MONITORING OF NATURAL INFECTION BY BOVINE VIRAL DIARRHEA VIRUS (BVDV) IN CATTLE HERDS

MONITORAMENTO DA INFECÇÃO NATURAL PELO VÍRUS DA DIARRÉIA VIRAL BOVINA (BVDV) EM REBANHOS BOVINOS[1]

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SUMMARY

Natural infection by bovine viral diarrhea virus (BVDV) was monitored in blood serum samples of three cattle herds. The samples were drawn in several harvests from each herd and submitted to virus neutralization test (VN) against BVDV-1 and BVDV-2. The non reactive samples to at least one of the genotypes and also those collected from calves younger than six months, were analyzed by reverse transcription polymerase chain reaction (RT-PCR). In two herds, BVDV was not detected in any blood sample and the number of reactive samples to VN test, especially young animals, decreased as the blood sample harvests were conducted. However, in the third herd, the infection remained during the monitored period, because BVDV was detected in two persistently infected animals (PI) and also in one transiently infected animal (TI). The cattle breeding system and intense movement of animals were favorable to the permanence of infection in this last herd. The dynamics of BVDV infection changed in the analyzed herds, highlighting the probable self clearance of BVDV in herd 1 and the risk factors related to transmission of BVDV, such as the frequent purchase of animals from different origins for herd 4, as well as the probable hypothesis that infection of herd 17 may have originated from the neighboring herd.

KEY-WORDS: Persistently infected animal. Risk factors. Self-clearance. Transiently infected animal.

RESUMO

A infecção natural pelo vírus da diarréia viral bovina (BVDV) foi monitorada em três rebanhos bovinos por meio de amostras de soro sangüíneo, obtidas em várias colheitas realizadas em cada rebanho, que foram submetidas ao teste de virusneutralização (VN) para o BVDV-1 e para o BVDV-2. As amostras não reagentes a pelo menos um dos genótipos e aquelas oriundas de bovinos com menos de seis meses de idade, reagentes ou não, foram analisadas pela reação em cadeia da polimerase precedida pela transcrição reversa (RT-PCR) para a pesquisa do BVDV. Em dois rebanhos o vírus não foi detectado em nenhuma amostra e a quantidade de animais reagentes ao vírus no teste de VN, principalmente nos animais jovens, diminuiu à medida que as colheitas foram realizadas. No entanto, no terceiro rebanho, a infecção permaneceu durante o período monitorado, pois o BVDV foi detectado em dois animais persistentemente infectados (PI) e também em um animal transitoriamente infectado (TI). O sistema de criação, bem como o intenso trânsito de animais, foram favoráveis à permanência da infecção nesse último rebanho. A dinâmica da infecção pelo BVDV foi variável nos rebanhos analisados, destacando a provável eliminação espontânea do BVDV no rebanho 1 e os fatores de risco relacionados à transmissão do BVDV, como a frequente aquisição de animais de diversas procedências pelo rebanho 4, assim como a provável hipótese da infecção do rebanho 17 ter originado a partir do rebanho vizinho.

PALAVRAS-CHAVE: Animal persistentemente infectado. Animal transitoriamente infectado. BVDV. Eliminação espontânea. Fatores de risco.

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INTRODUCTION

The bovine viral diarrhea (BVD) is a set of clinical and asymptomatic syndromes associated with infection by the bovine viral diarrhea virus (BVDV), a RNA virus that belongs to the genus *Pestivirus* of the family *Flaviviridae* (NETTLETON & ENTRICAN, 1995; BOLIN & RIDPATH, 1996). BVDV is classified into two different species (BVDV-1 and BVDV-2), both of which can exist in two different biotypes, cytopathogenic (CP) and non-cytopathogenic (NCP) (RIDPATH et al., 1994; TREMBLAY, 1996; RIDPATH, 2010).

A herd infected with BVDV has two potential sources of infection, the animal persistently infected (PI) and the animal transiently infected (TI) (HOUE, 1994; LINDBERG & HOUE, 2005). Persistent infection can be established either by BVDV-1 or BVDV-2 (LIEBLER-TENORIO, 2005), resulting from uterine infection by the NCP biotype of BVDV in the first 125 days of gestation (DEREGT & LOEWEN, 1995; GROOMS, 2004).

The PI animal is considered the main source of BVDV infection, as it constantly eliminates throughout its life high titers of virus in secretions and excretions (HOUE, 1995). On the other hand, the TI animal, that is, the one in the acute phase of illness, eliminates the virus for a few days or possibly weeks (THURMOND, 2005), but this animal can promote the persistence of the virus in the herd in the absence of PI animals (MOERMAN, 1993).

In order for BVDV to remain in the herd, a PI animal must get pregnant and pass on the infection to at least one calf (LINDBERG & HOUE, 2005). The BVDV can remain in a herd for several generations by transplacental infection, provided that there are susceptible animals in early pregnancy. If this condition is not fulfilled, the virus continues to circulate among other susceptible animals (TI) up to the moment when the infection ends in these animals, because they developed antibodies and fought the virus successfully (MOERMAN et al., 1993; LINDBERG & ALENIUS, 1999; SANDVIK, 2004).

