



Failure to Spread Bovine Virus Diarrhoea Virus Infection from Primarily Infected Calves Despite Concurrent Infection with Bovine Coronavirus

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SUMMARY

Previous reports on the spread of bovine virus diarrhoea virus (BVDV) from animals primarily infected with the agent are contradictory. In this study, the possibility of transmission of BVDV from calves simultaneously subjected to acute BVDV and bovine coronavirus (BCV) infection was investigated. Ten calves were inoculated intranasally with BVDV Type 1. Each of the 10 calves was then randomly allocated to one of two groups. In each group there were four additional calves, resulting in five infected and four susceptible calves per group. Virulent BCV was actively introduced in one of the groups by means of a transmitter calf. Two calves, susceptible to both BVDV and BCV, were kept in a separate group, as controls. All ten calves actively inoculated with BVDV became infected as shown by seroconversions, and six of them also shed the virus in nasal secretions. However, none of the other eight calves in the two groups (four in each) seroconverted to this agent. In contrast, it proved impossible to prevent the spread of BCV infection between the experimental groups and consequently all 20 study calves became infected with the virus.

Following infection, BCV was detected in nasal secretions and in faeces of the calves and, after three weeks in the study, all had seroconverted to this virus. All calves, including the controls, showed at least one of the following clinical signs during days 3–15 after the trial started: fever ($\geq 40^\circ\text{C}$), depressed general condition, diarrhoea, and cough. The study showed that BVDV primarily infected cattle, even when co-infected with an enteric and respiratory pathogen, are inefficient transmitters of BVDV. This finding supports the principle of the Scandinavian BVDV control programmes that elimination of BVDV infection from cattle populations can be achieved by identifying and removing persistently infected (PI) animals, i.e. that long-term circulation of the virus without the presence of PI animals is highly unlikely.

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INTRODUCTION

Bovine virus diarrhoea virus (BVDV) is endemic in many cattle populations, causing significant disease problems and economic losses. During 1993–96, control programmes aimed at eradicating BVDV without the use of vaccines were initiated in Sweden, Norway, Finland and Denmark (Husu & Kulkas, 1993;

Olsson *et al.*, 1993; Bitsch *et al.*, 1994; Waage *et al.*, 1994; Lindberg & Alenius, 1999). A similar voluntary control programme has also been started in the UK (G. Gunn, personal communication). In these schemes, high priority is given to control measures directed towards the livestock trade, in order to control the spread of BVDV between herds.

The control measures are focused on excluding the main transmitters of the infection, the persistently infected (PI) animals, from the market, and also on preventing trade with dams that carry PI foetuses. Also to be taken into account is the risk of introducing the infection to susceptible herds by

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other transmission routes without the initial presence of a PI animal. As animals with an acute BVDV infection can have a transient period of viral shedding (Kirkland *et al.*, 1991; Bolin & Ridpath, 1992), direct contact with such animals could be sufficient for transmission to occur. However, reports on the ability of acutely infected animals to serve as a vehicle for the spread of BVDV are contradictory. Attempts to transmit the virus by direct contact between primarily infected and susceptible cattle have proved unsuccessful (Niskanen *et al.*, 2000). Furthermore, animals that were experimentally infected by insemination, by parenteral injection or by rectal examination, did not transmit the infection to susceptible in-contact animals (Pritchard, 1963; Meyling & Jensen, 1988; Lang-Ree *et al.*, 1994). On the other hand, field studies in large herds have shown different results and seroconversions have occurred during extended time-periods (up to 2.5 years), although the presence of PI animals was not detected and no direct contact with PI cattle was found (Barber *et al.*, 1985; Brownlie *et al.*, 1987; Meyling *et al.*, 1990; Moerman *et al.*, 1993; Edwards, 1997). These differences in transmission rates may be explained in part by interacting immunosuppressive factors relating to the virus strain, the environment, and the management procedures.

Furthermore, primarily BVDV infected animals could possibly play a more significant role in the spread of BVDV infection in the presence of co-infections. For example, the rapid virus transmission reported by Alenius *et al.* (1991) may have been due to the fact that a concurrent bovine coronavirus (BCV) infection was present. Co-infecting agents may influence the infectivity of BVDV by modifying the immunological response of the host, or increase the amount of virus excreted by aggravating clinical signs such as cough, nasal secretions and diarrhoea.

