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DIFFERENCES IN EXPERIMENTAL VIRULENCE OF BOVINE VIRAL DIARRHOEA VIRAL STRAINS ISOLATED FROM HAEMORRHAGIC SYNDROMES

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ABSTRACT

In the late 1980s, a new hypervirulent and epidemic form of bovine viral diarrhoea virus (BVDV) infection appeared in North America. A similar but sporadic syndrome was later reported in Europe. To compare the pathogenic characters of the North American and European hypervirulent strains, we inoculated BVDV naïve calves with BVDV strains isolated from haemorrhagic syndromes originating in Belgium, France and the USA. The experimental procedure comprised daily clinical examination and measurement of blood and virological parameters.

The American BVD890/256 strain induced severe thrombocytopaenia, profuse diarrhoea and pneumonia in all calves, indicating that hypervirulent BVDV could be the primary infectious agent of pneumonia. Interestingly, a strong correlation was observed between the intense viraemia and a decreased platelet count. None of the European strains tested induced significant pathological signs, although isolated from cases presenting haemorrhagic syndrome.

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INTRODUCTION

Bovine viral diarrhoea (BVD) was first identified in 1946 (Olafson *et al*., 1946), and for 40 years acute BVD virus (BVDV) infection has been associated with a contagious mild to inapparent disease of cattle with low mortalities. In 1953, Ramsey and Chivers described another condition of cattle, called mucosal disease (MD), resulting from the superinfection by a cytopathic BVDV strain from an animal persistently infected with an antigenically similar non-cytopathic BVDV strain (Liess *et al.*, 1974; Brownlie *et al*., 1984). Unlike BVD, MD was, however, described as a highly fatal but sporadic disease. In the late 1980s, a new hypervirulent form of BVD, characterized by high morbidity and mortality rates appeared in North America (Corapi *et al.*, 1989). Affected cattle presented, besides severe hyperthermia and leucopenia, a thrombocytopaenia resulting in extensive bleeding lesions. Mortality rates sometimes reached over 30%, even in adults. Since that time, many cases of hypervirulent BVD have been reported in Canada and the USA (Rebhun *et al*., 1989; Pellerin *et al*., 1994; Bolin & Ridpath, 1992), including a respiratory form (Carman *et al*., 1998; Odeon *et al*., 1999). Experimental reproduction of hypervirulence has been reported with some strains (Corapi *et al.*, 1989; Bolin & Ridpath, 1995; Odeon *et al*., 1999).

Genetic sequencing of BVDV isolated from outbreaks of the hypervirulent form of the disease, revealed significant differences compared to conventional BVDV strains (Pellerin *et al.*, 1994). These differences led to the segregation of BVDV strains (Ridpath *et al.*, 1994) into a genotype 1, comprising many conventional strains and a genotype 2, which includes most hypervirulent strains. Phylogenic studies have shown that genotype 2 also comprises several low or moderately virulent strains. In Europe, the first clinical reports of haemorrhagic syndrome associated with BVDV were described in the early 1990s (Broes *et al.*, 1992; Thiel, 1993; Lecomte *et al*., 1996). Clinical signs comprised hyperthermia, diarrhoea and extensive bleeding lesions associated with a severe thrombocytopaenia. The European cases, however, differed from North American ones by the absence of an epidemic character. Moreover, the experimental reproduction of severe disease by inoculation of a European BVDV strain has never been reported (Hamers *et al.*, 1999).

To compare the pathogenic character of the North American and European so-called hypervirulent strains, we inoculated BVDV naïve calves with BVDV strains isolated from cases of haemorrhagic syndrome, originating in Belgium, France and the USA. Only the American BVD890/256 strain reproduced a thrombocytopaenic syndrome.

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MATERIAL AND METHODS

VIRUS

BVD890/256 is a clone of the American type II BVD890 strain (Bolin & Ridpath, 1992; Bolin & Ridpath, 1995). The strain was kindly provided by Professor S.R. Bolin and, after reception, was multiplied once in bovine turbinate cells prior to inoculation.

Culi 4 strain was isolated from a six-week-old Belgian blue calf, which died three days after onset of respiratory troubles. Postmortem examination revealed extensive haemorrhagic lesions on the heart, digestive tract, kidneys and cerebellum. Virus isolated from the spleen was multiplied once on Madin Darby Bovine Kidney (MDBK) cells prior to inoculation.