After an acute infection and the consequent BVDV elimination, the antibodies stay for a long time in blood serum (FREDRISKEN, 1999). Thus, the presence of BVDV infection in the herd can be detected by antibodies of young animals, also known as sentinels (SMITH & GROTELUESCHEN, 2004). Considering these variables in the dynamics of infection, this study aimed to monitor the natural BVDV infection of three cattle herds.

MATERIAL AND METHODS

Herds

Three herds suspected of being infected by BVDV were studied following methodology proposed by Pillars & Grooms (2002), which consists of detecting the antibodies in the blood sample of five calves, aged between 6 and 12 months that were not vaccinated against BVDV. Thus, the herd was considered reactive if at least three of five calves had neutralizing antibodies titers greater than 128 opposite a genotype of the virus, the samples were subjected to virus neutralization (VN) for both BVDV-1 and BVDV-2. On the other hand, when three of five calves had titers lower than 64 for both genotypes, the herd was considered negative. The monitored herds, identified as 1, 4 and 17, had at least three of five blood samples from calves, aged between 6 and 12 months, reactive to either BVDV-1 or BVDV-2, with titers higher than 128.

Herd 1, located in Machado county, Minas Gerais state, consisted of 336 Holstein dairy cattle under intensive breeding management. Herd 4, located in Poço Fundo, also Minas Gerais, consisted of 72 crossbred beef cattle under extensive breeding management. Herd 17, located in Pedregulho, São Paulo, consisted of 94 Girolando breed, a mixed (dairy and beef) cattle herd under semi-intensive management.

No measures were adopted to control the disease in herds 1 and 4. On the other hand, during this period, all animals older than 8 months in herd 17 were vaccinated, using a commercial vaccine containing inactivated BVDV-1 and BVDV-2, with a booster after 30 days of vaccination.

Samples

In the first step, paired blood samples were collected every 30 days from all animals of the three studied herds. Further blood samples were drawn, but at different times for each herd, as well as the number and class of animals tested per herd (Table 1). For herd 1, a total of 1,046 blood samples were drawn in 5 harvests over a 29 month period; for herd 4, 249 blood samples were drawn in four harvests over a 19 month period; and finally, for herd 17, 216 blood samples were drawn in three harvests during a 17 month period. Therefore, for all herds a total of 1,511 blood samples were drawn.

Blood samples were drawn using Vacutainer BD® tubes and, after harvesting, they were centrifuged at 1,080xg. Two 1.5-mL aliquots of blood serum were obtained from each sample and used to test for both BVDV-1 and BVDV-2. The samples were stored at -20ºC until used.

Serum test

All samples underwent the virus neutralization (VN) test to determine the presence of antibodies for both BVDV-1 and BVDV-2 (OIE, 2008). In the VN test, bovine kidney epithelial cells of the "Madin Darby bovine kidney" (MDBK) line, kept in Eagle MEM ("Minimal Essential Medium") Gibco® medium, supplemented with 10% fetal calf serum (SFB) Cultilab® free of *Pestivirus* and antibodies for BVDV**,** and employed the cytopathogenic strains (CP) of BVDV-1 (Singer) and of BVDV-2 (VS-253) were used. The blood serum samples, prior to testing, had been previously inactivated at 56ºC, during 30 minutes. The VN tests were performed using disposable

microtiter plates of 96 wells TPP®, and to the maintenance medium Eagle-MEM Gibco®, used for serum sample dilutions, was added a 1% penicillin (10.000UI mL^{-1}) and streptomycin (10.000ug mL^{-1}) Gibco® solution .

Duplicate serial dilutions from 1:10 to 1:5.120 were made for each serum sample tested. After adding the viral suspension containing 100$TCID_{50}$ (50% "tissue culture infective doses") of BVDV, the microplates were incubated in an incubator with atmosphere of 5% CO_2 at 37ºC. After 60 minutes, a suspension of cells MDBK containing 300.000 cells mL^{-1} in maintenance medium Eagle-MEM Gibco® with 10% SFB Cultilab® was added to plate wells. The plate was then incubated again at 37ºC, in 5% CO_2 atmosphere for 96 hours. Serum blood samples were considered reactive when they promoted neutralization of 100 $TCID_{50}$ of BVDV from 1:10 dilution. The reactive samples in the 1:5.120 dilution were tested again in duplicate up to 1:20.480 dilution. Antibodies titers were expressed as the reciprocal of the highest dilution in which virus neutralization was detected, and the final titer was the result of the geometric mean titers found in duplicates – GMT (THRUSFIELD, 1986).

BVDV research

Blood serum samples that were non reactive to at least one of the VN tests, either BVDV-1 or BVDV-2, and samples of calves younger than 6 months, reactive or not, were tested for BVDV by reverse transcription polymerase chain reaction (RT-PCR), using the protocol described by Pilz et al. (2005), with few modifications.

