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Livestock Diseases

Co-infection by *Neopora caninum* and bovine viral diarrhea virus in cattle from Rio Grande do Sul, Brazil, destined to exportation[1]

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ABSTRACT.- Alves M.E.M., Fernandes F.D., Monteiro F.L., Braunig P., Cargnelutti J.F., Flores E.F., Weiblen R. & Vogel F.S.F. 2020. **Co-infection by *Neospora caninum* and bovine viral diarrhea virus in cattle from Rio Grande do Sul, Brazil, destined to exportation.** *Pesquisa Veterinária Brasileira 40(8):593-597*. Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Avenida Roraima 1000, Santa Maria, RS 97105-900, Brazil. E-mail: fagnermedvet@gmail.com

Reproductive tests in cattle are of great economic importance, given the impact it can have on the production system and may be caused by agents. *Neospora caninum* and Bovine Viral Diarrhea virus (BVDV) are considered of great importance as reproductive and should be considered responsible for keeping animals persistently infected. The present study included 479 calf serum samples for export in the state of Rio Grande do Sul (RS). All samples were screened for BVDV by an ELISA antigen. BVDV antigen-positive ELISA samples were isolated from BVDV in cell culture. An indirect immunofluorescence (IFT) technique was used to detect anti-*N. caninum* antibodies. Of the 479 export-treated serum samples, 361 were positive for BVDV antigens by ELISA and/or viral isolation test (361/479-75.36%), and 109 IFT-positive samples for *N. caninum* (109/479-22.75%). Despite detection of antibodies anti-*N. caninum* did not differ statistically between naturally infected BVDV and non-BVDV infected animals suggesting that there is no interference of BVDV infection on infection or detection rate of animals with *N. caninum*, positive animals in viral isolation and high DO in BVDV-Ag ELISA. may present active disease and consequent immunosuppression, contributing to a potential reactivation of *N. caninum*.

INDEX TERMS: Coinfection, *Neospora caninum*, bovine viral diarrhea virus, cattle, Rio Grande do Sul, Brazil, exportation, BVDV.

RESUMO.- [**Coinfecção por *Neospora caninum* e vírus da diarreia viral bovina em bovinos do Rio Grande do Sul, Brasil, destinados à exportação.**] Testes reprodutivos em bovinos são de grande importância econômica, dado o impacto que podem ter no sistema de produção e podem ser causados por agentes. O *Neospora caninum* e o vírus da Diarreia Viral Bovina (BVDV) são considerados de grande importância como reprodutivos e devem ser considerados responsáveis por manter os animais persistentemente infectados. O presente estudo incluiu 479 amostras de soro de bezerro para exportação no estado do Rio Grande do Sul (RS). Todas as amostras foram rastreadas para BVDV por um antígeno ELISA. As amostras de ELISA positivas para o antigénio BVDV foram isoladas a partir de BVDV em cultura de células. Uma técnica de imunofluorescência indireta (IFT) foi utilizada para detectar anticorpos anti-*N caninum*. Das 479 amostras de soro tratadas para exportação, 361 foram positivas para antígenos de BVDV por ELISA e/ou teste de isolamento viral (361/479-75,36%) e 109 amostras positivas para IFT para *N. caninum* (109/479-22,75%). Apesar da detecção de anticorpos anti-*N. caninum* não diferiu estatisticamente entre animais infectados naturalmente BVDV e não BVDV sugerindo que não há interferência da infecção pelo BVDV na infecção ou taxa de detecção de animais com *N. caninum*, animais positivos em isolamento viral e alta DO em BVDV-Ag ELISA, pode apresentar doença ativa e consequente imunossupressão, contribuindo para uma potencial reativação de *N. caninum.*

TERMOS DE INDEXAÇÃO: Coinfecção, *Neospora caninum*, vírus da diarréia viral bovina, bovinos, Rio Grande do Sul, Brasil, exportação, BVDV.

