sheep are unknown, but PI sheep readily transmitted Border disease virus to calves kept in the same herd. Interestingly, viral RNA was not detected in blood and nasal swabs in any of the calves. This contrasts with the previous experiment in which we detected
BVD virus by isolation in cell culture in a majority of the orally inoculated animals (Hilbe et al., 2007). In our hands, real-time RT-PCR is more sensitive than cell culture isolation. Thus, viremia of Border disease virus may be of short duration and low titer in calves. Different from the previous experimental infection with BVD virus (Hilbe et al., 2007), we did not observe fever or changes in the leucocyte numbers in calves that seroconverted to Border disease virus. The same was true also for acutely infected sheep and goats (unpublished observation), which points to a low virulence also in these host species. In agreement with earlier reports, the PI sheep showed neurological signs of Border disease only in the first weeks of their lives (Løken, 1998; Krametter-Frütscher et al., 2010).

The skin lesions observed in some of the calves in both experiments deserve comment. In contrast to the mucosal disease-like lesions reported in sheep chronically or persistently infected with Border disease virus (Monies et al., 2004; Hilbe et al., 2009), the lesions observed in the calves were mild and transient. Moreover, the lesions were also much less severe than those in acute BVD recently described by Hessman et al. (2012). For several reasons, we are at a loss to explain the cause of these lesions. Firstly, we failed to isolate pestivirus, or to demonstrate pestiviral RNA, in any of the lesions analysed. Secondly, such lesions occurred also in some orally inoculated calves that did not seroconvert to Border disease virus, as assayed by ELISA and SNT. Due to their typical clinical picture, epidemiology and absence from Switzerland we can exclude a range of agents known to cause skin lesions, such as vesicular stomatitis and parapox viruses. All evidence obtained in our and in previous experiments (Torleiv Løken, personal communication) do not support the view that the lesions were directly caused by Border disease virus. Thus,
additional experiments will be required to determine the cause of these lesions, among them contact experiments between sheep uninfected by Border disease virus and calves originating from the same herds.

Bovines persistently infected with border disease virus have only rarely been observed in the field (Cranwell et al., 2007; Krametter-Frötscher et al., 2008; Strong et al., 2010). Although contact between bovines and sheep is not uncommon under the prevailing management conditions, we detected only 13 bovines PI with Border disease virus among 7,573 analyzed by sequencing in the course of the Swiss BVD eradication campaign (Bachofen and Stalder, in preparation). The ease of transmission of Border disease from PI sheep to seronegative cattle contrasts with the rarity of bovines found PI with Border disease virus in the field. In addition to a possible difference in the degree of adaptation to sheep vs. cattle as host species, a strong herd immunity to BVD virus may decrease the risk of persistent infection with Border disease virus in cattle. In crossed neutralization assays we previously observed up to tenfold antibody titer differences between representative strains of different BVD-1 virus subgroups (Bachofen et al., 2008) and this study shows up to 30-fold antibody titer differences between the homologous Border disease strain and a representative of the most frequent subgroup of BVD virus 1 found in our cattle population (see Figure 3C). Vaccination against BVD virus was very rare before, and has been banned since, the start of the eradication program. This suggests that natural infection of cattle with BVD virus may confer some degree of immunity also to Border disease virus. Before the start of BVD eradication, some 60% of the cattle population was seropositive to BVD virus (Rüfenacht et al., 2000), as compared to some 20% of the sheep seropositive to Border disease virus (Schaller et al., 2000;
Danuser et al., 2007). However, as the eradication program progresses, herd immunity to BVD virus decreases and more bovines will become susceptible for Border disease virus. While the biological consequences for a seronegative cattle population are difficult to judge at this time, infections with Border disease clearly pose a diagnostic problem for BVD eradication. Specifically, demonstration of freedom from BVD is based on absence of antibodies to BVD in the cattle population. To our knowledge, there exists no simple test that clearly differentiates, on the species level, antibodies to BVD virus from antibodies to Border disease virus. As indicated above and shown in the literature, both BVD- and Border disease viruses are antigenically highly diverse, with the antibodies to individual virus strains cross-reactive to varying degrees (Nettleton et al., 2008; Becher et al., 2003; Bachofen et al. 2008; Ridpath et al., 2010; Strong et al., 2010). The SNT clearly shows the difference in titer between antibodies to the Border disease virus strain used in our experiment and a representative strain of the most prevalent BVD virus subgroup present in the cattle population (Fig. 3C). However, due to its high resolution between antibodies to different subgroups of BVD virus (Bachofen et al., 2008), an SNT using just one strain of BVD-1 virus is not representative for the entire species. Moreover, the SNT is unsuitable for mass testing of sera because it is work-intensive and depends on cultured cells. The development of a simple test that allows a clear-cut and efficient differentiation of antibodies to BVD- and Border disease viruses is a high priority in BVD eradication. The ELISA used in our study is clearly less sensitive for detecting antibodies to Border disease virus than the SNT (compare Figs 3B and 3C). This ELISA detects mainly antibodies to the nonstructural protein NS3 of a BVD-1 virus (Wageck-Canal et al., 1998). Although significantly more conserved than E2, the main target protein of neutralizing
antibodies, the derived amino acid sequences of the NS3 of BVD- and Border
disease viruses are different between, and heterogeneous within, the two species of
pestivirus (Stalder, unpublished). Crossed ELISAs of immune sera to the different
subgroups of these two virus species would show if NS3 is a suitable antigen for
differentiation.

From the practical viewpoint of BVD eradication programs, it seems advisable to
minimize contact between sheep and cattle because of the complications due to
spillover of Border disease virus from sheep. This would also reduce the risk of
malignant catarrhal fever in bovines, a fatal disease which is caused by ovine herpes
virus-2, a virus carried virtually by all sheep (for a review, see Russell et al., 2009).

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Conflict of interest statement

The authors declare no conflicts of interest
Figure captions

**Figure 1.** Phylogenetic analysis of selected pestiviruses based on N<sup>pro</sup>.

The tree was constructed from the complete N<sup>pro</sup> coding sequences except for the two strains Aydinb/04 (EU930014) and Burdur (EU930014) (Shortening all sequences to 420 nucleotides did not change the structure of the tree). Sequences were obtained from Genbank and analyzed as described (Peterhans et al., 2010). Please note that the taxonomic position of some of the atypical pestiviruses and of some Border disease viruses is open, e.g. the HoBi like, Chamois and Turkey viruses. The position of the Border disease viruses used in this work is indicated by an arrow. Genbank accession #: CH-R4785: GU244490 (Npro), JQ994199 (5’UTR); CH-R4786: GU244489 (Npro), JQ994200(5’UTR).

**Figure 2.** Lesions.

A: erosion in the rostral palate of orally inoculated calf #7, B: erosions in calf #3 exposed to PI sheep.

**Figure 3.** Antibody response in calves.

A: Antibody response of orally inoculated calves; B: calves exposed to sheep persistently infected with Border disease; C: comparison of neutralizing antibody titer to Border disease virus CH-R4786 strain and BVD virus strain CH-04-01b of subgenogroup 1h.
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