The RNA extraction followed methodology recommended by Boom et al. (1990) using silica particles and guanidine isothiocyanate to purify the nucleic acids. For the extraction, 500µL of blood serum was used from each sample to be tested and the extracted RNA was eluted in water treated with DEPC (diethyl pyrocarbonate). The RT-PCR was performed using oligonucleotides sense primers 103 (5' TAG CCA TGC CCT TAG TAG GAC 3' – genomic position 103-124) and 372 antisense (5' ACT CCA TGT GCC ATG TAC AGC 3' – genomic position 372-392), designed from the sequence of the 5' untranslated region of the viral genome (5'UTR) of BVDV-1a NADL, which amplifies a product of 290 base pairs (pb) and shares homology between the maximum BVDV-1 and BVDV-2 (WEINSTOCK et al., 2001).

RT was performed using 9µL of the suspension containing the RNA and 20 pmol of the oligonucleotide primer antisense 372 that was denatured at 97ºC for 5 minutes and immediately placed on ice for 5 minutes. After that period, in each sample, it was added 8µL of the RT mix containing autoclaved ultrapure water, PCR buffer 10x Invitrogen™ (200mM tris-HCl pH 8,4; 500mM KCl), 0.25mM of each deoxyribonucleotide triphosphate (dNTP) Invitrogen™, 1.5mM of $MgCl_2$ Invitrogen™ and 60 units of reverse transcriptase enzyme "Moloney Murine Leukemia Virus Reverse Transcriptase" (M-MLV-RT) Invitrogen™, thus totaling a final volume of 20µL. After mixing, the cDNA synthesis was performed at 42ºC for 30 minutes, followed by a step of 5 minutes at 95ºC to inactivate the enzyme M-MLV-RT.

The PCR was performed using 5µL of cDNA and 45µL of the PCR mix, consisting of autoclaved ultrapure water, PCR buffer 10x (200mM tris-HCl pH 8,4; 500mM KCl), 0.25mM of each dNTP, 1.5mM of $MgCl_2$, 20 pmol of each oligonucleotide primer (sense 103 and antisense 372) and 2.5 units of the enzyme "Taq Platinum DNA polymerase" Invitrogen™, totaling a final volume of 50µL per sample. Samples of cDNA, together with the PCR mix, were homogenized and the reaction was conducted in a thermocycler (PTC – 200, MJ Research Co. Water Town, Ma) at 94ºC for 2 minutes, followed by 35 cycles of 1 minute at 94ºC (denaturation), 30 seconds at 59ºC (annealing) and 1 minute at 72ºC (extension), and one cycle at 72ºC for 7 minutes (final extension).

PCR products were analyzed by gel electrophoresis in 2% aragose Invitrogen™ with ethidium bromide (0,5mg/mL), in buffer TBE pH 8.4 (89mM tris; 89mM boric acid; 2mM EDTA), under steady voltage (100V) during approximately 60 minutes. On each gel, two channels were reserved for the positive controls (BVDV-1 and BVDV-2), applied on the same sample volume, and one channel for negative control (autoclaved ultrapure water). At the end of the period, the gel was observed under ultraviolet light using a BioRad® equipment and digitally photographed by "Electrophoresis Documentation and Analysis System" 290 KODAK®.

RESULTS

VN test

The VN test results for both BVDV-1 and BVDV-2 performed in 1,511 serum samples of herds 1, 4 and 17 and classified according to animal age groups (0 to 6 months, 6 to 12 months, 12 to 24 months and older than 24 months) are shown in Tables 2, 3 and 4.

RT-PCR

Virus detection by RT-PCR was performed in 566 serum samples that were not reactive to BVDV, or were not reactive to only one of the genotypes of the virus, and in samples from cattle younger than 6 months at the time of blood harvest. Of the total analyzed samples, 430 originated from herd 1; 36 from herd 4; and 100 from herd 17. BVDV was diagnosed only in herd 4.

In paired blood serum samples from the 1st and 2nd harvests, the virus was detected in 2 animals younger than 6 months, respectively, samples 04/58A, 04/58B, 04/72A and 04/72B, thus characterizing the occurrence of two PI animals (Figure 1 – Panel A). The same animals were not reactive to VN tests BVDV-1 and BVDV-2. However, in the third blood sample harvested in herd 4, BVDV was detected in the blood serum sample of cattle older than 24 months (sample 04/31C). Subsequently, in the blood serum sample from the 4th harvest of the same animal, the virus was

not detected, but the antibody titers (2560) for BVDV-1 and BVDV-2 were detected in this sample, thus characterizing a TI animal (Figure 1 – Panel B).

