Acta vet. scand. 1999, *40*, 253-262.

Serological and Molecular Diagnosis of Bovine Viral Diarrhoea Virus and Evidence of other Viral Infections in Dairy Calves with Respiratory Disease in Venezuela

By *C. Obando*[1,2], *C. Baule*[3], *C. Pedrique*[2], *C. Veracierta*[2], *S. Belák*[3], *M. Merza*[4] and *J. Moreno-Lopez*[1]

[1]Department of Veterinary Microbiology, Section of Virology, Swedish University of Agricultural Sciences, Uppsala, Sweden, [2]Instituto de Investigaciones Veterinarias, FONAIAP, Maracay, Venezuela, [3]National Veterinary Institute, and [4]SVANOVA Biotech, Uppsala, Sweden.

Obando C, Baule C, Pedrique C, Veracierta C, Belák S, Merza M, Moreno-Lopez J: Serological and molecular diagnosis of bovine viral diarrhoea virus and evidence of other viral infections in dairy calves with respiratory disease in Venezuela. Acta vet. scand. 1999, 40, 253-262. – An investigation based on 2 studies was carried out to assess the involvement of bovine virus diarrhoea virus (BVDV), bovine herpesvirus type 1 (BHV-1), and bovine respiratory syncytial virus (BRSV) in calf respiratory disease in dairy farms in Venezuela. In the first study, 8 farms were selected and paired serum samples from 42 calves with respiratory disease were tested by ELISA for antibodies to the 3 viruses. Seroconversion to BVDV, BHV-1, and BRSV was found to 5, 2, and 6 farms out of the 8, respectively. The proportion of calves that showed seroconversion to BVDV, BHV-1, and BRSV were 19%, 14%, and 26%, respectively. In the second study, another farm having previous serological evidence of BVDV infection was selected. The decline of maternal antibodies against BVDV was monitored in 20 calves and the half-life of maternal antibodies was 34 ± 12 days presumably indicating an early natural infection with BVDV. Furthermore, sera free of BVDV antibodies that were collected in studies 1 and 2 and were assayed for the presence of BVDV by nested RT-PCR. Two BVDV strains were detected and compared to those of ruminant and porcine pestiviruses. Both strains were assigned to subgroup Ib of type I BVDV. This investigation provides information on BVDV genotypes circulating in Venezuela and may contribute to the establishment of official control programmes against the viruses studied.

BRDC; maternal antibodies; BVDV; BHV-1; BRSV; PCR.

Introduction

Bovine respiratory disease complex (BRDC) is one of the major causes of economic losses in calves and growing cattle. The losses result not only from deaths but also from the cost of antibiotic treatment, reduced production, and poor growth performance of animals that recover (*Baker* 1990).

In general, it is accepted that the BRDC involves a sequential cascade of events initiated by stress that predisposes the animal to viral infections, which in turn facilitate rapid bacterial invasion, most frequently with *Pasteurella haemolytica* or *Pasteurella multocida* (*Jerico & Carter* 1985, *Allen et al.* 1991, *Giles et al.* 1991). Various viruses have been associated with BRDC, such as bovine viral diarrhoea

virus (BVDV), bovine herpes virus type 1 (BHV-1), bovine respiratory syncytial virus (BRSV), parainfluenza virus type 3 (PI-3), several serotypes of reoviruses, and bovine adenoviruses (*Hore* 1976, *Moreno-Lopez* 1979, *Stott et al.* 1980, *Yates* 1984). However, the role of many of these agents in BRDC is currently not fully understood (*Hjerpe* 1986, *Kennedy* 1996). The way in which BVDV is involved in the BRDC has been controversial. *Radostis & Townsend* (1989) considered that there was no substantial evidence to implicate BVDV in the pathogenesis of acute respiratory disease in cattle. In contrast, other researchers found that BVDV plays an important role in calf respiratory disease because of its ability to induce immunosuppression, which may increase the host's succeptibility to other pathogens (*Wray & Roeder* 1987, *Potgieter* 1988, *Baker* 1990). Among the viruses that affect the respiratory tract, BHV-1 and BRSV are generally considered to have the highest economical importance (*Kennedy* 1996, *Ellis* 1997).

