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MICROBIOLOGY

Effect of bovine leukemia virus infection and proviral load on the systemic profile of dairy heifers during the transition period

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RESUMO: O objetivo deste estudo foi avaliar o efeito do vírus da Leucose Bovina (BLV - BovineLeukosis Virus) sobre o perfil sistêmico em vacas primíparas no período de transição. Novilhas prenhas das raças Holandesa e Jersey (n=24) foram distribuídas em pares em dois grupos experimentais: (BLV+) e (BLV–). Os animais BLV+ foram classificados em subgrupos com alta e baixa carga proviral, de acordo com a mediana dos valores obtidos. Os animais foram avaliados nas semanas -3, -2, -1, parto (0), +1, +2 e +3. Amostras sanguíneas foram obtidas para análises hematológicas e bioquímicas, além da dosagem de haptoglobina. Na fase de triagem observou-se soroprevalência de 57,25% e 38.7%, respectivamente no rebanho e na categoria novilhas (38,7%). Em relação ao eritrograma, a concentração de hemoglobina corpuscular média (CHCM) foi a única variável que apresentou interação grupo*tempo, observando-se maiores valores no grupo BLV+ (BLV+ = 33,29 ± 3,39%; BLV- = 31,08 ± 2,31%). BLV teve efeito sobre a atividade da enzima aspartato aminotransferase (AST), com maiores valores no grupo BLV+. Os animais BLV+ apresentaram 4,96 mais chances de inflamação (haptoglobina ≥ 2,0 mg/dL). A concentração do fibrinogênio também foi maior no grupo BLV+ no parto e semana +1 pós-parto. O efeito da carga proviral sobre os leucócitos totais e número absoluto de linfócitos foi detectado. No nosso conhecimento, este é o primeiro estudo reportando o efeito do BLV sobre a saúde de vacas primíparas no período de transição, e a dependência da carga proviral sobre o perfil leucocitário e o perfil pró-inflamatório dos animais BLV+.
Palavras-chave: leucose, haptoglobina, leucocitose, linfocitose, novilhas, transição.

Efeito da infecção pelo vírus da leucose bovina e carga pró-viral sobre o perfil sistêmico de vacas primíparas no período de transição

ABSTRACT: This study aimed to evaluate the effect of bovine leukemia virus (BLV) on the systemic profile of naturally infected dairy heifers during the transition period. Pregnant Holstein and Jersey heifers (n=24) were distributed in pairs into two experimental groups: (BLV+) and (BLV–). Animals in the BLV+ group were divided into two subgroups based on the median BLV proviral load (high and low). The animals were then assessed at weeks –3, –2, –1, calving time (0), +1, +2, and +3. Blood samples were obtained for hematological and biochemical analyses, as well as haptoglobin measurements. Farm BLV screening revealed a herd BLV prevalence of 57.25% and heifer BLV prevalence of 38.7%. Mean corpuscular hemoglobin concentration was the only hematological variable for which group interaction was observed, with BLV+ cattle having higher values (33.29 ± 3.39%) than BLV– cattle (31.08 ± 2.31%). Aspartate aminotransferase activity was higher in the BLV+ heifers. The BLV+ group had greater incidence of inflammation (haptoglobin ≥ 2.0 mg/dL). Fibrinogen concentrations were also higher at weeks 0 and +1 in BLV+ heifers than in BLV– heifers. A high proviral load affected total leukocyte and lymphocyte count; however, this profile was not observed in the low proviral load and paired BLV– heifers. To our knowledge, this is the first study to report the impact of BLV infection on the health of dairy heifers during the transition period, demonstrating the effect of proviral load on white blood cell changes and early inflammation in infected animals.
Key words: leukosis, haptoglobin, leukocytosis, lymphocytosis, heifers, transition.

INTRODUCTION

Bovine leukemia virus (BLV) belongs to the Retroviridae family and to the genus Deltaretrovirus, which is genetically and antigenically similar to human T-lymphotropic viruses (GILLET et al., 2007). Vertical transmission of BLV can occur in utero or during delivery from infected dams to their offspring and by the administration of fresh colostrum and raw milk (RUIZ et al., 2018). However, the main route of BLV transmission is iatrogenic, via needles, palpation gloves, dehorning, and tattooing (BARTLETT et al., 2020; RUGGIERO & BARTLETT, 2019). The risk of transmission increases proportionally with proviral load (JULIARENA et al., 2007). Subclinical disease is the most common presentation of BLV infection, and approximately 30–40% of infected animals present with persistent lymphocytosis (PL). Less than 5% of infected cattle develop malignant lymphosarcoma (SCHWARTZ et al., 1994).

