A TRANSMISSION EXPERIMENT IN CALVES INFECTED WITH AN ASSUMED HYPERVIRULENT BOVINE VIRAL DIARRHOEA VIRUS TYPE 2C STRAIN

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Abstract—To assess the importance of transiently infected (TI) cattle in the epidemiology of bovine viral diarrhoea virus (BVDV) type 2 infections and to describe the clinical signs caused by such an infection, a transmission experiment was performed. Three calves were intranasally infected with a hypervirulent BVDV-2c field strain isolated during a severe BVDV outbreak in Germany and housed together with seven susceptible animals. The clinical signs of the BVDV infected animals varied from very mild disease (fever, loss of appetite) to severe watery and haemorrhagic diarrhoea and death. The clinical signs and the level of BVDV excretion depended on the degree of viraemia. The basic reproduction ratio (R_0) was estimated to be 0.49 (95% CI 0.06; 2.99), suggesting a limited viral spread using the BVDV-2c strain. This suggests that this BVDV-2 infection in TI animals resulted in limited transmission towards other animals.

Keywords—Bovine viral diarrhoea virus; Experimental infection; Horizontal transmission; Reproduction ratio; Virulence

1. Introduction

Cattle are infected with bovine viral diarrhoea virus (BVDV) either through a congenital infection (vertical transmission) or through a postnatal, acute infection (horizontal transmission). Vertical BVDV transmission from the second up to the fourth month of gestation can result in the birth of persistently infected (PI) calves (McClurkin et al. 1984, 158; Peterhans et al. 2010, 5). Acute BVDV infections of BVDV seronegative cattle result in transiently infected (TI) animals, which are viraemic for 10-14 days, starting 3 days post-infection (Lanyon et al. 2014, 202).

The transmission rate of an infectious disease can be expressed by its reproduction ratio, R. A special case is the basic reproduction ratio, R_0, defined as the mean number of secondary infections arising from one typical infectious case introduced in a fully susceptible population (Kroese and de Jong 2001, xxi; Lindberg and Houe 2005, 57; Velthuis et al. 2007, 203). R_0 is determined by the following parameters (Lindberg and Houe 2005, 57): the probability of transmission during a contact between an infectious and susceptible animal (β), the number of contacts per time period (k) and the duration of the infectious period (d).

Once colostrum-derived BVDV antibody titres have declined, PI animals continuously shed massive amounts of virus, resulting in high values for the parameters β and d (Lindberg and Houe 2005, 57). The amount of virus spread by TI animals is much lower because of a much shorter duration of the infectious period and the relatively low amounts of virus shed, resulting in low values for the parameters β en d. Therefore, PI animals are considered the main source of infection and can maintain the presence of BVDV in a susceptible population, while TI animals are generally considered to be of minor importance in spreading BVDV (Lindberg and Houe 2005, 59; Fulton et al. 2006, 126; Nickell et al. 2011, 35; Author year).

However, the virulence of BVDV strains is suggested to influence the amount and duration of virus shed by TI animals (Bolin and Ridpath 1992, 2161). Whereas the spread of low virulent strains is most likely to originate only from PI animals, it is suggested that highly virulent strains can be maintained within a susceptible population with only TI animals as source of infection (Ridpath et al. 2006, 202). Therefore transmission experiments with TI animals were conducted to test this hypothesis, hereby using BVDV strains isolated from individual cattle suffering from severe clinical BVDV-associated signs (Author year). The results of this study demonstrated a very limited viral spread from TI animals and confirmed the previous believe that TI animals only make a limited contribution to BVDV transmission.

In autumn 2012 and spring 2013 an outbreak of BVDV-2c in Germany and the Netherlands resulted in a high mortality rate, up to 80% in some veal calf herds (Doll and Holsteg 2013; Moen 2013). Since no PI animals were detected during this outbreak, it is suggested that BVDV has spread solely through TI animals (Doll 2013), which could indicate that TI animals may contribute substantially more to BVDV-2c spread than is generally assumed. The objective of the present study was to perform a transmission experiment according to the experiment performed by Author (year) with this BVDV-2c field strain isolated during the severe outbreak in Germany to quantify BVDV transmission by TI cattle.