Bovine coronavirus is a common bovine pathogen (Storz & Rott, 1980; Battaglia *et al.*, 1986; Paton *et al.*, 1998; Tråvén *et al.*, 1998) and co-infections with this virus and BVDV probably occur frequently. BCV is known mainly as an enteric agent, but this virus also causes respiratory tract infections in calves (McNulty *et al.*, 1984; Heckert *et al.*, 1990; Storz *et al.*, 1996, 2000). The disease is highly contagious, but calves commonly recover from uncomplicated BCV infections within a few days (Tråvén, 2000). The experiment described in this paper allowed us to study the transmission of BVDV, from calves simultaneously subjected to acute BVDV and BCV infection, to susceptible BCV-infected calves.

MATERIAL AND METHODS

Calves

Twenty-one conventionally reared calves, 18 males and three females, were obtained from two different closed dairy herds. The herds were certified as free from BVDV infection under the Swedish Control Scheme on BVDV (Alenius *et al.*, 1996). The calves were either Swedish Red and White breed (14 both sexes) or Friesian (four males) and aged between 59 and 165 days on arrival at the clinic. They were housed at the experimental unit for one week prior to the experiment, in order to avoid stress effects due to transportation and change of environment. All calves were shown to be free from BVDV, and were antibody-negative to BVDV and to BCV by tests undertaken both in the herd of origin and on the day of arrival. No other cattle were kept on the premises during the trial.

Viral sources and inoculation procedure

Foetal calf serum with a non-cytopathogenic BVDV Type 1 strain in a titre of 5×10^5 TCID₅₀ per mL was used to introduce BVDV infection. The inoculum was given intranasally, with 2.5 mL sprayed into each nostril.

The BCV infection was introduced to the experimental calves by contact with a BCV-inoculated transmitter calf, as previously described (Tråvén *et al.*, 2001). Briefly, BCV-containing faeces collected from cows during a winter dysentery outbreak were stored at -70°C with 10% DMSO before use. On day -1 , 100 mL of a 50% faecal suspension in phosphate-buffered saline was given through a stomach tube to a two-month-old calf that was kept in solitary in an outdoor pen. In addition, 3 mL of the faecal suspension was instilled into each nostril. Two days after inoculation this transmitter calf was introduced to the experimental calves.

Experimental design

Eighteen calves were matched into pairs according to age, sex, breed and herd of origin. The calves were then randomly allocated to one of two study groups (A and B) until each pair was represented by one calf in each group. Two additional calves were kept as uninfected controls (Group C). The three groups were housed in two separate units. Groups B and C were kept in the same unit but in separate pens divided by a floor-to-roof wall. The units had separate manure-shutters and their own extractor fans for evacuation of stable air. Fresh air entered the units via door openings. The units were closed with

a sliding door in order to reduce the risk of airborne transmission of BCV between groups B+C and A within the building.

On the day before the trial started, five calves from group A and B were randomly selected and put in an isolated pen. On day 0, they were inoculated intranasally with BVDV. The staff that performed all clinical examinations and sampling during the study did not know which calves had been inoculated with BVDV. On day 1, the calves were returned to their original groups. Then all the calves in group A were infected with BCV by placing them in contact with the BCV transmitting calf for 2 h. All calves in group A had nose-to-nose contact with the BCV transmitter twice during the contact period, and they were not allowed to lie down. The calves were repeatedly made to move around in the pen to increase the opportunity for transmission. The BCV transmitter did not defaecate during the 2 h in the pen. Directly after removal from group A the BCV transmitter left the experimental unit.

All personnel in contact with the calves changed their coats and boots and donned disposable gloves before entering each pen. To minimize the risk of BCV transmission to groups B and C, the staff worked, during any single day, with either group B or group C (always starting with group C) or with group A alone.

Sampling procedures

Serum samples – Blood samples were collected from each of the 20 study calves daily from day –1 upto and including day 17, and then on days 22, 29 and 36. All samples were drawn from the jugular vein using evacuated tubes (Becton-Dickinson). They were then centrifuged for 10 min at 3000 g. The sera were transferred to Ellerman tubes and stored at –20°C until analysis.

Nasal swabs and faecal samples – Nasal swabs (Virocult; Medical Wire & Equipment Co.) for BVDV detection were taken from each of the study calves on days 5, 7, 8 and 9, and for BCV detection on days 3 and 13 (2 and 12 days after BCV was introduced into group A).