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Proviral load may be associated with genetic factors (JULIARENA et al., 2008; MIRSKY et al., 1998) or be a consequence of long-term exposure observed in cows presenting with pronounced lymphocytosis during the transition period (WISNIESKI et al., 2020). Even cattle without PL can have a high proviral load (HPL).

BLV infection alters the mechanism of apoptosis and proliferation of infected immune cells in cows. In particular, it mainly targets B lymphocytes, disrupting the function and distribution of lymphocyte subsets in the blood (MIRSKY et al., 1998). The proportion of B:T cells in BLV+ animals is often non-proportional, with a discernible bias toward the B population (BARTLETT et al., 2013). Additionally, the innate immune response is also compromised by BLV (BLAGITZ et al., 2017). DELLA LIBERA et al. (2015) reported that infected cows had reduced milk neutrophil function, marked by decreased phagocytosis and production of reactive oxygen species, as well as decreased the viability of B cells present in milk, especially in PL animals. Infected cattle are more susceptible to clinical disorders and co-infection, which can compromise milk production and longevity (BARTLETT et al., 2013; EMANUELSON et al., 1992), as well as impair milk production following immunization against other pathogens (PUENTES et al., 2016).

The transition period is critical for dairy cattle. It is a period of metabolic distress, characterized by metabolic and oxidative stress, which results in a proinflammatory and immunosuppressive state in cattle. This scenario may be worse for cattle infected with BLV (GOMES et al., 2017). Few studies have reported the impact of BLV infection on dairy cows during the transition period, and to the best of our knowledge, no study has reported the effect of BLV on the health parameters of dairy heifers during the transition period. In our previous study (GOMES et al., 2017), we described the profile of BLV+ multiparous cows during the transition period, which was characterized by lymphocytosis between weeks –3 and partum, due to the increase in B cells (CD21+), followed by a decrease in the leukocyte population in the first 3 weeks after calving. Furthermore, the percentage of BLV+ cattle increased from dry-off (38.9%) to delivery (43.6%). However, this descriptive study did not include a control group of BLV– cattle. In addition, most BLV studies have not evaluated the proviral load in heifers during the transition period, which seems to be the main marker for the immune imbalance and induction of low proviral load (LPL) in infected cattle.

We hypothesize that the effect of BLV on health occurs during early infection and can manifest in the first transition period of young primiparous cattle, in addition to the possible influence of individual responses based on the proviral load of BLV. This study aimed to evaluate the effect of BLV on the health of young dairy heifers during the transition period using a set of laboratory examinations, such as red blood cells (RBC) and white blood cells (WBC) parameters, biochemical biomarkers, and acute-phase proteins.

MATERIALS AND METHODS

Farm, animals, and management

This study was conducted in a commercial dairy farm located in Rio Grande do Sul State, Brazil (latitude 29°28'14.0"S, longitude 51°33'53.1"W, altitude 484 m). The farm has 648 Holstein and Jersey breed animals, with 230 lactating cows producing 6,100 L of milk per day (an average of 26.2 L per cow). The heifers were maintained in pastures until they reached a reproductive age of approximately 13–14 months. They received weekly doses of prostaglandins until they were in heat, followed by artificial insemination. Every 14 d, pregnancy diagnostic ultrasonography was performed. Animals were maintained in the pasture for up to 2 months from expected calving and transferred at 60 and 30 d prepartum to the free-stall barn and maternity compost barn, respectively. The compost barn consists of a large area covered with sawdust, wood-cutting waste, and composted manure. After calving, the animals were reintroduced into the free-stall system with mattress bedding, automatic forced ventilation, and sprinklers to avoid heat stress. During the experimental period, the animals were fed diets formulated to meet the requirements recommended by the National Research Council (2001).

Initial screening and inclusion criteria

All animals in the herds older than 90 d (n=613) were screened serologically for BLV using enzyme-linked immunosorbent assay-blocking ELISA (BLV Compact 2.0®, Ingenasa, Ingezim) to determine the general herd prevalence. Animals under the age of 3 months were not tested to avoid maternal antibody interference with the serological test results.