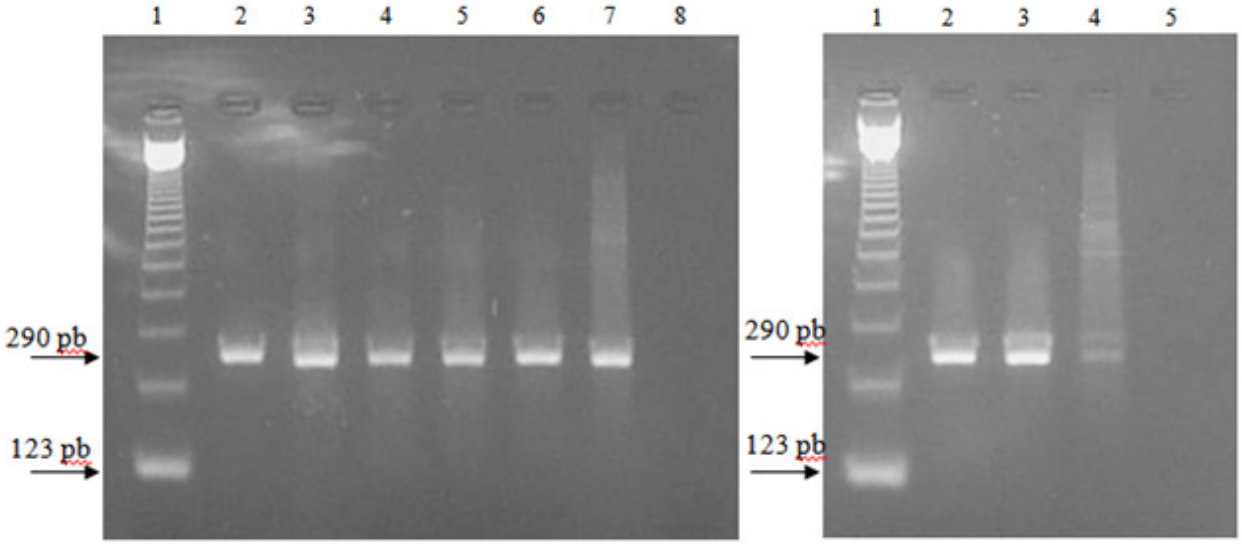
DISCUSSION

During the monitoring period, different results were found among the studied herds and it could be verified that some factors such as herd rearing system and marketing of livestock were associated with the presence of BVDV infection (QUINCOZES et al., 2007). Tables 2, 3 and 4 show the results for VN tests for BVDV-1 and BVDV-2, for herds 1, 4 and 17, respectively. The analysis of herds considered BVDV infected by VN test results for both genotypes.

The number of animals in herd 1 reactive to BVDV in the VN tests decreased over time (Table 2). Not all animals of the herd were analyzed in the last two blood sample harvests; the presence of antibodies was investigated only in animals that indicated if virus transmission was occurring inside the herd, that is, sentinel animals (PILLARS & GROOMS, 2002). Sentinel animals consisted of the groups aged up to 12 and 24 months at the time of the 4th and 5th blood harvests, respectively. On the other hand, the young animals whose blood samples were reactive to BVDV included also animals younger than 6 months old and, therefore, these antibodies could come from the colostrum (DUBOVI, 1996).

The VN test results of herd 1 conducted on blood samples of 5 harvests showed evidence of self-clearance of BVDV (LINDBERG & ALENIUS, 1999). The self-clearance of the virus is evidenced mainly by the failure to detect the amplification of the viral genome BVDV in the RT-PCR, conducted in 430 blood serum samples from the 5 harvests that were not reactive to BVDV or were from animals younger than 6 months old. In addition, all animals older than six months at the time of the first two blood harvests that were not reactive to BVDV, remained non-reactive in the 3rd harvest. Also, few animals older that six months that were reactive in the first two blood harvests with low antibody titers became non-reactive in the 3rd harvest.

The ratio of non-reactive animals among harvests was more evident when younger animals in the age brackets 6 to 12 months and 12 to 24 months were analyzed (Table 2). The number of non-reactive



Panel A **Panel B**

Figure 1. Analysis to detect the BVDV, by electrophoresis on 2% agarose gel with ethidium bromide, the products (290 pb) amplified by RT-PCR in paired samples of blood serum of herd 4.
Panel A - (1) Molecular weight marker DNA "ladder" 123 pb Invitrogen®; (2) cytopathogenic strain BVDV-1 Singer; (3) cytopathogenic strain BVDV-2 VS-253; (4) sample 4/58 A; (5) sample 4/58 B; (6) sample 4/72 A; (7) sample 4/72 B; (8) negative control – autoclaved ultrapure water.
Panel B - (1) Molecular weight marker DNA "ladder" 123 pb Invitrogen®; (2) cytopathogenic strain BVDV-1 Singer; (3) cytopathogenic strain BVDV-2 VS-253; (4) sample 4/31 C; (5) negative control – autoclaved ultrapure water.

Table 1 - Total blood samples taken to monitor the natural BVDV infection in herds 1, 4 and 17 according to time interval (months), category and the number of animals analyzed.

herd	samples	harvests					Total samples/herd
		1ª	2ª	3ª	4ª	5ª	
1	Interval (months)*	0	1	16	17	29	1.046
	samples (n)	336	333	257	109	11	
	Analyzed animals	Entire herd	Entire herd	Entire herd	All cattle born in the interval between the 2nd and 3rd harvest and those with less than six months in the 2nd harvest	cattle between 6 and 12 months	
4	Interval (months)*	0	1	7	19	-	249
	Samples (n)	72	76	92	09	-	
	Analyzed animals	Entire herd	Entire herd	Entire herd	All non-reactive cattle in the 3rd sampling	-	
17	Interval (months)*	0	1	17	-	-	216
	samples (n)	94	94	28	-	-	
	Analyzed animals	Entire herd	Entire herd	All cattle aged less than 8 months old**	-	-	
Total							1.511

(-) harvest not performed
* Interval (months) for the 1st harvest
** Herd vaccinated after the 2nd harvest, samples were collected only from non-vaccinated animals in the 3rd sampling.