The diagnosis of bovine respiratory infections by virus isolation is usually difficult and often requires several blind passages in cell culture (*Potgieter & Aldridge* 1977). Therefore, serological tests, such as ELISA and the direct detection of viral genomes by PCR, are useful tools for the identification of the causative agent, whether or not the viruses are inactivated (*Florent & de Marneffe* 1986, *Belák & Ballagi-Pordány* 1991).

In dairy cattle herds in Venezuela, as in many other countries, calf mortality is one of the most important causes of economic losses. A study carried out on 2 656 new-born calves in the central-west region of the country showed a rate of 7.8% mortality during the first 3 months of life (*Pedrique et al.* 1990). Another study in the same region indicated that respiratory infections and diarrhoea are the most frequent syndromes associated with calf mortality (*Obando* 1994).

The role of the viruses discussed above in cattle diseases has mainly been studied in Europe, North America, and Australia, and relatively few studies have been performed in other continents or sub-continents, such as Central and South America. The aim of the present studies was to investigate the occurrence of BVDV, BHV-1, and BRSV infections in dairy calves with respiratory diseases in Venezuela, with particular focus on BVDV.

Materials and methods

Farms, animals, and sampling procedures

Study 1. Eight dairy farms (A to H) with 100 to 300 pure or crossbred Holstein cattle and without recent history of vaccination against viruses of the BRDC (BVDV, BHV-1, and BRSV) were selected. The calves in these farms are given free access to colostrum for 3 or 4 days. All calves were identified by ear numbers and clinically examined for respiratory symptoms, such as depression, anorexia, increased respiratory rate, temperature above 39 °C, nasal discharge, coughing, and, in some cases, diarrhoea. Acute and convalescent serum samples were collected 3 or 4 weeks apart from 42 calves (1 to 90 days old) showing at least 4 of the above respiratory symptoms.

Study 2. Another dairy farm (1) with more than 4 000 pure Holstein cattle was selected, based on previous serological evidence of BVDV infection and a history of calf respiratory disease as an important problem during the last 3 years. Twenty Holstein calves were sampled at 15 days after birth and then every 15 days, until they were 3 months old and material antibodies against BVDV was monitored by ELISA:

Serology

The serum samples, which were to be analysed

in Sweden, were inactivated at 65 °C for 3 h and transported according to current Swedish regulations (*Obando et al.* 1999). The particularity did not allow to use samples for isolation of viruses in cell culture, so diagnosis of viral infection was based on the assessment of specific antibody response, as measured by ELISA.

In the first study, paired sera collected from 42 animals with acute respiratory disease were tested for antibodies to BVDV, BHV-1, and BRSV. The commercial indirect ELISA kits, developed by SVANOVA Biotech (National Veterinary Institute, Uppsala, Sweden), were used according to the manufacturer's instructions. Serum samples with ELISA-corrected optical density (OD) values equal to or greater than 0.20 were considered positive for antibodies to BVDV, BHV-1, and BRSV. To minimize inter-assay variation, all paired sera were assayed in one trial. A calf was considered recently infected with a particular virus if it was seronegative in the first sample (acute phase) and seropositive when tested the second time (convalescent sera) or alternatively, when the convalescent sera showed an increase of at least twice the cut-off value (*Florent & de Marneffe* 1986).

In the second study, serum samples collected every 15 days from 20 Holstein calves were tested by ELISA only for specific IgG antibodies to BVDV.

Nested PCR for detection of BVDV RNA

RNA extraction. Twenty-five serum samples negative for antibodies to BVDV (study 1) and 7 serum samples from calf C-117 (study 2), which was negative for antibodies to BVDV, were used as the source of RNA for the nested PCR.