Culi 6 strain was isolated from a three-month-old Belgian blue calf which died within a few hours of the appearance of dyspnoea. Postmortem findings included extensive bleeding lesions in the muscles, digestive tract, bladder, peritoneum, pleura and thymus. Virus isolated from the spleen was inoculated either as filtered spleen extract or after one multiplication cycle on MDBK cells.

L256 strain originated from France and was isolated from the leucocytes of a 26-month-old heifer presenting with thrombocytopaenia associated with epistaxis, gingival, vulvar and conjunctival haemorrhages. L256 strain was passaged twice on MDBK cells before inoculation.

None of the culi 4, culi 6 or L256 strains showed cytopathic effects in cell culture. Genetic typing by selective RT-PCR amplification of the 5′UTR end (Letellier *et al*., 1999) revealed that culi 4, culi 6 and L256 belong to the genotype I.

All viral suspensions were titrated after inoculation to confirm infectious titres of inoculums. Additionally, strains Osloss nc and CD87 (kindly provided by Professor E.J. Dubovi) were used for the seroneutralization tests.

CALVES AND INOCULATION SCHEME

Two successive series of nine and seven Danish crossbred calves, aged between 27 and 52 days, and negative for BVDV antibodies and antigen, were housed individually in isolation facilities. In the first series, calves were inoculated by pairs, respectively with L256, culi 4 and culi 6 strains passaged once in cell culture. Another pair of calves was inoculated with the culi 6 spleen extract while the last calf served as control. In the second series, six calves were inoculated with strain BVD890/256 while one calf served as control. The inoculation titre and route are presented in Table I.

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Table 1. *Inoculation scheme used for experimental inoculation of calves*

	Number of calves	*Mean age at day 0*	*Strain*	*Cell culture passage*	*Dose and route*
Series 1	2	48	L256	2	7.6×10^6 $CCID_{50}$ I.N. +3.6×10^6 $CCID_{50}$ I.V.
	2	47	Culi 4	1	1.7×10^6 $CCID_{50}$ I.N.
	2	43	Culi 6	1	9.5×10^5 $CCID_{50}$ I.N.
	2	44	Culi 6	0	5.7×10^3 $CCID_{50}$ I.V.
	1	51	Control	–	–
Series 2	6	45	BVD890/256	1	4.2×10^5 $CCID_{50}$ I.N.
	1	39	Control	–	–

Légende de la table. Viral titres were determined by titration of viral suspension after inoculation of calves. I.N.: intranasal route; I.V.: intravenous route. Age is in days. Cell culture passage indicates the number of passages in cell culture between isolation (or reception for strain BVD 890/256) and inoculation.

CLINICAL EXAMINATION AND SAMPLING

All calves were examined before inoculation on day -3 and day 0. After inoculation, the animals were examined daily. Clinical examination comprised measurement of rectal temperature, control of feed intake, diarrhoea, respiratory signs, behaviour, assessment of gingival, nasal and ocular mucosa. Blood samples and nasal swabs were taken either daily (second series) or every other day (first series), as shown in Table II.

Calves presenting with severe dyspnoea, dehydration, excessive weakness or any other serious health deterioration were euthanased.

Table 2. *Mean thrombocyte counts (x 1000/μ L)*

	First series					Second series	
	L256 (n = 2)	Culi 4/1 (n = 2)	Culi 6/0 (n = 2)	Culi 6/1 (n = 1)	Control (n = 1)	*Control (n = 1)*	*BVD890/256 (n = 6)*
day – 3	1477	827	821	1200	1004	637	738
day – 2	–	–	–	–	–	–	–
day – 1	–	–	–	–	–	586	693
day 0	1336	779	880	1121	968	561	684
day + 1	–	–	–	–	–	574	726