2. Material and methods

The experimental design was identical to the protocol of the previously described transmission experiments (Author year). Ten calves were checked for the absence of BVDV- RNA and antibodies using real-time RT-PCR (RT-qPCR) and virus neutralization (VN), respectively. At the time of infection, the animals were aged between 109 and 141 days, respectively. A BVDV-2c field strain (NRW 14-13) isolated during the BVDV outbreak in Germany (Jenckel et al. 2014, 6984) was cultivated on Madin-Darby Bovine Kidney cells (two passages) and a titre of 1.3 x 10^6 tissue culture infective dose/mL

($TCID_{50}$/mL) was obtained. After an acclimatization period of 14 days, three randomly chosen calves were isolated from the other calves and inoculated with BVDV-2c through intranasal inoculation of 5.0×10^6 $TCID_{50}$. After 2 days of separation the inoculated animals were housed together in one box with the seven contact animals and BVDV spread and clinical signs were daily recorded. When no infectious animals were left (i.e. all blood samples and nasal swabs were negative by RT-qPCR), all BVDV seropositive animals were removed and the non-infected contact animals were used in a second infection experiment that started 63 days after the first inoculation. All calves were slaughtered 50 days after the start of this second experiment.

The clinical examination (see Appendix A), sample collection, virus isolation (VI), RT-qPCR, virus neutralization (VN), haematology and estimation of R_0 were performed as previously described (Author year). An animal was considered infected if positive test results were obtained for RT-qPCR, VI and VN. R_0 was estimated by maximum likelihood (MLE) according to the final size method (Velthuis et al. 2007, 207). Fever was defined when the rectal temperature was > 39.0 °C, the one-sided upper limit of the 95% CI obtained for the average rectal temperature of all calves during the last 3 days before inoculation.

3. Results

Following the first inoculation, blood samples of all three inoculated animals were positive in RT-qPCR (from 2 up to 17 days post inoculation [dpi]) and VI (from 3 up to 11 dpi), but differences in the degree of viraemia were noticed (Fig. 1a-b). One inoculated animal ("Inoculated 1", Fig. 1) died 17 dpi and was still RT-qPCR-positive at that moment. After the first inoculation 2 out of 7 contact animals were infected.. The first contact infected animal became positive in RT-qPCR and VI at 11 dpi and 16 dpi, respectively (Fig. 1a-b), while the second contact infected animal was RT-qPCR-positive between 24 and 38 dpi (Fig. 1b).

The experimental infection was repeated by inoculating 2 out of 5 remaining BVDV negative susceptible animals, but none of the three remaining contact animals became infected. Compared to the first inoculation a lower degree of viraemia was obtained and the viral titre could not be determined. Nevertheless, both inoculated calves were positive in VI (Fig. 2a) and the nasal swabs were RT-qPCR-positive between 2 and 10 dpi (Fig. 2c).

BVDV-infection was confirmed by the development of neutralizing antibodies in all five inoculated animals (from 9 dpi on) and both contact infected animals (from 18 dpi and 30 dpi on). The remaining susceptible animals were BVDV seronegative until the end of the experiment.