The RNA was extracted by using the guanidinium-thiocyanate phenol chloroform method described by *Chomiczynski & Sacchi* (1987). Briefly, 150 μl of each sample diluted 1:5 in PBS were vigorously mixed with 400 μl of a lysis solution (4 M guanidinium-thiocyanate, 25 mM sodium citrate, 0.05% w/v sarcosyl, and 0.1 M 2-mercaptoethanol) and left on ice for 10 min. This mixture (500 μl) was extracted twice with an equal volume of a 1:1 v/v solution of acidic phenol:chloroform and once with chloroform. The aqueous phase was precipitated in 2 volumes of cold 95% ethanol and 0.1 volume of 3 M sodium acetate at –20 °C overnight. RNA was pelleted by centrifugation at 10 000 x g for 30 min, and the pellet was resuspended in 10 μl diethyl pyrocarbonate-treated water.

Synthesis of cDNA. Mixtures consisting of 5 μl extracted RNA, 1 μl random hexamers (0.02 U; Amersham Pharmacia Biotech, Uppsala, Sweden) and 5 μl water were denatured at 65 °C for 5 min and then immediately chilled on ice for 5 min. Subsequently, the following components were added: 1 μl RNAguard ribonuclease inhibitor (24 U; Amersham Pharmacia Biotech, Uppsala, Sweden), 5 μl of the first-strand reaction buffer (0.25 M Tris-HCl, pH 8.3; 0.375 M KCl; 15 mM $MgCl_2$), 2.5 μl of each dNTP (2 mM; Amersham Pharmacia Biotech), and 1 μl of Moloney murine leukemia virus reverse transcriptase (200 U; Gibco, BRL, Bethesda, MD, USA). Sterile water was added to give a final volume 25 μl. The reaction mixture was incubated at 37 °C for 90 min. The synthesised cDNA was immediately used for PCR or stored at –20 °C until it was used.

Polymerase chain reaction (PCR)

A 296-bp DNA fragment of the 5' non-coding region (NCR) of the BVDV genome was amplified in a first round of PCR, and then a 169-bp DNA fragment was amplified from the first round of PCR products.

The primers used were reported by *Elvander et*

al. (1998): primers OPES 13a (5' GCTAGCCATGCCCTTAGTAGGA 3') and OPES 14a (5' ATCAACTCCATGGCCATTTACAGC 3') for the first round of PCR amplification, and primers OPES 11 (5' TGAGTACAGGGTAGTCGTCAGTGGTTCG 3') and OPES 12a (5' GGCCTTTGCAGCACCCTATCAG 3') for the second round of PCR amplification.

The first PCR was run as follows: 5 cycles with denaturation at 94 °C for 45 s, annealing at 53°C for 1 min and extension at 72 °C for 1 min. This was followed by 30 cycles with denaturation at 94 °C for 45 s annealing at 48 °C for 45 s, and extension at 72 °C for 1 min. An additional final extension step at 72 °C for 7 min was included. For the second PCR, the thermoprofile was 5 cycles with denaturation at 94 °C for 45 s. annealing at 57 °C for 1 min, and extension at 72 °C for 1 min, followed by 30 cycles with denaturation at 94 °C for 45 s, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min. An additional final extension step at 72 °C for 7 min was also included.

PCR products were analysed by electrophoresis on 2% agarose gels stained with ethidium bromide and visualised on an UV transiluminator.

Sequencing of the PCR products and phylogenetic analysis

The PCR products were purified by either the QIA quick PCR Purification kit or the Jetsorb DNA Purification kit (both from Quiagen, Germany), the latter after excision of the products from agarose gels. The PCR products were sequenced from both strands in an Abi Prisma sequencer.

The nucleotide sequences derived for the 119-base pair amplicons from the 5' NCR were aligned and compared to the corresponding region of sequences of pestiviruses of bovine, ovine, and porcine origin published by others. Nucleotide sequence comparisons and phylogenetic analysis were done with DNASTAR software package (DNASTAR, Madison, WI, USA).