After initial serological screening, 24 Holstein (n=12) and Jersey (n=12) heifers between 23 and 26 months of age that were close to calving were included in this study. The animals were divided into BLV+ (n=12) [Holstein BLV+ (n=6), Jersey

BLV+ (n=6)] and BLV– [Holstein BLV– (n=6), Jersey BLV– (n=6)] groups. The animals were tested for specific antibodies at weeks –3, 0, and +3, and digital polymerase chain reaction (PCR) was performed at weeks –3 and +3. The same criteria for BLV– animals were expected to yield negative results in both tests at the same time points (at the beginning and end of the transition period). Positive and negative heifers were matched in pairs according to date of birth, breed, and expected pregnancy. We sampled 16 animals (eight pairs) between October 2018 and February 2019 and eight animals (four pairs) between July and September 2019, corresponding to winter and spring seasons. Multiparous cows were excluded from the experiment because of difficulty in detecting and selecting animals older than 36 months that tested negative for BLV. Nine heifers were excluded from the experiment because of abortion (n=1), positive PCR results with negative serology results (n=2), anticipated calving (n=3), and your corresponding pair (n=3).

Blood samples

Heifers and young primiparas from both groups were sampled weekly at –3, –2, –1, 0 (calving), 1, 2, and 3 weeks' time. Whole blood and serum were collected in three tubes containing K3 ethylenediamine tetraacetic acid (EDTA) and two tubes containing a clot activator (Vacutube, Labor Import, China) by coccygeal venipuncture using a 25 × 8 mm needle (BD Vacutainer, Brazil) in a vacuum system. Serum and plasma were centrifuged at 1,000 x *g* for 10 min in tubes containing clot activator and K3 EDTA, respectively, and stored in 2 mL microtubes at –20 °C.

Dropletdigital PCR for BLV quantification

Nucleic acids were extracted from whole blood samples using the commercial kit MagMAX™ CORE Nucleic Acid Purification Kit (Applied Biosystems) in accordance with the manufacturer's standard protocol. The nucleic acids were purified from individual samples and total DNA was measured using qubit ® following standard manual instructions.

Droplet digital PCR (ddPCR) was performed using the QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The HEX probe and primer sequences were designed to amplify a partial segment of the BLV env gene (DE BRUN et al., 2022). The primer sequences were F: 5′-CAG TGA CTG GGT TCC CTC TGT C-3′, R: 5'-AGG GCG AGR CCG GGT CCA GAG-3' and the probe was: HEX 5'-CCC TCC CTG GGC TCC CGA RA-3'BHQ1. Briefly, 1 µL of DNA was mixed with 1 µL of each primer (10 µM) + 0.5 µL the HEX probe (10 µM) + Bio-Rad 2 × Supermix was emulsified, and the droplets were transferred to a 96-well plate (Eppendorf, Hauppauge, NY, USA). PCR was performed in a C1000 touch thermocycler (Bio-Rad) with the following conditions: initial denaturation for 10 min at 95 °C, then 40 cycles for 30 s at 94 °C and 1 min at 58 °C, and a final deactivation of the enzyme for 10 min at 98 °C. Finally, fluorescent droplets were used to determine the number of resulting positive events and analyzed using QuantaSoft version 1.7.4 software (Bio-Rad). The fluorescence detection threshold was determined manually based on the negative controls. The BLV proviral load was determined as copies/µL of genomic bovine DNA, and each sample was analyzed in duplicate (DE BRUN et al., 2022). The results are expressed as the average of two measurements. The median was traced to identify animals with LPL/HPL.

Hematology

Hematological cells and compounds were evaluated using an automatic hematological reader (Mindray BC 2800 Vet), and leukocyte subsets were differentiated using microscopic blood smear Giemsa staining.

Biochemical biomarkers

The activities of serum aspartate aminotransferase (AST) (Labtest®ref: 109) and gamma-glutamyl transferase (GGT) (Labtest® ref: 105) were measured using an automated wet chemistry analyzer (Smart 200, Biotecnica®) and commercial kits (LAB-TEST®), according to the manufacturer's standard protocol. Serum beta-hydroxybutyrate (BHB) (Randox® ref: RB1008), cholesterol (Randox® ref: CH2655), and triglyceride (Randox® ref: CH3810) levels were measured using a second automated wet chemistry analyzer (LabMax 240, Labtest, Tokyo, Japan). The total plasma protein (TPP) levels were estimated using a total protein refractometer (Vet RHC-300 ATC; Contec).