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day + 2	1194	668	785	1044	886	–	–
day + 3	–	–	–	–	–	–	–
day + 4	1010	671	725	937	868	604	578
day + 5	896	625	715	922	781	562	586
day + 6	–	–	–	–	–	509	558
day + 7	914	732	728	983	976	583	456
day + 8	–	–	–	–	–	562	322
day + 9	895	735	726	896	719	483	168
day + 10	–	–	–	–	–	401	87
day + 11	948	788	778	964	719	501	63
day + 12	924	657	710	896	646	510	97
day + 13	–	–	–	–	–	500	129
day + 14	982	774	743	801	635	499	99
day + 15	–	–	–	–	–	490	166
day + 16	1002	748	751	708	556	–	–
day + 17	–	–	–	–	–	–	–
day + 18	958	731	503	717	602	510	522
day + 19	–	–	–	–	–	488	635
day + 20	–	–	–	–	–	404	682
day + 21	934	828	801	748	593	–	–

n: number of calves inoculated. For strain BVD890/256, individual values as low as 20 (x 1000/μ L) platelets were recorded on several occasions (data not shown).

BLOOD PARAMETERS

Within 4 h of collection, blood samples were examined for red blood cells (RBC), leucocytes, packed cell volume (PCV), haemoglobin, and thrombocytes. Cell counts were performed on a Coulter Counter ZM and thrombocytes were measured on a Technicon sorter. For all euthanased calves, bone marrow cytology on proximal femur aspirate (Relford, 1991) was undertaken to assess megacaryocyte morphology.

Figure 1. *Mean rectal temperature in °C (dotted line) and NS3 (p80) antigen assay (plain line). Control values are those from the control animal of the second series; BVD 890/256 values are mean values of all animals (n = 6) inoculated with strain BVD890/256. Antigen assay, expressed in optical density, was performed with Serelisa BVD – MD AG mono indirect test (Synbiotics).*

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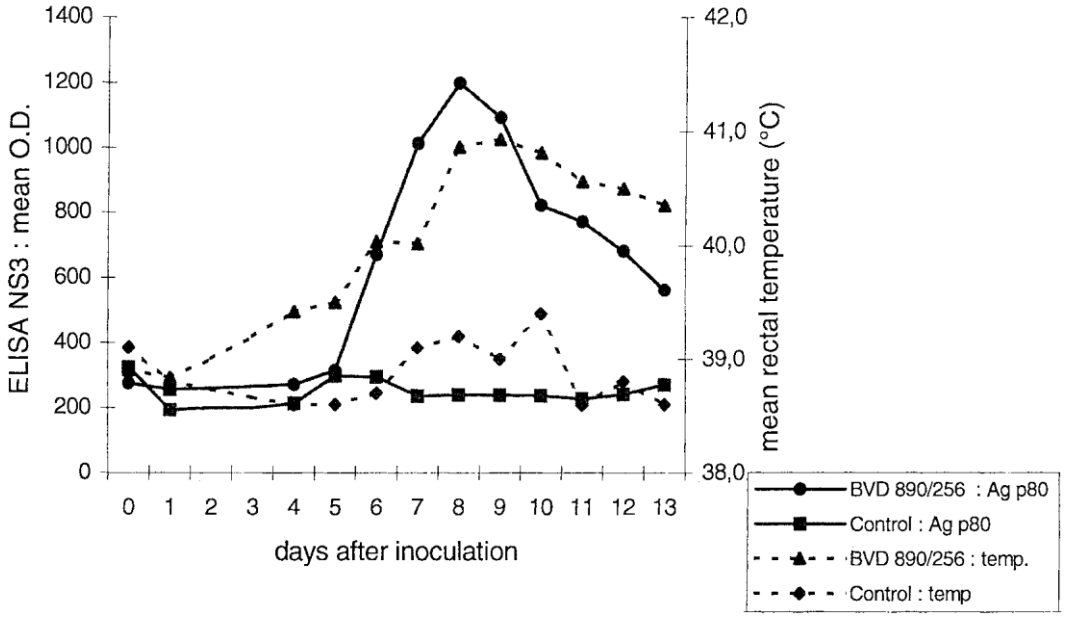

VIROLOGICAL PARAMETERS

Detection of viral antigens in buffy coat was performed with Serelisa BVD – MD AG mono indirect test (Synbiotics), according to the manufacturer's recommendations.

Detection of virus in nasal swabs was done by viral isolation in cell culture followed by indirect immunofluorescence assay, as described by Boulanger *et al*. (1991). In the first series, virus isolation tests were also performed on buffy coats.