Table 2 - Results of VN testing for BVDV-1 and BVDV-2 performed on serum samples from herd 1, according to age group at different harvesting times.

Age bracket	BVDV	VN	Harvests				
			1ª	2ª	3ª	4ª	5ª
0-6 mo.	BVDV 1	Total number of samples	39	42	23	34	3
		Reactive	24 (61,54%)	21 (50%)	12 (52,17%)	22 (64,70%)	0
		Non- reactive	15 (38,40%)	21 (50%)	11 (47,83%)	12 (35,30%)	3 (100%)
	BVDV 2	Reactive	24 (61,54%)	21 (50%)	13 (56,52%)	24 (70,59%)	0
		Non-reactive	15 (38,40%)	21 (50%)	10 (43,48%)	10 (29,41%)	3 (100%)
6-12 mo.	BVDV 1	Total number of samples	47	46	31	31	8
		Reactive	31 (65,96%)	28 (60,87%)	1 (3,23%)	1 (3,23%)	0
		Non- reactive	16 (34,04%)	18 (39,13%)	30 (96,77%)	30 (96,77%)	8 (100%)
	BVDV 2	Reactive	30 (63,83%)	29 (63,04%)	1 (3,23%)	1 (3,23%)	0
		Non-reactive	17 (36,17%)	17 (36,96%)	30 (96,77%)	30 (96,77%)	8 (100%)
12-24 mo.	BVDV 1	Total number of samples	65	64	52	44	-
		Reactive	51 (78,46%)	49 (76,56%)	9 (17,31%)	8 (18,18%)	-
		Non- reactive	14 (21,54%)	15 (23,44%)	43 (82,69%)	36 (81,82%)	-
	BVDV 2	Reactive	48 (73,85%)	44 (68,75%)	9 (17,31%)	7 (15,90%)	-
		Non-reactive	17 (26,15%)	20 (31,25%)	43 (82,69%)	37 (84,10%)	-
> 24 mo.	BVDV 1	Total number of samples	185	181	151	-	-
		Reactive	175 (94,60%)	174 (96,13%)	116 (76,82%)	-	-
		Non- reactive	10 (05,40%)	7 (03,87%)	35 (23,18%)	-	-
	BVDV 2	Reactive	176 (95,14%)	170 (93,92%)	128 (84,77%)	-	-
		Non-reactive	09 (04,86%)	11 (06,08%)	23 (15,23%)	-	-
Total		Total number of samples	336	333	257	109	11
	BVDV 1	Reactive	281 (83,63%)	272 (81,68%)	138 (53,70%)	31 (28,44%)	0
		Non- reactive	55 (16,37%)	61 (18,32%)	119 (46,30%)	78 (71,56%)	11 (100%)
	BVDV 2	Reactive	278 (82,74%)	264 (79,28%)	151 (58,75%)	32 (29,35%)	0
		Non-reactive	58 (17,26%)	69 (20,72%)	106 (41,25%)	77 (70,64%)	11 (100%)

(-) harvest not performed for the age group.

Table 3 - Results of VN testing for BVDV-1 and BVDV-2 performed on serum samples from herd 4, according to age group at different harvesting times.

Age bracket	BVDV	VN	Harvests			
			1ª	2ª	3ª	4ª
0-6 mo.	BVDV 1	Total number of samples	7	11	1	-
		Reactive	5 (71,43%)	9 (81,82%)	0	-
		Non- reactive	2 (28,57%)	2 (18,18%)	1 (100%)	-
	BVDV 2	Reactive	5 (71,43%)	9 (81,82%)	0	-
		Non-reactive	2 (28,57%)	2 (18,18%)	1 (100%)	-
6-12 mo.	BVDV 1	Total number of samples	4	4	14	1
		Reactive	4 (100%)	4 (100%)	13 (92,86%)	1 (100%)
		Non- reactive	0	0	1 (7,14%)	0
	BVDV 2	Reactive	4 (100%)	4 (100%)	13 (92,86%)	1 (100%)
		Non-reactive	0	0	1 (7,14%)	0
12-24 mo.	BVDV 1	Total number of samples	5	5	18	-
		Reactive	5 (100%)	5 (100%)	17 (94,44%)	-
		Non- reactive	0	0	1 (5,56%)	-
	BVDV 2	Reactive	5 (100%)	5 (100%)	17 (94,44%)	-
		Non-reactive	0	0	1 (5,56%)	-
> 24 mo.	BVDV 1	Total number of samples	56	56	59	8
		Reactive	54 (96,43%)	55 (98,21%)	54 (91,53%)	3 (37,50%)
		Non- reactive	2 (3,57%)	1 (1,79%)	5 (8,47%)	5 (62,50%)
	BVDV 2	Reactive	54 (96,43%)	55 (98,21%)	53 (89,83%)	3 (37,50%)
		Non-reactive	2 (3,57%)	1 (1,79%)	6 (10,17%)	5 (62,50%)
Total		Total number of samples	72	76	92	9
	BVDV 1	Reactive	68 (94,44%)	73 (96,05%)	84 (91,30%)	4 (44,44%)
		Non- reactive	4 (5,56%)	3 (3,95%)	8 (8,70%)	5 (55,56%)
	BVDV 2	Reactive	68 (94,44%)	73 (96,05%)	83 (90,22%)	4 (44,44%)
		Non-reactive	4 (5,56%)	3 (3,95%)	9 (9,78%)	5 (55,56%)