Results

Serological findings

Study 1. Seroconversion to BVDV was found in calves from 5 farms (A, C, D, F, and H), to BHV-1 from 2 farms (D and F), and to BRSV from 6 farms (A, C, D, E, F, and H). None of the calves from farm E showed antibodies to BVDV and BHV-1, but 2 did show antibodies to BRSV, and 1 seroconverted. In all of the calves, when tested the first time, 23 (55%) were found to be seropositive to BVDV, BHV-1 and BRSV seropositivity was detected in 12 (29%) and 32 (76%), respectively (Table 1). Eight out of 42 calves (19%) seroconverted to BVDV, and 4 (50%) of these calves were seronegative against this pestivirus during the acute phase. Six calves (14%) in 2 herds, seroconverted to BHV-1, and all of them were found to be without antibodies against this herpesvirus when sampled the first time. Eleven out of 42 (26%) seroconverted to BRSV, and 64% of these calves were seronegative against this pneumovirus during the acute phase.

Finally, concomitant seroconversion to 2 viruses, BVDV and BHV-1 or BVDV and BRSV were detected in 4 calves (farms A and F). There was no concomitant serovoncersion to BHV-1 and BRSV. A concomitant seroconversion to all of the selected viruses was found in 1 calf (farm D).

Study 2. BVDV-IgG antibody titres measured in 17 out of 20 calves that demonstrated no detectable increase in antibody levels during the 90-day period of the experiment showed a half-life of 34 ± 12 days. However, BVDV-Ab titres in 1 calf (C-124) rose steadily until 45 days of age, then began to decay until 60 days of age, when a plateau was reached. Similarly, another calf (C-120) showed a steady decline in BVDV-

Table 1. Serological status of viruses tested by ELISA in paired sera from dairy calves with respiratory diseases.

Farm	Age (days)	n	Virus								
			BVDV			BHV-1			BRSV		
			1st	2nd	nS	1st	2nd	nS	1st	2nd	nS
A	45-60	2	1	2	2	1	1	0	2	2	2
B	10-41	3	2	2	0	3	1	0	3	2	0
C	24-50	6	4	4	1	3	1	0	3	3	1
D	01-37	8	5	4	1	2	1	1	5	6	4
E	45-90	3	0	0	0	0	0	0	2	2	1
F	65-90	9	5	7	3	2	7	5	7	5	1
G	51-90	4	4	3	0	1	0	0	4	3	0
H	60-90	7	2	2	1	0	0	0	6	4	2
Total		42	23 (55)	24 (57)	8 (19)	12 (29)	11 (26)	6 (14)	32 (76)	27 (64)	11 (26)

1st Sampled during the acute phase.
2nd Sampled 3 or 4 weeks apart.
nS Number of seroconverted calves.
() Percentage.

Ab titres, but the slope for the decay was less steep than that obtained for the rate of decline. Finally, only a low level of antibodies to BVDV (OD value: 0.27), close to the cut-off point, was detected in calf C-117 at 15 days after birth (Fig. 1).

Detection of BVDV RNA and genotyping

BVDV RNA called Ven 32 and Ven 38 (see Fig. 2) were detected in one acute serum from farm F and in both acute and convalescent sera from another calf from farm H, respectively (study 1). No BVDV RNA was detected in negative serum samples from calf C-117 (study 2). The nucleotide sequence analysis of the 2 strains indicated (according to the nomenclature for BVDV proposed by *Pellerin et al.* (1994) that they could be assigned to type I BVDV, subgroup Ib (Fig. 2).