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INTRODUCTION

Cattle reproductive diseases have great economic importance due to productive losses as return to estrus, abortion, long interval between births, birth of weak and unviable animals, birth reduction and increase in females discard rate (Dubey et al. 2007, Neta et al. 2010, Lanyon et al. 2014, Lilenbaum & Martins 2014). Diverse etiological agents are related to bovine reproductive diseases and the most frequent are *Neospora caninum* (NC), Bovine Viral Diarrhea virus (BVDV), *Leptospira* spp., Bovine Herpesvirus type 1 (BoHV-1), *Brucella* spp., *Campylobacter* spp., *Tritrichomonas* spp., *Chlamydia abortus*, *Coxiella burnetii* (Kirkbride 1992, Morris et al. 2018, Olmo et al. 2018, Softic et al. 2018). Among these agents NC and BVDV have significant importance because they to promote reproductive losses and have the ability to establish persistent infections (Chernick et al. 2018).

Bovine pestivirus are worldwide important pathogens of cattle promoting reproductive, enteric, hemorrhagic and/or respiratory disorders (Dubovi 1994, Pellerin et al. 1994, Flores et al. 2000, Fulton et al. 2002). Three species of pestivirus are recognized affecting bovine: Bovine viral diarrhea virus types 1 (BVDV-1, or Pestivurus A), Bovine viral diarrhea virus types 2 (BVDV-2, or Pestivirus B) and HoBi-like (Pestivirus H). The maintenance of BVDV (and HoBi-like) in herds occurred mainly by persistent infected (PI) animals, that are BVDV immunotolerant. These animals are produced when seronegative cows are infected by BVDV at 40 to 120 days of pregnancy, and after birth, calves allow the virus replication in a variety of tissues and shed virus in secretions and excretions for their lifetime (McClurkin et al. 1984). Immunosuppression is a common consequence of BVDV infection in adult animals that facilitates secondary infections and disease severity (Brownlie 1990).

NC is a protozoan belonging to the phylum Apicomplexa and family Sarcocystidae (Dubey et al. 1999), it is widely distributed in the world and considered one of the main agent causing abortion in bovines (Dubey & Schares 2011). The definitive hosts are domestic and wild canids (Gondim 2006) and NC is capable of infecting a wide range of intermediate hosts including bovine. In bovine, infection occurs via horizontal or vertical, and the vertical transmission is the most important in epidemiological aspect (Williams et al. 2009). Infection of pregnant cows promotes embryonic mortality, return to estrus, abortion, birth of weak animals, birth of animals with nervous signs and/or healthy but persistently infected animals (PI). PI calves are the main responsible for the maintenance of the agent in the herd and therefore they are extremely important in the epidemiology of the disease (Dubey et al. 2007). Besides that, transplacental infection by *N. caninum* may occur at any gestational stage and also may occur in subsequent pregnancies of persistently infected females due to the protozoa reactivation (Davison et al. 1999, Dubey et al. 2007).

BVDV frequently determine immunosuppression that contributes to subsequent infection by other agents or reactivation of latent agents and this viral infection also contributes to the severity of infection caused by other agents (Baker 1995). NC may be a secondary cause of abortion and also may potentialize abortion caused by other agents (Mineo et al. 2006, Asmare et al. 2012). BVDV and *N. caninum* co-infection is reported in other studies and suggested that should be related to higher rates of reproductive losses and consequently economic losses (Thurmond et al. 1997).

Thus, due the economic impact related to productive and reproductive losses caused by the infection with these two agents in herds, the present study aimed to determine the frequency of antibodies anti-*N. caninum*, BVDV antigen and the co-infection by *N. caninum* and BVDV in animals naturally infected destined to exportation.

MATERIALS AND METHODS

The current study included 479 sera samples of beef calves destined to exportation from Rio Grande do Sul state (RS). All samples were screened for BVDV by an antigen ELISA. Positive samples for BVDV antigen in ELISA were submitted to BVDV isolation in cell culture. Indirect immunofluorescence technique (IFT) was used for antibodies anti-*N. caninum* detection.

Samples. A total of 479 samples of beef sera were taken from calves destined for export was maintained under refrigeration (4°C) until analyses.

Antigen ELISA. All 479 sera samples (50µl) were submitted to BVDV antigen ELISA (ELISA BVDV-Ag) using IDEXX BVDV Ag/Serum Plus (code 9943830) and/or IDvet ID Screen BVDP80 Antigen Capture (code BVDAGP80-10P) kits. All procedures were performed according manufacture instructions. Samples were considered positive to BVDV antigen when corrected OD ration (OD sample - OD negative) was >0.3 and/or the S/P ration was ≥0.2, when IDEXX kit and IDVet kit were used, respectively.