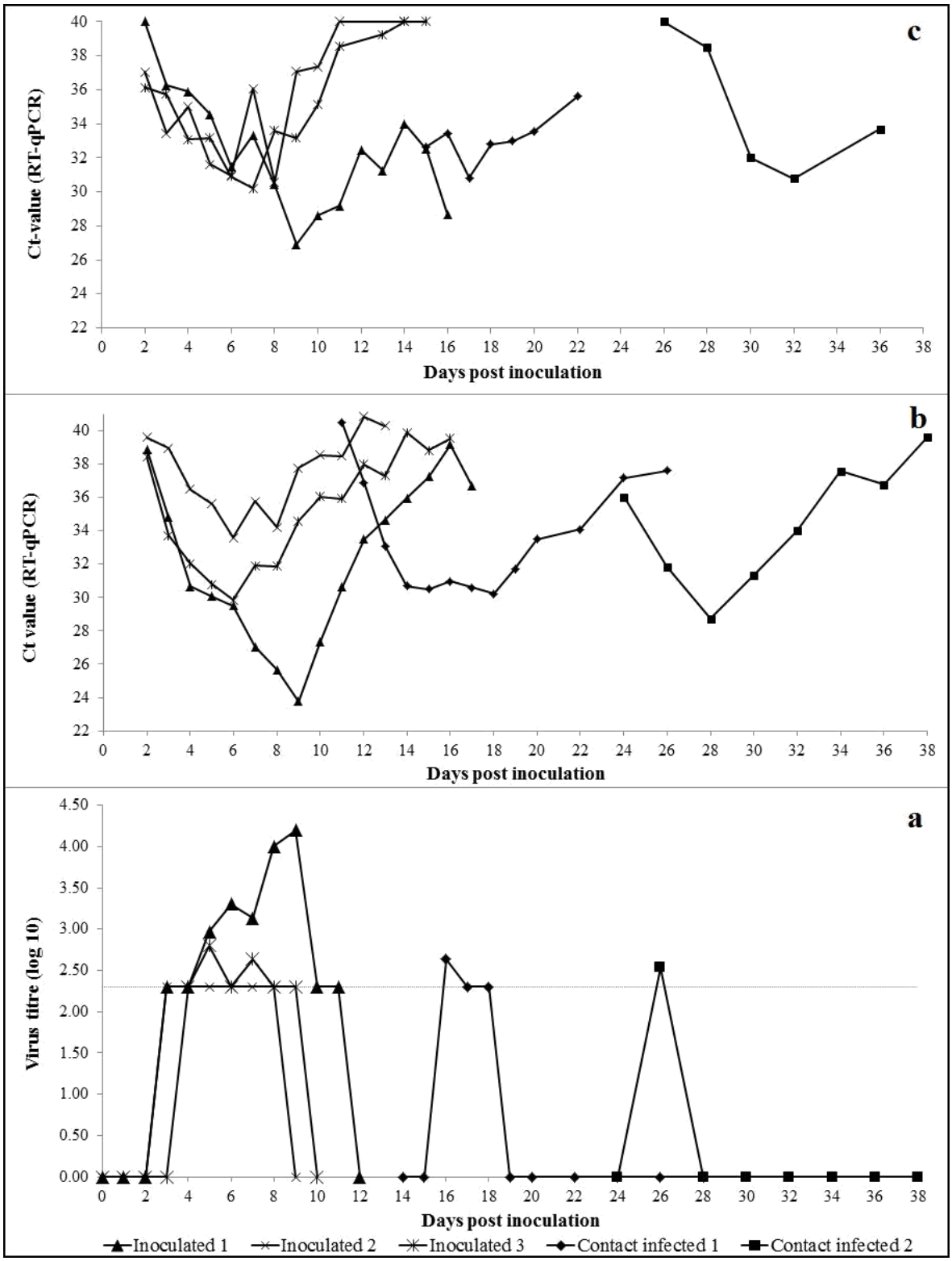

Fig. 1. Virological results from the first inoculation (three inoculated animals and seven susceptible animals). a) Viral titres (log10 $TCID_{50}$/mL) of positive blood samples of the inoculated animals and both animals infected by contact. The dotted line represents the detection limit (2.30). Samples represented on the detection limit are positive in virusisolation, but the viral titre was insufficiently high to determine a viral titre. b) Ct-values of RT-qPCR-positive blood samples. A lower Ct-value indicates a higher amount of viral RNA present in the sample. c) Ct-values of RT-qPCR-positive nasal swabs.