(-) harvest not performed for the age group.

Table 4 - Results of VN testing for BVDV-1 and BVDV-2 performed on serum samples from herd 17, according to age group at different harvesting times.

Age bracket	BVDV	VN	Harvests		
			1ª	2ª	3ª
0-6 mo.	BVDV 1	Total number of samples	9	10	17
		Reactive	5 (55,56%)	6 (60%)	10 (58,82%)
		Non- reactive	4 (44,44%)	4 (40%)	7 (41,18%)
	BVDV 2	Reactive	5 (55,56%)	6 (60%)	7 (41,18%)
		Non-reactive	4 (44,44%)	4 (40%)	10 (58,82%)
6-12 mo.	BVDV 1	Total number of samples	6	6	11
		Reactive	3 (50%)	3 (50%)	0
		Non- reactive	3 (50%)	3 (50%)	11 (100%)
	BVDV 2	Reactive	2 (33,33%)	2 (33,33%)	0
		Non-reactive	4 (66,67%)	4 (66,67%)	11 (100%)
12-24 mo.	BVDV 1	Total number of samples	30	30	-
		Reactive	17 (56,67%)	17 (56,67%)	-
		Non- reactive	13 (43,33%)	13 (43,33%)	-
	BVDV 2	Reactive	19 (63,33%)	18 (60%)	-
		Non-reactive	11 (36,67%)	12 (40%)	-
> 24 mo.	BVDV 1	Total number of samples	49	48	-
		Reactive	42 (85,71%)	42 (87,50%)	-
		Non- reactive	7 (14,29%)	6 (12,50%)	-
	BVDV 2	Reactive	45 (91,84%)	43 (89,58%)	-
		Non-reactive	4 (8,16%)	5 (10,42%)	-
Total		Total number of samples	94	94	28
	BVDV 1	Reactive	67 (71,28%)	68 (72,34%)	10 (35,71%)
		Non- reactive	27 (28,72%)	26 (27,65%)	18 (64,29%)
	BVDV 2	Reactive	71 (75,53%)	69 (73,40%)	07 (25%)
		Non-reactive	23 (24,46%)	25 (26,60%)	21 (75%)

(-) harvest not performed for the age group.

animals in these age brackets, mainly in the last three harvests, was much higher than in the first two. Specifically in the 5th harvest, when blood samples were collected only from animals up to 12 months, no animal was positive for BVDV at that time (PILLARS & GROOMS, 2002). Relating the results of VN tests of herd 1 to the infection stages of BVDV as proposed by Houe (1995), it can be suggested that PI infected animals were removed around the time of the first two harvests.

In paired samples of the 1st and 2nd harvests from animals from age group 0 to 6 months, the majority of them had antibody titers lower in the 2nd harvest, including some animals no longer reactive, thus suggesting that the antibodies were from colostrum. However, in eight animals, antibody titers remained constant in the two harvests, and in one case the antibody titer was 4 times higher in the 2nd harvest. From this group of animals younger than 6 months at the time of the first two harvests, those eight animals were the only ones reactive to BVDV at the time of the 3rd and 4th sample harvests (data not shown).

However, in contrast to the setting stage of BVDV infection in the herd mentioned above, the infection source was present in the occasion of the 1st blood harvest, since three of eight reactive calves were approximately 30 days old. Moreover, this source of infection would probably be an animal TI, because an PI animal would infect a larger number of animals (SEKI et al., 2006). The BVDV diagnosis by RT-PCR would possibly detect PI animals easier than TI animals, since they constantly exhibit and eliminate high titers of the virus (HOUE, 1995), while TI animals eliminate low titers of BVDV virus and for a few days (MOERMAN et al., 1993; THURMOND, 2005).

On the other hand, the colostral antibodies may remove all free virus from the blood serum of infected calves up to 6 months old, which would result in false-negative BVDV tests (BEZEK & MECHOR, 1992; DUBOVI, 1996; SALIKI et al., 1997; STOKSTAD & LOKEN, 2002). Perhaps for this reason, BVDV was not detected by RT-PCR in any blood serum sample of the first two harvests.