Virus isolation. Virus isolation was performed into monolayers of MDBK cells by inoculating sera samples obtained from positive BVDV antigen ELISA (50µl/well of 24-wells plate). Samples were submitted to 3 passages of 5 days each. The presence of viral antigens was assessed by submitting inoculated cells (at passage 3) to an indirect fluorescente antibody assay (IFA), using a pool of BVDV-specific monoclonal antibodies (MAbs) as primary antibodies (Corapi et al. 1990, Kreutz et al. 2000) and anti-mouse conjugated with fluorescein as secondary antibody. Samples were evaluated at 400x magnification under epifluorescence microscope (Carl Zeiss - Germany, HBO 50/AC, Axiolab) using a 465-495nm excitation and 515-555nm emission filter.

Indirect immunofluorescence technique (IFT) for NC antibodies. The IFT was used to detect immunoglobulins-G against NC in the serum of animals. The IFT evaluation was performed using microscopy slides containing fixed tachyzoites of the NC-1 strain of NC. Sera samples were diluted 1:50 in PBS (pH 7.2) and incubated for 30 min at 37°C in a humid dark chamber. The secondary antibody used was bovine anti-IgG-fluorescein isothyocianate (FITC) conjugate for 30 min at 37°C in a humid dark chamber. Serum sample known as positive or negative was used as a control. Samples were evaluated at 400x magnification under epifluorescence microscope (Carl Zeiss - Germany, HBO 50/AC, Axiolab) using a 465 495nm excitation and 515-555nm emission filter. We considered positive samples reactions that showed a peripheral or diffuse fluorescence in the tachyzoites surface, in contrast to apical or polar fluorescence that were considered negative samples (Paré et al. 1995).

Statistical analysis. Comparisons between the direct ELISA results for BVDV, BVDV isolation and the detection of anti-NC antibodies were analyzed using the Chi-square test.

RESULTS AND DISCUSSION

From the 479 sera samples of calves destined to exportation, 361 were positive for BVDV antigens in ELISA and/or viral isolation test (361/479-75.36%), and 109 positive samples in IFT for NC. (109/479-22.75%). The detection of anti-*Neospora caninum* antibodies in animals naturally infected by BVDV was 23.27% (84/361) and 21.18% (25/118) in animals negative for BVDV antigen (Table 1).

The occurrence of anti-NC antibodies detected in the present study when the total serum samples were analyzed (22.75%) is in accordance with serological studies realized in some Brazilian regions/states, as Goiás (27%) (SANTIN et al., 2017) and Paraná (13.2%) (Snak et al. 2018). There was not statistical difference in the frequency detection of antibody anti-NC in animals naturally infected with BVDV (23.27%) and not infected (21.18%), suggesting that in the bovines tested, the frequency of animals persistently infected with *N. caninum* should not be influenced by previously BVDV infection.

Studies conducted by He et al. (2004) in Australia and Lassen et al. (2012) in Estonia also did not found correlation between BVDV infection and *Neospora* spp. However, Chi Duong et al. (2008) found association between the presence of anti-*N. caninum* and anti-BVDV antibodies in cows from small farms in Vietnam and this association between the occurrence of *Neospora* spp. and BVDV antibodies was been previously described by Björkman et al. (1996) in Sweden. In Brazil, Melo et al. (2004) detected anti *Neospora* spp. and anti-BVDV antibodies in milk from cows, demonstrating co-existence between these two agents in the analyzed herd. Therefore, the levels of BVDV or NC infection and co-infection are associated with herd characteristic as size, beef or milk production system and sanitary conditions (Thurmond et al. 1997).