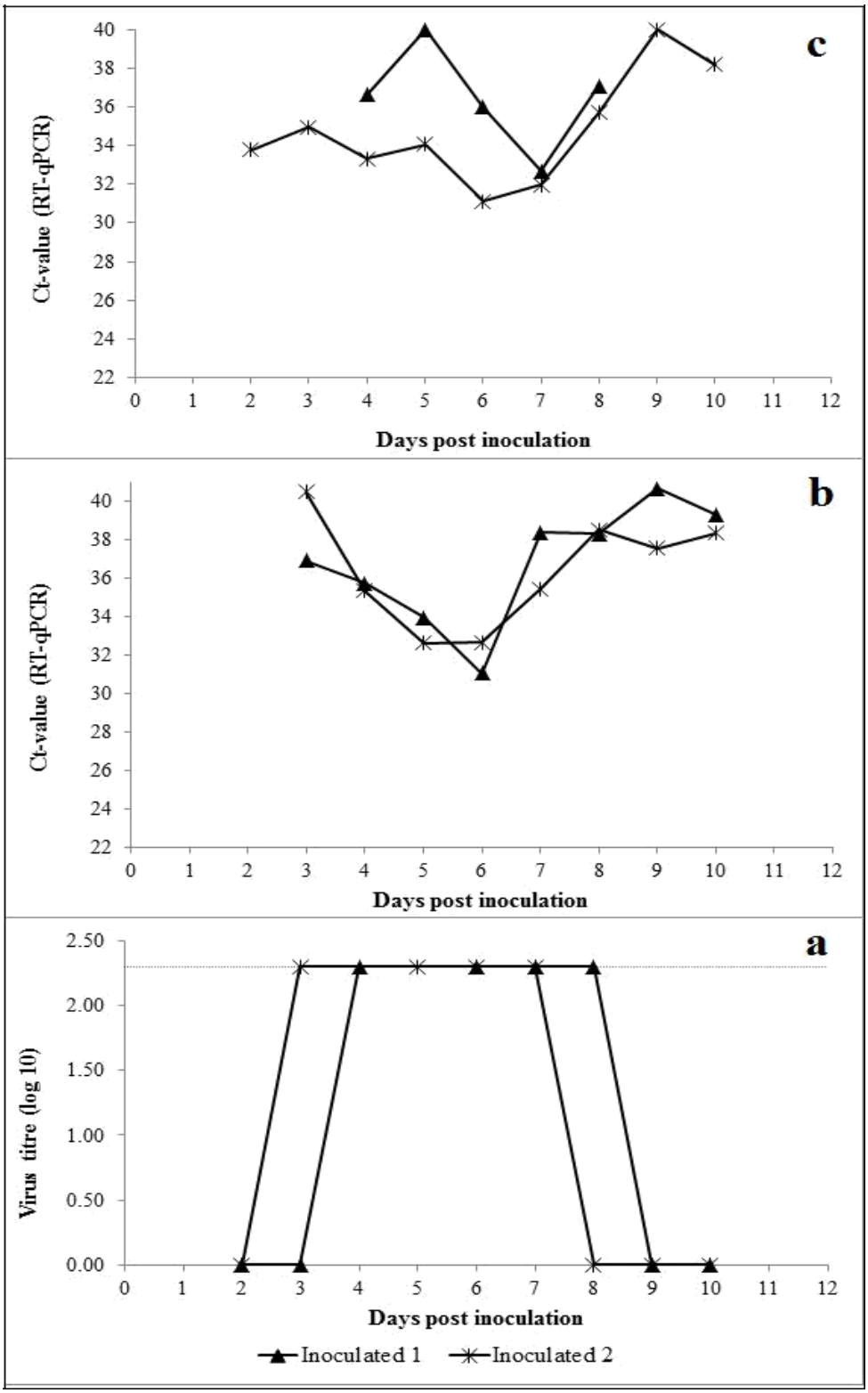

Fig. 2. Virological results from the second inoculation (two inoculated animals and three susceptible animals. a) Viral titres (log10 $TCID_{50}$/mL) of positive blood samples of the inoculated animals. The dotted line represents the detection limit (2.30). Samples represented on the detection limit are positive in virus isolation, but the viral titre was insufficiently high to determine a viral titre. b) Ct-values of RT-qPCR-positive blood samples. A lower Ct-value indicates a higher amount of viral RNA present in the sample. c) Ct-values of RT-qPCR- positive nasal swabs.

The combined MLE of R_0 for the first and second inoculation was 0.49 (95% CI 0.06; 2.99). Although statistically not significantly different from 1, this point estimate indicated that one typical infectious case introduced in a fully susceptible population would cause on average only 0.49 secondary infections and suggests that infection with TI animals infected with this BVDV-2c strain would theoretically fade out.

Following the first inoculation, the inoculated calves had a loss of appetite for 6 days starting 7 dpi (appetite score = 3). Together with fever between 4 and 11 dpi, loss of appetite was the only clinical sign that was noticed for one inoculated animal ("Inoculated 2", Fig. 1). The two other inoculated animals developed severe watery diarrhoea 8 dpi for 6 days (faeces consistency score = 2). While one of these animals ("Inoculated 3", Fig. 1) completely recovered 14 dpi, the other ("Inoculated 1", Fig. 1) became recumbent 14 dpi (posture score = 2, lying down score = 4) and died 17 dpi. The first and second contact infected animal both developed a haemorrhagic diarrhoea (faeces consistency score = 3) between 21 and 26 dpi and between 30 and 36 dpi, respectively. Both animals also lost appetite (appetite score = 3) during 3 consecutive days, but completely recovered 28 and 38 dpi, respectively.