Discussion

This work presents evidence that infections or coinfections with BVDV, BHV-1, and BRSV were present in the dairy calves during acute respiratory disease. This result was expected because of the high prevalence of antibodies to these viruses found in beef cattle in Venezuela (*Obando et al.* 1999) and because of studies that suggest the involvement of these viruses in the so-called predisposing causes of acute BRDC (*Thomas et al.* 1977, *Stott et al.* 1980, *Radostis & Townsend* 1989, *Ellis* 1997). Detection of BVDV RNA in sera and seroconversion to this virus in calves with respiratory disease provides evidence of BVDV infection in these animals. Even though BVDV RNA was detected in 1 calf during the acute phase of the disease, it was not possible to consider BVDV the primary pneumopathogen since seroconversion to both BVDV and BRSV was detected in this animal. Furthermore, pneumopathogenicity may vary among BVDV strains (*Potgieter et al.* 1985).

BVD viruses are divided into 2 genotypes, BVDV type I and BVDV type II, on the basis of 5' NCR sequencing. BVDV type I comprises

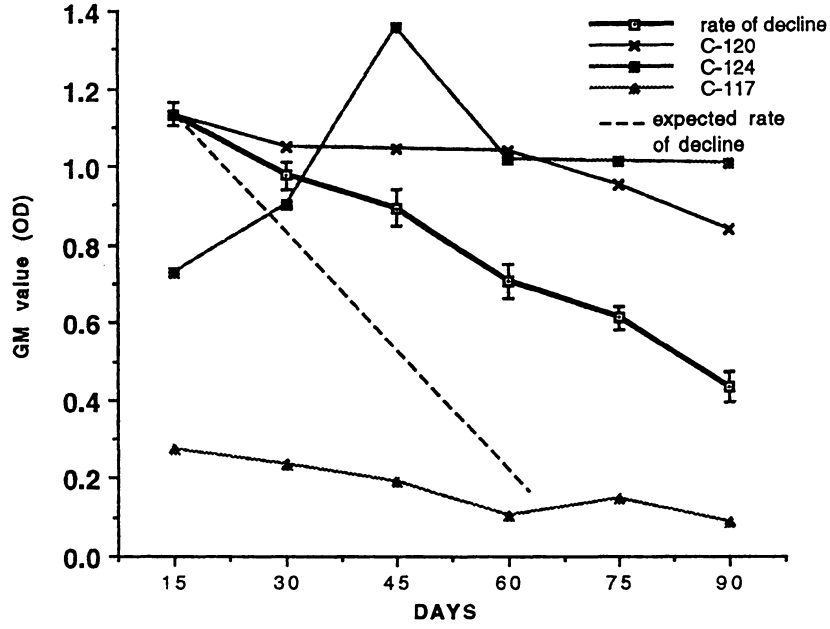

Figure 1. The decline of maternal antibodies against BVDV in 20 calves. The obtained and expected rate of antibody decline as well as the particular serological profile in 3 calves are shown.

the majority of strains characterised so far, while BVDV type II includes strains isolated in connection with Haemorhagic Syndrome in North America (*Ridpath et al.* 1994, *Pellerin et al.* 1994). The comparative nucleotide sequence analysis of the 5' NCR of the RNA from the two BVDV strains detected here showed that both strains belong to genotype I.

Table 1 shows that seroconversion to BRSV was found most frequently (26%), followed by seroconversion to BVDV (19%), and, less frequently, seroconversion to BHV-1 (14%). In addition, most of the calves that seroconverted to BHV-1 were from farm F, indicating that this virus might not be a common factor predisposing calves to respiratory diseases in the farms studied here. Although our results were obtained from a small number of animals, they are in agreement with results from other studies on calves with respiratory disease that show more frequent seroconversion to BRSV than to BVDV or BHV-1 (*Martin et al.* 1989, *Allen et al.* 1992). Even though BHV-1 infection was not common, 100% of the cases detected occurred in calves without antibodies to this virus. These findings might be due to the virus having been recently introduced into those herds; however, BHV-1 antibodies derived from colostrum seem to suppress the immunoresponse. *Menanteau-Horta et al.* (1985) reported that calves given BHV-1 vaccine at 84 days of age in the presence of maternal antibodies did not show seroconversion. In contrast, the same calves seroconverted after immunisation with BVDV vaccine, although maternal antibody titres were higher than they were for BHV-1.