Acute-phase proteins

Fibrinogen was evaluated at seven time points (–3 to +3 weeks) in both BLV+ and negative animals. Heat-denaturing techniques were used to determine fibrinogen concentration, according to MILLAR et al. (1971). Haptoglobin (HP) concentration was assessed at weeks – 3, 0, and +3 using a turbidometric assay according to the procedures described by RAMOS et al. (2021).

Statistical analysis

Statistical package for the social sciences (SPSS Statistics for Windows, Version 19.0, IBM Corp, Armonk, NY, USA) was used for statistical analyses. Data distributions were initially analyzed using the profile observed in histograms, q-plots, and homogeneity variance tests. Variables presenting a nonlinear distribution were subjected to numerical transformation. After data linearization, a mixed model was used to evaluate the effects of infection status (BLV+ and BLV–), time (weeks –3, –2, –1, 0, +1, +2, +3), and group × time interaction on health parameters of dairy heifers during the transition period. Unstructured, autoregressive, and component symmetric covariant matrices were tested, and the models were selected according to the lower value of the Akaike information criterion. Comparisons between groups at each time point were performed using the t-test only when group and interactions were detected in the mixed model procedure. The same statistical analysis was performed to compare primiparous cattle with HPL or LPL and the corresponding BLV– cattle.

A logistic regression model for repeated measures was generated, considering infection status (BLV+ and BLV–) as an independent variable and animals with inflammation (HP > 2 mg/dL), ketosis (BHB > 1.2 mmol/L), and hepatic damage (AST > 132 U/L) (HUTCHINSON et al., 2020) as the dependent variables. The Spearman correlation between the viral load and hematological and biochemical parameters at weeks –3 and +3 were analyzed using R studio version 4.0.5 (R studio, Boston, USA). Differences were considered significant at $P < 0.05$, and those at $0.05 < P \leq 0.10$ were considered tendencies.

RESULTS

Serological screening

The general prevalence of BLV was 57.25% (n=351/613) and 38.7% in the heifer group (> 90 d) (76/124).

Hematological profile

Table 1 shows the effects of BLV, time, and the interaction of BLV group × time on the hematological profile of dairy heifers during the transition period. BLV infection did not affect blood parameters, and the interaction between time × BLV group was only detected in the mean corpuscular hemoglobin concentration group (P=0.02). The means of the experimental group were contrasted at each time point, and higher values were observed for the BLV+ (33.29 ± 3.39%) group than the BLV– group (31.08 ± 2.31%) at week +2 (P=0.01).

The time-analysis of the hemogram components within each experimental group are presented in Supplementary table 1 (available at https://zenodo.org/records/10146114).The effect of time was detected for most RBC parameters, except for MCV and MCHC. The time-analysis within each experimental group shows the effect of the transition period for RBC count and hemoglobin concentration in the BLV– group. RBC and hemoglobin concentration peaked on calving day, followed by a drop in the post-partum period, with the lowest value detected on week +2. The number of eosinophils increased from week –3 to week –1, followed by an intense fall on week 0 (calving day) which persisted until the final day of the experiment (week + 3).

For the BLV+ group, transition period was observed to affect the RDW. The RDW variable had an increase among week –3 to week +1, followed by an intense drop in the subsequent weeks, with the lowest value observed on week +1.

The effect of time was observed in the BLV– and BLV+ groups through the number of platelets. Overall, a gradual increase in the number of platelets was observed from week –3 up to week +3.

Biochemical biomarkers profile

Table 1 shows the effects of BLV, time, and the interaction of BLV group × time on the biomarker profiles of dairy heifers during the transition period. The effect of infection status was detected for AST, in addition to the tendency of increased acute phase proteins in the BLV groups. Interaction between time x group was found for triglycerides (P=0.02) and fibrinogen (P=0.04) parameters. The time effect was observed for all biochemical variables, except fibrinogen.