Following the second inoculation few clinical signs were noticed. Both animals lost appetite for 2 days beginning at 7 dpi, were depressed (posture score = 2) and showed nasal discharge (nasal discharge score = 1) 8 dpi. All inoculated and contact infected animals showed fever (> 39.0 °C) during infection, with duration of pyrexia varying between 5 and 8 days. Compared to the RT-qPCR-negative calves leucocyte and thrombocyte counts were significantly lower in RT-qPCR-positive animals during both inoculations (Table 1).

Table 1. Comparison of haematology between RT-qPCR-negative and RT-qPCR-positive animals during both inoculations. During the first inoculation 3 inoculated animals infected 2 out of 7 susceptible animals in contact. A second inoculation was performed 63 days later, during which none of the 3 susceptible animals were contact infected by the 2 inoculated animals.

	RT-qPCR-negative		RT-qPCR-positive		*p*-value[a]
	Estimate	95% CI	Estimate	95% CI	
First inoculation					
Leucocytes (1000/µL)	9.0	8.0; 10.1	6.4	5.2;7.6	< 0.001
Thrombocytes (1000/µL)	486	394; 578	338	240;436	< 0.001
Packed cell volume (%)	29.7	27.1; 32.3	30.2	27.6;32.9	0.29
Haemoglobin (g/dL)	11.1	10.1; 12.1	11.3	10.3;12.3	0.14
Second inoculation					
Leucocytes (1000/µL)	8.8	7.2; 10.3	6.6	4.9;8.3	< 0.001
Thrombocytes (1000/µL)	487	295; 679	346	115;547	0.002
Packed cell volume (%)	27.4	25.8; 29.1	26.7	24.8;28.7	0.33
Haemoglobin (g/dL)	10.5	9.7; 11.3	10.4	9.5; 11.3	0.84

[a] Linear mixed model with "RT-qPCR-positive" (no/yes) and "Animal" as fixed and random effect, respectively (SAS 9.4, SAS Institute).

4. Discussion

Although pronounced clinical signs such as diarrhoea and death were observed during this study, these clinical observations were not as severe as encountered during the outbreak in Germany (Doll and Holsteg 2013). Several different BVDV field strains were isolated during this outbreak (Jenckel et al. 2014, 6984), which indicates that the BVDV-2c

strains were genetically highly variable. Two of these field strains were used in experimental infections. Results from the infection with the NRW 14-13 strain are described in the current study, while Jenckel et al. (2014, 6983) experimentally infected 8-week-old calves with NRW 19-13-8. Both strains were considered to have a high level of virulence (Jenckel et al. 2014, 6984). During the latter study all animals (n = 8) died acutely or had to be euthanized within 11 days. Given the high genetic variability in these BVDV-2c strains, changes in the genome with shifts in strain virulence following virus cultivation can possibly explain the difference in morbidity and mortality observed during our study on the one hand and encountered in the field and during the experimental infection of Jenckel et al. (2014, 6984) on the other hand. To avoid genetic changes as much as possible, the second passage on cell culture of the isolated virus was used. Another explanation for the observation of more mild clinical signs could be the age of calves, since age appears to have a key influence on the clinical manifestations associated with BVDV infection: more pronounced clinical signs are observed in younger animals (Hamers et al. 2003, 239). While in our study the calves were aged between 16 and 20 weeks and between 25 and 29 weeks at the moment of the first and second inoculation, respectively, the calves in the study of Jenckel et al. (2014, 6983) were only 8 weeks old. However, also adult cattle suffered from severe clinical disease during the outbreak in Germany (Doll and Holsteg 2013).

The clinical signs of the BVDV infected animals in the present study were varying from very mild disease to severe watery and haemorrhagic diarrhoea and death. The severity of the clinical signs was associated with the degree of viraemia. Animals with a higher viral titre (VI) and lower Ct-values (RT-qPCR) showed more severe clinical signs. This result is in agreement with comparable observations made by Walz et al. (2001, 1097-98). The amount of virus shed is likely to depend on the propensity of a strain to replicate (Bolin and Ridpath 1992, 2162) and could thus also be related to the degree of viraemia. The patterns of the RT-qPCR results of the nasal swabs and blood samples were indeed similar (Fig. 1b-c, Fig. 2b-c).