Seroneutralization tests : sera from all calves were tested for neutralizing antibodies against two references strains: Osloss nc was used as the type 1 reference strain while CD87 served as the type 2 reference strain. Seroneutralization tests were conducted in microtitre plates containing confluent monolayers of calf testicular cells. All sera were tested in duplicate in twofold dilution, starting at dilution 1:4, against approximately 300 $CCID_{50}$ of virus. The end-point dilution was read by indirect immunofluorescence staining, using a pool of monoclonal antibodies against E0, E2 and NS3 as primary antibody (Boulanger *et al.*, 1991).

RESULTS

For both series, control calves remained healthy throughout the experiment. No BVDV antigen nor BVDV antibodies were detected in either of the two controls.

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CLINICAL FINDINGS

First series (European stains): as already published elsewhere (Hamers *et al*., 1999), none of the infected calves presented any significant clinical sign and rectal temperatures always remained within physiological limits (Fig. 1).
Second series (BVD890/256): all infected calves developed severe clinical signs comprising profuse diarrhoea starting 10 ± 0.9 days after inoculation (DAI), associated with anorexia; severe weakness was seen in three of the six infected calves. In four calves, diarrhoea persisted until euthanasia. All infected calves presented with important hyperthermia (> 40°C), as shown in Figure 1, starting on average 6.7 ± 1.5 DAI and persisting until euthanasia. All animals also developed respiratory signs comprising coughing and, in two, severe dyspnoea. For two calves, the deterioration in health was so severe that euthanasia was performed at 13 DAI. Another three calves were slaughtered for the same reason at 19 DAI while the last inoculated calf fully recovered one month after inoculation. The control calf was slaughtered at 19 DAI to serve as postmortem control.

Table 3. *Neutralizing antibody titre: mean of maximum individual values against Osloss nc (type 1) and CD 87 (type 2) strains*

	Number of calves	*Inoculated strain*	*Mean highest neutralizing antibody titre*	
			Against Osloss nc	*Against CD 87*
Series 1	2	L256	7	4.75
	2	Culi 4	7.25	5.5
	2	Culi 6#1	6	7.5
	2	Culi 6#0	7	3
	1	Control	<2	<2
Series 2	6	BVD890/256	<2	7.9
	1	Control	<2	<2

Antibody titres are in log2 of dilution. Sera tested from dilution 1:4 to 1:512.

Figure 2. *Mean thrombocyte counts according to strain inoculated: results are presented as percentages of the thrombocyte count at day 0.*

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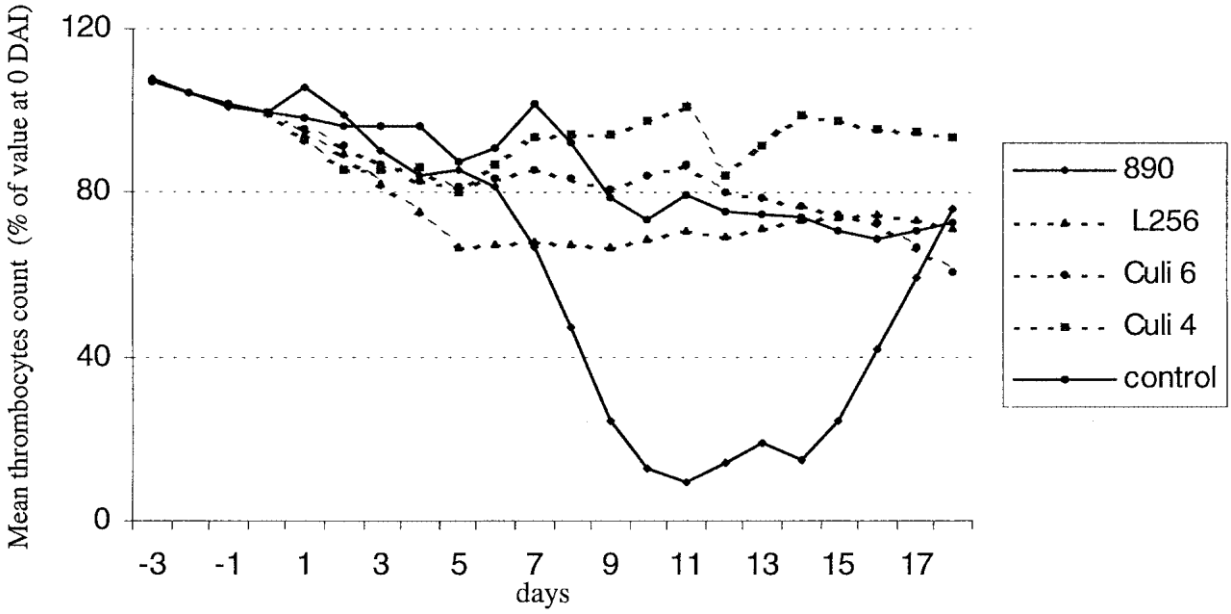