Faecal samples for BCV detection were taken directly from the rectal ampulla of all calves on day –1, days 0–15, 17, 22, 29 and 36. Both nasal swabs and faecal samples were stored at –70°C until analysis.

Clinical examinations, analyses of clinical data and treatments with antibiotics

The study calves were subjected to clinical examinations from day –1 upto and including day 15. At examination the following parameters were assessed: body temperature, general condition, faecal consistency, respiratory rate and sounds on auscultation. In addition, the presence of cough or any conjunctival or nasal discharge was recorded.

The degrees of diarrhoea, coughing, respiratory sounds on auscultation, nasal and conjunctival discharge were converted to numeric values. The values ranged from 1 to 3, corresponding to a mild, moderate, and severe degree. Similarly, body temperatures between 40.0–40.5°C, 40.6–41.0°C and >41.0°C and respiratory rates between 51–80, 81–110 and >110 per minute were assigned the numerical values 1, 2 and 3. Individual scores for general condition, body temperature, diarrhoea and respiratory tract involvement were calculated. For example, for a specific calf, all the daily values for body temperature were summed to produce a cumulative body temperature score for that calf. The respiratory score included scores for respiratory rate, respiratory sounds on auscultation, nasal and conjunctival discharge and coughing. The Wilcoxon rank-sum (Mann–Whitney) test as applied in Stata software (Stata Corp.) was used to test whether the difference in distribution of clinical scores was significant between BVDV- and BCV-infected calves *versus* calves infected with BCV only.

Detection of antibodies to BVDV and BCV, isolation of BVDV and detection of BCV-antigen

Indirect enzyme-linked immunosorbent assays (ELISAs) (Svanova Biotech) were used to determine seroconversions to BVDV and BCV (Juntti *et al.*, 1987; Alenius *et al.*, 1991). Samples generating an absorbance value ≥ 0.20 were regarded as positive for BVDV and BCV antibodies. The presence of BVDV on nasal swabs was detected in an immunoperoxidase test (Meyling, 1984). BCV antigen on nasal swabs and in faecal samples was detected using a single-coated antigen ELISA (Svanova Biotech). Samples generating an absorbance value ≥ 0.20 , were regarded as positive for BCV. The Wilcoxon rank-sum (Mann–Whitney) test was used to see whether there was a significant difference in the number of days with detectable BCV faecal shedding between dually infected calves and calves infected with BCV only.

RESULTS

BVDV shedding and antibody responses

All 10 calves that were intranasally inoculated with BVDV became infected with the virus and seroconverted to BVDV between days 15 and 36 post-infection (p.i.). However, no seroconversion was detected in the other eight calves in groups A and B (Fig. 1). The two control calves also remained seronegative to BVDV throughout the observation period.

BVDV was detected in nasal swabs on day 5 p.i. from six of the 10 calves intranasally inoculated with the virus, two in group A and four in group B. Two of the four positive calves in group B were also positive on day 7 p.i., and one was still positive on days eight and nine p.i. (Table I). BVDV was not isolated from any other nasal swab.

BCV shedding and antibody responses

All 20 study calves, irrespective of trial group, became infected with BCV. The mean absorbance values in each group on days 0, 14, 17 and 22 are

shown in Table II. All but three calves had seroconverted to BCV by day 12 or 14 p.i. One more calf in each group (A and B) had seroconverted when tested on day 21 and the remaining calf (in group B) showed seroconversion on day 16 p.i. These three calves had all been inoculated with BVDV.

BCV was detected in nasal swabs and/or faecal samples from 15 calves altogether. Faecal shedding was detected in six calves in group A, three in group B and in both calves in group C. The calves shed BCV for upto four days in faeces (Table I). The faecal shedding of BCV occurred about two days later in groups B and C than in group A. In total, nine of 10 calves infected with BCV alone shed BCV in faeces, while only three of 10 calves infected with both agents shed BCV in faeces at detectable levels. The number of days of faecal shedding of BCV in faeces was significantly lower ($P < 0.05$) in the dually infected calves than in the calves infected with BCV only. Four calves in group A and seven in group B shed BCV in nasal secretions on day three and/or day 13. The control calves both shed BCV in nasal secretions on day 13 (Table I).