Several factors could have contributed to the self-clearance of BVDV infection in herd 1, such as the death of a PI animal, for example. Moreover, as in many dairy herds, the animals were reared in lots, which restricts contact among all animals of the herd (LINDBERG & HOUE, 2005); male calves were discarded soon after birth, and this would reduce in 50% the probability of a PI new animal to be inserted in the herd (LINDBERG & ALENIUS, 1999; GUNN et al., 2004); and the acquired immunity of young animals would have been sufficient to prevent fetal infection and generation of PI animal when they became adults (MUÑOZ-ZANZI et al., 2004). The sale of heifers that were part of replacement breeding herd was a fairly common practice. Therefore, another hypothesis is that the marketing of heifers could also have contributed to the self-clearance of BVDV, as these heifers were most likely the ones to get pregnant with PI animals.

The characteristic of long-term antibody titers in the animals (LINDBERG & ALENIUS, 1999) was observed in the older animals of herd 1. In the first two harvests, all the cows older than five years were reactive to BVDV, and the cows between 8 and 10 years old had the highest antibody titers compared to all others (data not shown).

The economic losses caused by BVDV in herds where the virus is endemic are considered moderate, but remain constant (SANDVIK, 2004). This was also observed in herd 1, where according to the owner, the occurrence of reproductive changes, especially in heifers, and the difficulty to raise calves up to 6 months old due to lung and enteric infections was a constant problem. The owner also reported that the eight calves that were seropositive in the first two harvests and the only animals of the said age group that were reactive to BVDV in the third harvest, had also developmental delay compared to other animals of the same herd. It is likely that all changes were due to BVDV infection, which can act not only as the primary infectious agent, but also as an agent that favors secondary infections (BROCK, 2004; KOZASA et al., 2005).

The analysis of herd 4 showed that more than 90% of the animals were reactive to BVDV in 3 of 4 harvests, and it almost reached the entire herd in the 2nd harvest, with 96% of animals seropositive (Table 3). The BVDV detection by RT-PCR was performed in 36 samples and the virus was detected in five samples. Specifically, four samples from the first two harvests of animals younger than 6 months, one was a heifer (samples 04/58A and 04/58B) and the other a calf (samples 04/72A and 04/72B) (Figure 1 – Panel A). Both animals were PI, since BVDV was detected in samples of the same animal collected during an interval of at least three weeks (HOUE, 1995). According to the classification proposed by Houe (1995), this herd was at the stage where PI animals older than 4 months are diagnosed in herds with over 90% of the animals reactive to BVDV.

In the third harvest, BVDV was also detected in a blood sample of an adult animal non-reactive to VN test (sample 04/31C) (Figure 1 – Panel B). However, the sample of the same animal from the 4th harvest, displayed neutralizing antibodies against the virus and titer 2,560. This animal was classified as TI because at the time of the 3rd harvest it had an acute infection, therefore, the virus was controlled and antibodies were detected in the next harvest (MOERMAN et al., 1993; LINDBERG & ALENIUS, 1999; SANDVIK, 2004).

Herd 4 is a typical example of the Brazilian cattle herd, where beef cattle is raised extensively, all animals are part of the same large lot, with great transit of animals, that is, the constant commercialization of animals creates great circulation of animals between herds, thus favoring the spread of BVDV. This herd consisted of animals from different origins, which also constitutes an important risk factor in the transmission of BVDV (SOLIS-CALDERON et al., 2005).

The PI calves detected in this study were born to two heifers of one of these herds. They were purchased 12 months prior to the first blood sample harvest and were probably pregnant at the time. Therefore, these

heifers may have been infected with BVDV either prior being bought or after being inserted in the herd. If the latter occurred, the source of infection could have been a PI animal in the herd that had perhaps been subsequently sold, or a TI animal in the herd when the heifers were in early pregnancy. Finally, the fact is that the heifers were infected, which caused transplacental infection and the birth of two PI calves.

With respect to non-reactive animals in the 1st and 2nd harvests, besides the two PI infected animals, two animals older than 24 months were also non-reactive to the virus in the first harvest. However, one of them became reactive in the 2nd harvest and the other in the 3rd harvest. Probably, these animals were purchased shortly before the completion of the first harvest and when placed in the herd came into contact with PI animals. Moreover, the intense contact between the animals in the herd with PI infected animals was noticed by the high antibody titers (10,240) detected in animals of different age groups, therefore, indicative of recent infections and/or resulting from the constant stimulus promoted by BVDV.

Between the 2nd and 3rd harvests in herd 4, the PI heifer died, some cows and heifers were sold, as well as other animals were purchased. Soon after the 2nd harvest, one lot of steers was purchased and placed in the herd, and close to the date of the 3rd harvest, few heifers were also purchased. The VN tests performed in the samples from the 3rd harvest showed that all recently acquired steers were reactive to BVDV, and that all the cattle from the 2nd harvest remained infected, with the exception of the PI animal. However, from the batch of newly introduced heifers, two heifers were reactive while nine were non-reactive to the virus. BVDV was detected by RT-PCR in one blood sample of this group that was not reactive to VN test (sample 04/31C) (Figure 1 – Panel B).