Seroconversion to 2 or 3 viruses was detected in

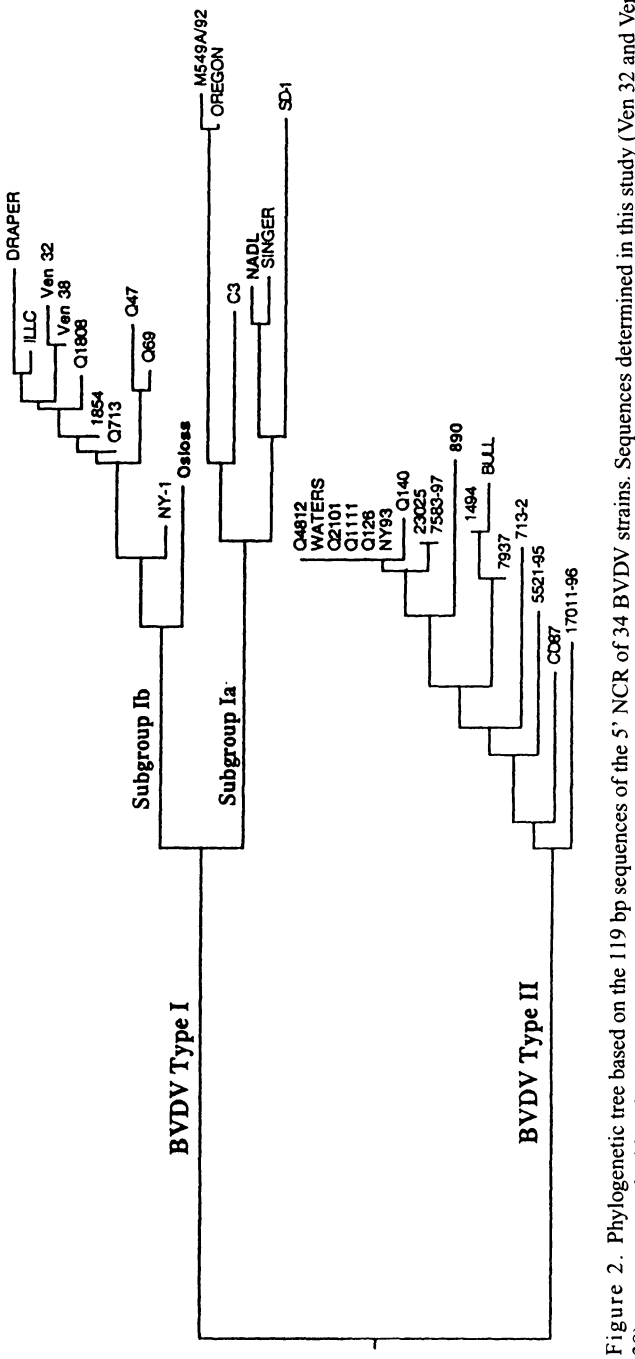

Figure 2. Phylogenetic tree based on the 119 bp sequences of the 5' NCR of 34 BVDV strains. Sequences determined in this study (Ven 32 and Ven 38) were compared with published sequences of bovine, ovine and porcine origin.

5 animals, suggesting that these viruses may concomitantly infect young calves.

This finding has previously been reported for herds during outbreaks of BRDC (*Moreno-Lopez* 1979, *Richer et al.* 1988, *Straub* 1994). Although the reliability of serological examinations of BRSV in calves less than 6 months old is limited (*Uttenthal et al.* 1996). The presence of active immune response was still detected, probably because 64% of the reacting calves were seronegative to BRSV at the first sampling and the remaining calves had low antibody titres. Large increases in antibody titre to BRSV detected by immunofluorescence and virus neutralisation tests in calves 3 to 6 months old with respiratory diseases have been reported (*Adair* 1986).