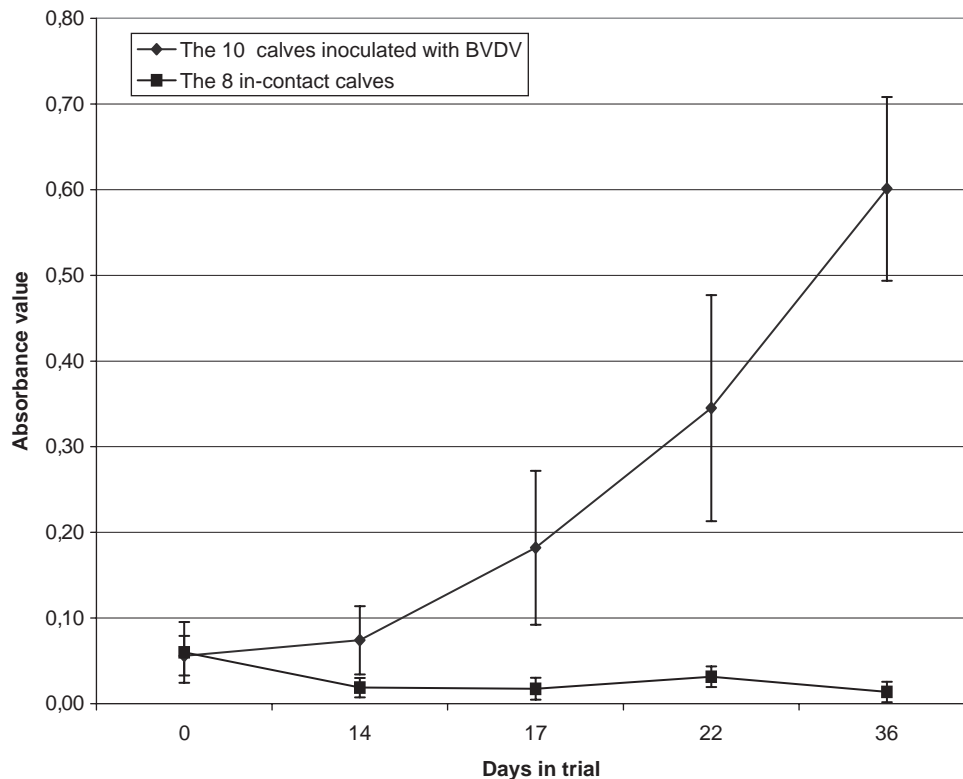


Fig. 1. BVDV antibodies *in sera* (diluted 1 : 25) from 10 calves inoculated with the virus and eight calves in close contact with these primarily infected calves. Antibodies to BVDV was measured with an indirect ELISA and the samples generating an absorbance value ≥ 0.20 were regarded as positive. The results are expressed as mean absorbance values at 450 nm \pm SD.

Table I

Presence of BVDV in nasal swabs (+), BCV in nasal swabs (●) and in faecal samples (○) in calves following infection with both viruses, or with BCV only. Five calves each in both groups A and B were inoculated intranasally with BVDV. BCV was introduced into group A by a transmitter calf orally and intranasally inoculated with the agent. Nasal swabs were taken from each calf on days 5, 7, 8 and 9 for detection of BVDV and on days 3 and 13 for detection of BCV. Faecal samples were collected daily throughout the study period

Calves	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13
<i>Group A</i>															
1 ^a							○	○	○	○					
2 ^a							+								
3 ^{a,b}															●
4 ^a															
5 ^a							+			○					
6								○		○					
7 ^b									○	○					●
8									○						●
9					●					○					●
<i>Group B</i>															
10 ^a					●		+		+						●
11 ^a					●		+								
12 ^a							+								
13 ^{a,b}					●								○		
14 ^{a,b}					●		+		+	+	+				
15 ^b					●										
16 ^b					●					○	○				●
17									○	○	○	○			
18										○					
<i>Controls</i>															
19										○					●
20											○				●

Days in trial

^a Calves inoculated with BVDV;

^b Calves treated with antibiotics.

Clinical disease

All calves, including the controls, showed clinical signs following the infections. Signs including fever ($\geq 40^\circ\text{C}$), depressed general condition, diarrhoea and cough were observed between days three and 15. The median values of the clinical scores for general condition were 4.0 and 2.5 ($P=0.07$) respectively, for calves infected with both BVDV and BCV and for calves infected with BCV only (Table III). The corresponding values for body temperature were 7.5 and 3.5 ($P=0.11$).