POST-MORTEM EXAMINATION

None of the animals from the first series nor the control calves had any significant macroscopic lesion. In contrast, all five euthanased animals from the second series showed macroscopic lesions: four calves had signs of dehydration, three had haemorrhagic lesions on the spleen, heart, urinary bladder, abomasum or mesentery, and all exhibited multiple lesions of acute to subacute pneumonia, with oedema and consolidation. Multiple focal abscesses were observed in two of them.

BLOOD PARAMETERS

First series: blood parameters of all calves always remained within physiological limits. The mean thrombocyte counts are shown in Figure 2.

Second series: with exception of calf N°15, all animals infected with BVD890/256 showed a severe thrombocytopaenia (< 100 000 T/mm^3), starting at 10 DAI and persisting for 4.8 $\pm$ 1.3 days (Fig. 2). With exception of calf N°15, all infected animals had a leucopenia starting at 9 DAI and persisting for six days. Leucopenia was principally associated with a dramatic reduction in neutrophils (on average, <250 neutrophils/mm^3 at 10 DAI). Calf N°15, however, had a leucocytosis (21 000 leucocytes/mm^3 at 11 DAI), probably related to the extensive purulent acute pneumonia.

From about 13 DAI, three of the six infected calves presented with low PCV values (<20%) combined with low haemoglobin dosage (<6 g.L^{-1}) and RBC counts. Moreover, these three calves were all clinically dehydrated. However, one of them did not present any macroscopic lesion of bleeding. Megacaryocytic morphology, evaluated by cytological analysis of bone marrow aspirates appeared normal.

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VIROLOGICAL FINDINGS

First series: virus was detected neither in the buffycoat nor in nasal swabs. ELISA Detection of NS3 (p80) antigen always remained negative although seroconversion occurred in all animals (Table III). Seroneutralization tests demonstrated a rise in neutralizing antibodies starting on average at 16 DAI against both Osloss nc and CD87 strains. Neutralizing titres were usually lower against CD 87 strain than against Osloss nc strain.

Second series: results of ELISA antigen detection in buffy coat are presented in Figure 1. BVDV NS3 antigen was detected in all infected calves from day 6, with a maximum at 8 DAI. Values for calf N°15 were very high while those of calf N°12 remained rather low. With the exception of calf N°12, BVDV was isolated from nasal swabs of all animals on two to eight occasions (data not shown).

Neutralizing antibodies against CD87 strain were detected on average as early as 12 DAI (Fig. 3), whereas seroneutralization tests remained negative against the Osloss strain.

DISCUSSION

The purpose of this study was to compare, through experimental inoculation, the virulence characters of several BVDV strains isolated from clinical haemorrhagic syndromes in Belgium, France and the USA. None of the European tested strains, all belonging to genotype I, produced significant clinical signs. Moreover, neither viraemia nor viral excretion was detected. However, titration of viral suspensions after inoculation and the strong seroconversion observed by seroneutralization tests against both type I and type II BVDV strains, confirmed that the calves had received infectious inoculums. This result suggests that high virulence is not an intrinsic feature of these strains, although isolated from cases presenting with haemorrhagic syndrome. Inoculation of the BVD 890/256 American strain produced, in all infected animals, such severe clinical signs that five of the six inoculated calves had to be euthanased. Hyperthermia, severe diarrhoea and respiratory signs were observed in all animals. Postmortem examination revealed extensive pulmonary lesions and macroscopic bleeding lesions were seen in three animals, although these were more discrete than expected from published reports (Corapi et al., 1989; Bolin & Ridpath, 1992). Blood parameters were profoundly modified: thrombocytopaenia was particularly marked and anaemia was also observed. Antigen detection revealed an intense viraemia as well as viral excretion in almost all infected calves, starting at 6 DAI. A strong and rapid seroconversion was observed by seroneutralization tests against type II strain, but no neutralizing antibodies against type I strain were detected in these animals.