The half-life of passively acquired BVDV antibodies has been reported to be around 21 days (*Brar et al.* 1978, *Pálfi et al.* 1993, *Menanteau-Horta et al.* 1985). In this study, the half-life in 17 out of 20 calves was found to be 34 ± 12 days. The delay in decline of antibody levels might be a consequence of actively produced antibodies against BVDV infections since no vaccine against this virus was used. *Howard et al.* (1989) reported that the decline of maternal antibodies to BVDV appears to be slow and does not give the usual half-life of 21 days when natural infection has occurred. The serological profile of calf C-124 might indicate an early natural infection with BVDV, even in the presence of specific maternal antibodies. However, infection in the later stage of pregnancy could also be possible, according to the serological profile of calf C-120 (Fig. 1). In addition, 4 (50%) of the calves that seroconverted to BVDV also had antibodies at the first sampling. These observations might indicate that passively acquired BVDV-IgG antibodies do not make calves completely resistant to natural BVDV infections. *Bolin et al.* (1991) reported that new-born calves with colostral antibodies or vaccinated feedlot calves might be susceptible to diseases induced by certain antigenic variants of BVD viruses. Differences in neutralising antibody titres against specific isolates of BVDV are detected in serum from convalescent cattle. The low level of BVDV antibodies in calf C-117 during the sequential study (90 days) could be the consequence of a failure of passive immunoglobulin transfer and not an indication of immunotolerance to BVDV, as initially suspected, since BVDV RNA was not detected in the serum samples. Moreover, the lack of active immune response to BVDV during the sampling period indicates that this calf was not exposed to the virus.

In spite of these results, it was not possible to conclude that a certain virus or viruses were determinant causes of a particular disease condition because control calves were not included and because association between serological findings and pathological lesions of diseased animals was not performed. In addition, seroconversion to viruses of the BRDC can occur in groups of cattle without occurrence of respiratory disease (*Allen et al.* 1992, *Scott* 1997). However, these results do suggest that BVDV, BHV-1, and BRSV infections are, to some extent, involved in the cases of calf respiratory disease studied here. Epidemiological studies of these viruses are necessary before appropriate sanitary control measures can be implemented in Venezuela.

Acknowledgements

This work was supported by the Swedish Foundation for International Cooperation in Research and Higher Education (STINT), SVANOVA Biotech, and FONAIAP. The authors thank Israel Peña, SASA-Apure (MAC), for valuable help, Stefan Alenius for critical reading of the manuscript.

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Sammanfattning

Serologisk och molekylar diagnostik av bovint virus diarre virus samt påvisande av andra virusinfektioner hos kalvar med respiratoriska sjukdomar i Venezuela.

Två studier genomfördes för att påvisa bovint virus diarré virus (BVDV), bovint herpesvirus typ 1 (BHV-1) och bovint respiratoriskt syncytial virus (BRSV) hos kalvar med luftvägsinfektioner. I den första studier, parserum från 42 kalvar i åtta besättningar testades med ELISA för antikroppar mot de 3 virus. Serokonverteringar för BVDV, BHV-1 och BRSV kunde påvisas i 5, 2 och 6 av respektive 8 besättningar. Procentuellt sätt kalvarna som serokonverterade vid BVDV, BHV-1 och BRSV var 19, 14 och 26 procent till respektiva virus.

I den andra studier en ny besättningen med BVDV problem selekterades och halvering av maternala antikroppstiter mot BVDV undersöktes i 20 kalvar. Titrarna halverades efter 34 ± 12 dagar som indikerar en tidigt infektion med BVDV. Dessutom BVDV fri sera från djur I studier 1-2 testades med RT-PCR för att påvisa närvaro av BVDV-RNA. Två BVDV stammar av typ I subgrup IB detekterades. Resultaterna indikerar att BVDV genotyper cirkulerar i Venezuela och resultaterna kommer att bidra till genomförande av officiell kontroll av de undersökta virus.

(Received January 28, 1999; accepted April 28, 1999).

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