In total, 15 calves had diarrhoea. Eight of those were dually infected and seven were infected with BCV only. The diarrhoea lasted for 1–5 days, with median clinical scores of 5.0 and 2.0 ($P=0.14$) for

calves with dual and single infection, respectively (Table III).

The respiratory tract involvement showed a similar pattern for all calves irrespective of their coinfection status. On days 5–6 and 15, the calves had an increased respiratory rate and increased respiratory sounds on auscultation. In addition, conjunctival and nasal discharges were present and the calves were coughing. The median values of the clinical scores reflecting respiratory tract involvement were 59.0 and 53.5 respectively, for dually infected calves and for calves infected with BCV only (Table III).

Six calves that had extended periods of fever (6–10 days) were treated with antibiotics (benzylpenicillin procaine) for 3–7 days between days 13 and 20.

Table II

Antibodies to BCV in sera (diluted 1 : 25) from 20 calves following infection. A calf orally and intranasally infected with BCV introduced the infection into group A. An indirect ELISA was used to determine the level of antibodies and samples generating an absorbance value ≥ 0.20 were regarded as positive. The results are expressed as mean absorbance values determined at 450 nm \pm SD

Group	Mean absorbance value (SD)			
	Day 0	Day 14	Day 17	Day 22
A (n=9)	0.01 (0.01)	0.48 (0.30)	0.74 (0.38)	0.99 (0.42)
B (n=9)	0.02 (0.06)	0.21 (0.13)	0.71 (0.42)	1.04 (0.35)
C (n=2)	0.01 (0)	0.33 (0)	0.82 (0.21)	1.26 (0.04)

Table III

Clinical scores for general condition, body temperature, diarrhoea and respiratory tract involvement. General condition, body temperature, respiratory rate and degree of diarrhoea, coughing, respiratory sounds on auscultation, conjunctival and nasal discharge were scored as described in Material and Methods. Then, the individual scores were calculated for these parameters by adding up the daily values for each calf. For example, for a specific calf, all the daily values for body temperature were added to produce a cumulative body temperature score for that calf

Calves infected with	Clinical scores			
	General condition Median (range)	Body temperature ($^{\circ}$ C) Median (range)	Diarrhoea Median (range)	Respiratory tract involvement Median (range) ¹
BVDV and BCV (n=10)	4.0 (1–13)	7.5 (2–23)	5.0 (0–22)	59.0 (39–77)
BCV (n=10)	2.5 (0–7)	3.5 (1–18)	2.0 (0–10)	53.5 (30–109)

¹Includes respiratory rate, respiratory sound, nasal and conjunctival discharge and coughing.

The two infectious statuses were equally represented among these calves, but four of them belonged to group B (Table I).

DISCUSSION

In this study there was no transmission of BVDV from calves simultaneously subjected to acute BVDV and BCV infection, whereas all study calves became infected with BCV. These BVDV results are in agreement with previously published studies (Lang-Ree *et al.*, 1994; Niskanen *et al.*, 2000) and confirm the experiences from ongoing BVDV-control programmes that the virus is not easily transmitted to susceptible cattle by primarily infected animals. On the other hand, the results clearly show that introduction of a BCV-infected animal can result in a rapid spread of BCV to all susceptible animals within a unit. This is consistent with the high morbidity described in diarrhoea outbreaks associated with

BCV (Durham *et al.*, 1989; Saif, 1990; Alenius *et al.*, 1991).

All 10 calves inoculated with BVDV became infected, as shown by seroconversion, and six of them were found to shed virus in nasal secretions. Susceptible in-contact calves were exposed to the acutely BVDV-infected calves and to their excretions by being housed together in the same pens throughout the experiment. Furthermore, BCV was introduced which should, in theory, facilitate the transmission of BVDV by causing increased nasal secretions, cough and diarrhoea, clinical signs that were indeed shown by the dually infected calves. In addition, co-infection as with BCV in this trial may reduce the non-specific resistance and consequently lower the BVDV dose required for infection of the BVDV-susceptible in-contact calves. However, the timing of the co-infection may be crucial. The introduction of BCV one day after the BVDV inoculation was chosen to ensure maximum onset of