During the outbreak in Germany TI animals with a high viral titre (genomic load almost equivalent to that in PI animals) and long-lasting virus shedding (up to 8 weeks) were reported (Doll 2013). Moreover, during the experimental infection executed by Jenckel et al. (2014, 6984) very high viral loads in blood and organs (Ct-values < 20 in RT-qPCR) were shown in all animals. In a study comparing Ct-values of PI and TI animals, it has been shown that only 1 out of 57 TI animals obtained a Ct-value below 25, while only 1 out of 17 PI animals had a Ct-value above 25 (Hanon et al. 2014, 159). These results indicate that in the present study, very similar to the observations encountered in the field (Doll 2013), the animal "Inoculation 1" (Fig. 1) obtained a genomic load 8-9 dpi almost equivalent to that in PI animals, which indicates that this animal, at least for 1-2 days, has substantially spread BVDV in the environment. At the moment of its death (17 dpi) the animal was still RT-qPCR-positive, but negative in VI, which suggests that viral shed had stopped. The duration of viral shed was thus much shorter than 8 weeks as observed in the field (Doll 2013). To study the role of these TI animals with a high viral load, the transmission experiment could be repeated using a 'one to one' approach (one infective and one susceptible, repeated five times), since this method is preferred to study the most relevant characteristics of R_0 (Kroese and de Jong 2001, xxv).

Despite the presence of this animal with a high viral titre and four additional inoculated calves during two inoculations, a limited BVDV transmission was demonstrated. Nevertheless, since the nasal swabs of all BVDV infected animals (both inoculated and contact infected) were RT-qPCR-positive, it can be assumed they all had the potential to spread BVDV (Fig. 1c; 2c). This result supports the general assumption that TI cattle, unless infected in early gestation, have little contribution to

the overall BVDV spread, as already demonstrated before (Niskanen et al. 2000, 96; Niskanen et al. 2002, 254; Author year).

This result is in contrast with the observations in the field, where it is suggested that BVDV has spread solely through TI animals (Doll 2013). Nevertheless, thorough evidence is needed to prove that it is not due to PI animals that have escaped identification (Lindberg and Alenius 1999, 200; Lindberg and Houe 2005, 59; Laureyns et al. 2010, 22), as this is also suggested for the German outbreak (Jenckel et al. 2014, 6990). It might for instance have been the case that PI animals died before they were identified as PI. Furthermore, BVDV can also spread when PI animals are aborted or stillborn (Lindberg et al. 2004, 465). Other factors that may have hampered viral spread, such as the experimental conditions, under which animals are likely to be less challenged by other pathogens and environmental conditions have already been discussed (Author year).

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Appendix A: Clinical scoring system

Characteristic	Score
Posture (apathy)	0 = The calf is standing or lying down with the head held high. 1 = The calf is standing with the head kept down. 2 = The calf is lying down with the head on the ground. 3 = The calf is in lateral decubitus. When lying down, 0 = the calf stands up itself. 1 = the calf stands up when approaching. 2 = the calf stands up when touching. 3 = the calf needs a clear stimulus to stand up itself. 4 = the calf does not stand up itself, but does when pulled up. 5 = the calf does not stand up itself and does not keep standing when pulled up.
Nasal discharge	0 = No nasal discharge 1 = Clear nasal discharge 2 = Mucopurulent nasal discharge 3 = Blood
Coughing	0 = No coughing 1 = The calf coughs once 2 = The calf coughs occasionally (< 10 times during stable visit) 3 = The calf coughs regularly (> 10 times during stable visit)

Characteristic	Score
Respiration rate	0 = 20-24 per minute 1 = >40 per minute 2 = >80 per minute 3 = >80 per minute + difficulty to breath
Behaviour	0 = The calf comes towards the fence or looking at the person performing the clinical examination. 1 = The calf is standing with other calves. 2 = The calf is walking alone in the stable. 3 = The calf is standing alone and is not interested in the environment.
Appetite	0 = The calf starts eating immediately. 1 = The calf hesitates to eat, but finally eats everything. 2 = The calf hesitates to eat and does not eat much. 3 = The calf hesitates to come to the fence and does not eat much. 4 = The calf hesitates to come to the fence and does not eat. 5 = The calf does not come to the fence.
Faeces consistency	0 = Normal faeces consistency 1 = Fluid faeces consistency 2 = Watery faeces 3 = Blood in the faeces
Rectal temperature = … °C	