The time-analysis of the biochemistry parameters within each experimental group are presented in Supplementary file 2 (available at https://zenodo.org/records/10146114). AST values were constantin the BLV– group during the transition period; however, the BLV+ group differed between time periods. AST in the BLV+ group was higher from weeks –3 up to week +2 than week +3 post-partum. GGT varied with time only in the BLV– group, with higher values observed in the pre-partum than post-partum period. BHB had time variations in both experimental groups, with high values during the post-partum period. Triglyceride concentration was lower in the post-partum than pre-partum period in both experimental groups, while cholesterol concentration exhibited opposite behavior. BLV+

Table 1 - Effect of bovine leukemia virus (BLV) on the red blood cells, white blood cells, and biochemical biomarkers in dairy heifers through the transition period.

	----------Groups----------		----------P-value----------		
	BLV+ (n=12)	BLV- (n=12)	Time	Groups	Time × Groups
Red blood (cell/µL)	5.98±1.39	5.96±0.69	0.00	0.67	0.88
Hemoglobin (g/dL)	9.74±1.42	9.53±1.4	0.00	0.47	0.37
Hematocrit (%)	29.81±4.08	29.19±4.98	0.00	0.56	0.59
MCV (fL)	50.7±5.73	49.71±3.88	0.22	0.32	0.49
MCHC (%)	32.72±2.31	32.23±1.99	0.53	0.23	0.02
RDW (%)	18.04±11.02	16.52±0.93	0.00	0.50	0.22
Leukocytes (× 10^3/µL)	17.9±9.24	13.16±3.92	0.34	0.18	0.88
Neutrophil (× 10^3/µL)	5.36±3.24	4.75±2.42	0.25	0.67	0.74
Lymphocytes (×10^3/µL)	11.56±8.1	7.81±2.6	0.64	0.39	0.31
Monocytes (× 10^3/µL)	0.20±0.3	0.17±0.20	0.41	0.64	0.97
Eosinophil (× 10^3/µL)	0.76±1.01	0.42±0.43	0.00	0.08	0.47
Platelets (× 10^3/µL)	368.7±174.67	302.89±114.91	0.00	0.13	0.98
AST (U/L)	77.73±31.57	72.87±53.71	0.01	0.02	0.21
GGT (U/L)	23.41±9.5	21±6.67	0.01	0.25	0.19
BHB (mmol/L)	0.45±0.19	0.45±0.19	0.00	0.83	0.71
Triglycerides (mg/dL)	18.63±12.59	18.83±12.83	0.00	0.77	0.02
Cholesterol (mg/dL)	78.89±18.8	71.52±17.75	0.00	0.17	0.75
TPP (g/dL)	6.68±0.62	6.57±0.76	0.00	0.53	0.59
Haptoglobin (mg/dL)	8.81±15.96	3.35±3.49	0.00	0.09	0.95
Fibrinogen (mg/dL)	354.88±204.37	371.43±174.64	0.32	0.60	0.04

Legend:MCV, Mean corpuscular volume; MCHC, Mean corpuscular hemoglobin concentration; RDW, Red cell distribution width, AST, Aspartate aminotransferase; GGT, Gamma-glutamyl transferase; BHB, β-Hydroxybutyrylation; TPP, Total plasma protein. Statistical difference was considered when the P-value ≤ 0.05. The effects of group, time, and group × time interaction were determined using a mixed model procedure.

and BLV– groups had similar variation for TPP, with higher values on weeks –3 and weeks +2 and +3. The acute phase protein fibrinogen had variations only in the BLV+ group, peaking on weeks +2 and +3.

Figure 1 shows the prevalence of heifers presenting with inflammation based on a cut-off of HP >2. During the transition period, the BLV+ group had a higher prevalence of animals with HP >2 mg/dL (79.4%) than the BLV– group (43.8%) with an odds ratio of 4.959 (IC95%: 1.114–22.080) for inflammation to occur in the BLV+ group than in the BLV– group. Regarding ketosis and hepatic damage, no association between the groups (BLV+ and BLV–) was observed in the logistic model because the data of all animals remained within acceptable range limits.

Effect of BLV proviral loads on hematological and biochemical parameters.

Proviral load for each BLV+ and BLV– heifer established at weeks –3 and +3 are shown in table 2. BLV+ heifers were distributed into HPL and LPL groups by assessing the median values obtained in this study (3.28 copies/µL of genomic bovine DNA).