clinical signs from the BCV infection at the time when BVDV excretion was expected, i.e. 4–9 days after BVDV inoculation (Bruschke, 1998). Also, BCV was introduced before the expected interferon Type 1 response to the BVDV infection on days 3–4 (Tråvén *et al.*, 1991), in order to minimize the inhibitory effect on BCV replication. By the inadvertent transmission of BCV to group B, we also had an opportunity to study the effect of BCV infection introduced 2–3 days after the BVDV infection, as suggested by BCV shedding in faeces. Yet no transmission of BVDV occurred in either group. However, the introduction of BCV before BVDV was not studied in this experiment. It is possible that the susceptibility of the calves to the BVDV challenge could have been increased by a preceding BCV infection. Factors contributing to the failure of BVDV transmission may have been low viral excretion by the primarily infected calves, and low virulence of the non-cytopathogenic BVDV strain used. The variation in virulence, as reflected by clinical signs, between BVDV isolates is considerable. For example, animals infected with certain BVDV Type 2 strains show serious illness and have high virus titres in serum for a prolonged period of time (Corapi *et al.*, 1990; Bruschke, 1998; Carman *et al.*, 1998). It is also possible that the spreading of the virus could have been demonstrated if a cytopathogenic biotype of BVDV strain had been used in the experiment. Furthermore, the transmission of BVDV might have been more successful if the calves, both transmitters and susceptible in-contact calves, had been exposed to environmental, nutritional and/or transportation stresses.

BCV transmission from the transmitter calf to the calves in group A probably occurred by contact with nasal secretions and/or by aerosol, since faecal material was not introduced into the pen of group A. Despite the rigorous measures taken to prevent the spread of BCV infection, all calves (including groups B and C) became infected with this virus. BCV infection is highly contagious and infected calves frequently propagate the virus via the enteric and respiratory tracts simultaneously (Saif *et al.*, 1986; Heckert *et al.*, 1990). We tried to prevent the spread of BCV (via both direct and indirect routes) between groups by housing the calves in two separate units (group A in one unit and groups B and C in separate pens in another unit). The units were located approximately 30 m apart in separate corridors. They also had separate manure-shutters. Furthermore, the personnel taking care of and examining the animals only worked with calves in one of the units during

any one single day and protective coats and boots were worn when entering each pen. In addition, the units had separate air ducts with extractor fans to evacuate stable air. Even so, air may still have been circulating within the building. Early BCV-shedding (day 2) was detected in nasal secretions, indicating replication of the virus in the upper respiratory tract. This supports our hypothesis that the rapid spread of BCV between units was due to aerosol transmission.

All calves infected exclusively with BCV became diseased, which was regarded clinically as a sign of alimentary and respiratory tract involvement, i.e. diarrhoea, nasal and conjunctival discharge and coughing. BCV was found in nasal secretions and in faeces of the calves and the infection was confirmed by seroconversions in all calves. However, the faecal excretion of BCV was significantly lower ($P < 0.05$) in the dually infected calves than in calves infected with BCV only. This could indicate a direct or indirect interference of BVDV with BCV replication, possibly mediated by BVDV-induced interferon Type 1 (Tråvén *et al.*, 1991). A virulent field strain of BCV, originating from an outbreak of winter dysentery in adult cows, was used to introduce BCV infection in the experimental calves. The clinical disease shown by the calves is consistent with previously published experimental studies demonstrating that the outcome of the BCV infection is largely unaffected by both the origin of the virus (cow or calf isolate) and the route of inoculation (nasal or oral) (Saif *et al.*, 1986; El-Kanawati *et al.*, 1996; Tråvén *et al.*, 2001).

In conclusion, all of the study calves became infected with BCV, whereas none of the calves in close contact with the 10 primarily BVDV-infected calves did. We conclude that spread of BCV infection is very difficult to prevent within a cow-house. To prevent the spread of BCV between herds, all direct and indirect contacts with herds with current or recent outbreaks of diarrhoea among cows and/or calves should be avoided. Furthermore, we suggest that spread of BVDV infection is not likely to occur during periods when the only source of virus is primarily infected animals. Consequently, BVDV does not seem to be a very contagious agent capable of causing acute epizootic diarrhoea in cattle herds, provided no PI animals are introduced into the herds. Therefore, it seems highly likely that the early reports of BVDV in connection with outbreaks of acute epizootic diarrhoea actually described a scenario where other enteric pathogen(s), for example BCV, co-existed with BVDV but escaped detection due to lack of appropriate virus diagnostic methods.

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