The presence of anti-*Neospora* spp. in animals that did not ingest colostrum and non-vaccinated indicates the occurrence of persistent infection (Dubey et al. 2007). Therefore, all animals that have antibodies to *Neospora* spp. diagnosed are persistently infected. The detection of BVDV virion or viral antigens indicates both acute infection (transiently infected animals - TI) or persistent infection (persistently infected animals - PI) (Bachofen et al. 2010). Therefore, serologically positive animals for *Neospora* spp. and positive for BVDV antigen in viral isolation or ELISA-Ag tests are considered to be co infected by these two agents. The viral and parasitological coexistence should be related to a series of epidemiological factors mainly the immunosuppression determined by BVDV (Melo et al. 2004).

Detection frequency of anti-NC. in positive- BVDV animals from viral isolation was 18.75%, whereas positive - BVDV in ELISA-Ag and negative in viral isolation were 23.7% (Table 2). Concomitant infection by two agentes should be related to birth reduction rate, PI and TI animals usually suffer of immunosuppression caused by BVDV infection, which probably facilitate secondary infection by other agents, as *N. caninum* (Asmare et al., 2012). Pregnant cows co-infected with *N. caninum* and BVDV (female TI, female harboring PI calf, or female PI) would have higher rates of abortion and return to estrus than monoinfected cows and this occurs because co-infections potentialize the negative reproductive effects in cattle caused by both agents (Bjorkman et al. 2000, Wouda et al. 1998, Quinn et al. 2004).

Analyzing OD results obtained in ELISA, the detection frequency of anti-Neospora spp. antibodies is higher when OD is above 1.01 (Table 3). Higher OD in direct anti-BVDV ELISA is related to higher antigen detection and consequently higher probability of BVDV PI animals (Cornish et al. 2005). When BVDV isolation in cell culture was possible, it was observed that from the six positive animals for both agents BVDV and *N. caninum*, five had high OD in ELISA (Table 3). Analyzing the negative samples, a similar tendency is observed.

Monoinfection or co-infection with *N. caninum* and BVDV is associated with reproductive losses at any stage of gestation (Wouda et al. 1998) and despite of the detection of antibodies anti-*N.caninum* did not differ statistically between animals naturally infected by BVDV and not BVDV infected suggesting that there is not interference of BVDV infection in the infection or detection rate of animals with *N. caninum*, animals positive in viral isolation and with high OD in BVDV-Ag ELISA may

Table 1. Detection of anti-*Neospora caninum* in animals naturally infected by BVDV

ELISA BVDV-Ag	Anti-*Neospora caninum* IgG			
	Positive		Negative	
	Number	Percentual (%)	Number	Percentual (%)
Positive (361)	84	23.27	277	76.73
Negative (118)	25	21.18	93	78.82
Total (479)	109	22.75	370	77.25

Table 2. Detection from viral isolation

BVDV viral isolation	Anti-*Neospora caninum* IgG			
	Positive		Negative	
	Number	Percentual (%)	Number	Percentual (%)
Positive	6*	18,75	26	81,25
Negative	78	23,70	251	76,3
Total	84	100	277	100

Table 3. Results obtained in ELISA

ELISA BVDV-Ag	Positive BVDV isolation		Negative BVDV isolation		TOTAL	anti-*Neospora caninum* IgG	
	anti-*Neospora caninum* IgG		anti-*Neospora caninum* IgG				
OD	+	-	+	-		+	-
Lower 0.3*	-	-	25	93	118	25	93
0.3-1.0	1	8	57	194	260	58	202
1.01 – 2.0	0	4	14	36	54	14	40
Above 2,01	5	14	7	21	47	12	35
TOTAL	6	26	78	251	361	109	370

* Negative in ELISA BVDV antigen.

present active disease and consequently imunnosupression as disease consequence contributing to a potential *N. caninum* reactivation. Although the samples in this study were obtained from animals destined to exportation, and the reproductive history of the original properties is unknown, the results suggested the occurrence of infection with *N. caninum* and/or BVDV and that should be related to reproductive problems and consequently economic losses.

CONCLUSIONS

The present study reinforces the importance and occurrence of *Neospora caninum* and BVDV as pathogens infecting bovines and although the demonstrated low occurrence of co-infection, these agents are circulating in bovine herds and consequently causing damage to health, reproduction and animal production.

Further research should be conducted in animals infected by the two agents to more clearly determine the importance of BVDV and NC co-infection in the reproductive rates of cattle.

Conflict of interest statement.- There are no conflicts of interest.

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