Severe outbreaks of diarrhoea accompanied with thrombocytopaenia have already been observed with certain BVDV strains (Corapi et al., 1989; Pellerin et al., 1994; Bolin & Ridpath, 1995), whereas other BVDV strains have been described as responsible for severe respiratory troubles (Carman et al., 1998; Odeon et al., 1999). In our experiments, we observed a sequential apparition of these three signs in all animals infected with BVD890/256, showing that a unique hypervirulent BVDV

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strain can induce both thrombocytopaenic and respiratory syndromes. Circumstances which favour the expression of a respiratory syndrome over bleeding signs remain to be investigated.

We found that viraemia and virus shedding were easily detected in most calves inoculated with BVD890/256, whereas calves inoculated with the three European strains always remained below detection limits. Differences in viraemia and virus shedding have already been reported even within an homologous cytopathic and non-cytopathic pair of BVDV strains (Lambot et al., 1998). Moreover, neutralizing antibodies appeared earlier in the BVD890/256 inoculated calves. This could indicate, as observed by Bolin and Ridpath (1992), that BVD890/256 multiply in vivo at a high rate.

Our observation that BVDV type I inoculation induces neutralizing antibodies against both virus types whereas that type II inoculation only induces anti type II neutralizing antibodies is consistent with the observation of Paton et al. (1999) who reported on fetal protection in sheep. Seroneutralization results should not be considered as fully predictive of immunological cross-protection, but do indicate that BVDV type I strains could induce broader cross-protection than type II strains. How BVDV produces thrombocytopaenia is not yet fully understood: as previously reported (Rebhun et al., 1989; 1994; Bolin & Ridpath, 1992), microscopic examination did not reveal any significant alteration of megakaryocytes or of other marrow cells. This finding and the rapidity of both the initial decrease in platelet count and the subsequent recovery, suggest that thrombocytopaenia results from a peripheral consumption of platelets rather than from a production default. Other authors have shown that during viraemia, BVDV particles may be associated with circulating platelets (Corapi et al., 1989). This association is, however, not constant and may be occasionally observed with classical – non-hypervirulent – strains (Bolin & Ridpath, 1992). On the other hand, the thrombocytopaenia phase was preceded by a peak of viraemia and platelet count recovery appeared to be closely related to the apparition of neutralizing antibodies and concomitant decrease of viraemia (Fig. 3). High titres of virus in the blood could, therefore, be required to produce the thrombocytopaenia.

BVDV classical strains have been reported to intervene in respiratory troubles (Howard et al., 1989; Baker, 1995), possibly in association with other pathogens. In many cases, however, this does not seem to be accompanied by gross pathological changes (Bielefeldt-Ohmann, 1995). Moreover, BVDV antigens are regularly identified in healthy lungs. The severity of macroscopic lung lesions observed in our experiment with BVD 890/256 is consistent with the observation of others (Carman et al., 1998; Odeon et al., 1999) and suggests that BVDV hypervirulent strains could be, under certain circumstances, the primary infectious agent of severe pneumonia. Experiments under specific pathogen-free conditions are, however, required to demonstrate fully the lung pathogenicity of hypervirulent BVDV.

To our knowledge, this study is the first attempt to reproduce experimentally the thrombocytopaenic syndrome with BVDV strains of European origin, and the successful experiment with BVD 890/256 demonstrates the validity of our experimental conditions. Our results indicate that the European tested strains (culi 4, culi 6 and L256) are less virulent than BVD 890/256. This finding is supported by the sporadic number of clinical cases compared to the epidemic character associated with BVD 890 outbreaks, and this might be correlated to genetic

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differences in the strains. In Europe, BVDV of low virulence may be fortuitously isolated from haemorrhagic syndromes but it is important to differenciate BVDV strains that are hypervirulent, per se, from strains that have been isolated from sporadic haemorrhagic syndromes. In a retrospective analysis, Broes et al. (1992) found a correlation between haemorrhagic syndrome and BVDV infection. It is, therefore, suggested that induction of sporadic haemorrhagic syndrome by BVDV type I requires the presence of a number of co-factors, whereas in the epidemic form of the syndrome, BVDV is the primary cause of the disease.

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