Coincidentally, on the day of the 3rd harvest, the PI animal that by then was already a steer and was being held together with the other animals in the herd, was sold. The removal of the source of infection from the herd could be realized at the time of the 4th harvest, since from the nine sample of heifers non-reactive to BVDV by the VN test of the previous harvest, only four became reactive to the virus and, among them, the one where BVDV was detected by RT-PCR in the 3rd harvest (sample 04/31C), which characterized a TI animal. Since part of the heifers remained non-reactive, virus transmission through the TI infected animal was not very efficient (MOERMAN et al., 1993; LINDBERG & ALENIUS, 1999; SANDVIK, 2004; THURMOND, 2005).

When these heifers were placed in the herd, some of them were less than 60 days pregnant, and others were still not pregnant. Even though the PI animal, which is the main source of BVDV infection (HOUE, 1995), was removed, some of them had already been exposed to the virus and could have suffered fetal infection and be pregnant with PI calves. If this occurred, the herd would remain infected by PI animals to be born later (WITTUM et al., 2001).

About 70% of the animals of herd 17 were reactive to BVDV for the VN tests of the first two harvests (Table 4). The third harvest was conducted only in animals as old as 8 months, since the remaining animals in the herd were vaccinated against BVDV after the 2nd blood sample harvest. Of these animals, none was positive, with the exception of those younger than 4 months, and possibly the antibodies detected were of the colostral origin. BVDV was not detected by RT-PCR in any of the serum samples analyzed.

The results of both VN tests and RT-PCR, when correlated to the BVDV infection stages proposed by Houe (1995), suggested that possibly the PI animals were removed around the time of the first two blood sample harvests, as it happened in herd 1. The results of the VN tests of all serum samples from three harvests in herd 17, as well as the non-amplification of the viral genome of BVDV by RT-PCR, also provided evidence of the probable occurrence of self-clearance of BVDV (LINDBERG & ALENIUS, 1999).

The distribution of reactive animals of the first two harvests were very homogeneous among different age groups, but with higher incidence in adult animals (Table 4). These results showed that BVDV spread in the herd, because animals reactive to the virus were detected in all lots. However, the antibody titers present in the reactive samples were not as high as the ones detected in the other studied herds, with the maximum titer of 640 (data not shown).

Since there were infected animals in the entire herd, the source of infection was probably a TI animal. However, the hypothesis of infection by a TI animal should not be discarded, since they can also promote virus persistence in the herd (MOERMAN et al., 1993).The area of the property intended for rearing cattle was not extensive and the lots were close to each other. As this cattle herd was reared under semi-extensive management, the animals had access to the pastures and one of the lots was in contact with the neighboring animals since they were separated by a fence only. This contact would be an important risk factor for transmission of BVDV between herds (SMITH & GROTELUESCHEN, 2004).

Prior to the first harvest, the owner informed that the occurrence of reproductive changes in the herd had increased considerably during the last year, and that heifers were the most compromised animals of the herd. This information suggested that the agent responsible for these reproductive changes was the BVDV, since in newly infected herds, reproductive changes are the first consequence noticed (SANDVIK, 2004). Only lactating cows were commercialized, heifers remained in the herd, and when destined to breeding, they would be more likely to suffer fetal infection, because they were younger animals with fewer opportunities of previous contacts with BVDV.

If the virus was introduced in the herd by a TI animal, the infection could have ceased (MOERMAN et al., 1993), but persistent infection could also have been established (LINDBERG & HOUE, 2005). Thus, there could have been a transition period between acute infection of some animals and subsequent birth of PI calves (HOUE, 1994), since PI animals were not detected in the herd during monitoring period. It is important to mention that among the studied herds,

only herd 17 vaccinated animals older than 8 months against BVDV. However, the vaccination happened after the 2nd harvest, which would prevent only fetal infections from that moment (BROCK, 2004; SMITH & GROTELUESCHEN, 2004).

It is interesting to note the changing dynamic of BVDV infection that happened in the studied herds (SANDVIK, 2004). At the same time that reproductive changes were very evident in herd 17, the occurrence of BVDV in herd 4 was not even suspected before the tests and the productive and reproductive changes that happened in herd 1 were moderate and constant, thus suggesting that the infection was present in the herd for a considerable period. It is also important to highlight the probable spontaneous elimination of BVDV by herd 1 and the risk factors related to BVDV transmission, such as the frequent purchase of animals of different origins for herd 4, as well as the likely possibility that infection of herd 17 originated from the neighboring herds.

However, the main risk factor associated with the introduction of BVDV in a herd is the acquisition of cows pregnant with PI fetuses (BROCK, 2004). Possibly all three monitored herds contributed to the spread of the disease to other herds, since pregnant cows were traded in all of them. Perhaps, many producers who acquired pregnant cows in this condition from the monitored herds, acquired a "Trojan horse" (LINDBERG et al., 2002) and began to present different clinical forms of BVD after the birth of PI calves (BROCK, 2004). Herd 4 displayed extremely favorable conditions to spread the BVDV to other herds, the detection of PI animals showed the presence of viral activity; the constant transit of animals represented the constant availability of animal susceptible to infection, and marketing of animals of various ages, from calves to pregnant females, was the definitive step for transmitting the virus to other herds.

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