Effects of proviral load on the hematological and biochemical parameters of dairy heifers during the transition period are presented in table 3 and table 4. There was no effect of infection status or interaction group x time for hematological and biochemical variables comparing BLV+ cattle presenting with LPL and their BLV– counterparts, except for the high values of AST in BLV+ heifers. Primiparous dairy cattle with an HPL had a higher count of total leukocytes (P=0.01) and lymphocytes (P=0.03) than their corresponding BLV– counterparts. No interaction was observed between the groups and time for any hematological and biochemical variables.

DISCUSSION

This study investigated the effect of BLV on the health status of dairy heifers during the transition period. The herd serum prevalence of BLV was 57.25%, with lower prevalence for heifers

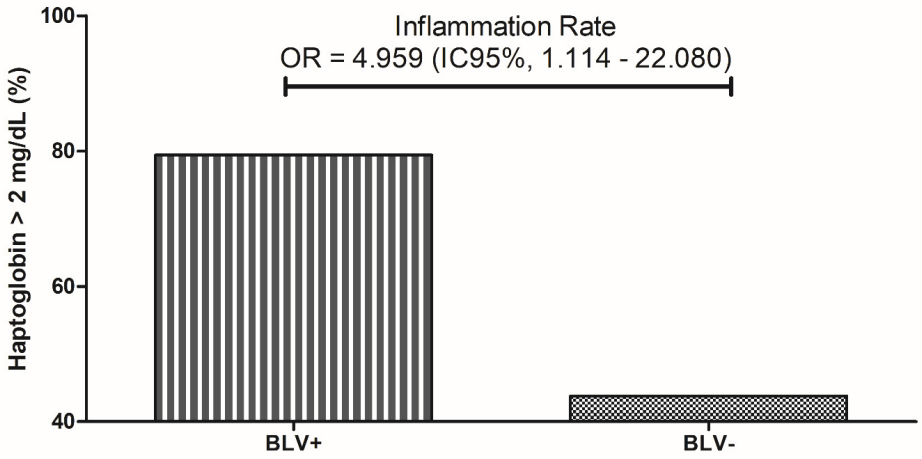

Figure 1 - Frequency of inflamed dairy cattle in the bovine leukemia virus (BLV) infected (BLV+) or non-infected (BLV–) groups during the transition period.

(38.7%), which allows for the composition of the BLV– group in our study. The prevalence found in our study is greater than those reported in other Brazilian studies in different regions, ranging from 9.2–56.3% (BAPTISTA FILHO et al., 2019; PUENTES et al., 2016; FLORES et al., 1988b). These differences can be attributed to the gap in recent BLV prevalence research and the diagnostic methods used to detect infection, since most previous studies used agar gel immune diffusion, which is less sensitive than ELISA (BAPTISTA FILHO et al., 2019).

In this study, serology for BLV was defined for animals over 90 d of age, due to the high titers of maternal antibodies in the first months of life. In contrast, there is evidence reporting the persistence of maternal antibodies after 3 months (DITTRICH,

Table 2 - Classification of heifers in low (n=6) and high proviral load (n=6) of bovine leukemia virus groups in dairy heifers through the transition period.

			----------Week----------		
	Animal ID[1]	Breed	-3	+3	Average
			----------Proviral load (copies/µL)[2]----------		
Low proviral load	475	Jersey	0.14	1.20	0.67
	917	Jersey	0.30	0.21	0.25
	946	Jersey	1.30	0.26	0.78
	511	Holstein	0.07	0.30	0.18
	641	Holstein	0.18	0.27	0.22
	694	Holstein	2.50	3.90	3.20
High proviral load	716	Jersey	2.50	4.20	3.35
	844	Holstein	3.60	3.80	3.70
	688	Jersey	7.70	7.90	7.80
	714	Holstein	10.60	17.10	13.85
	517	Holstein	13.30	19.00	16.15
	858	Jersey	20.70	19.40	20.05
	-	-	-	-	3.28

[1] ID, Identification.
[2] Results are expressed in copies/µL of genomic bovine DNA.

Table 3 - Effect of low proviral load in the bovine leukemia virus (BLV)+ group on hematological and biochemical healthy requirements in dairy heifers during the transition period.

Variables	BLV+ (n=6)	BLV-(n=6)	Time	Group	Time × Group
Red blood cell/µL	6.25±1.76	6.06±0.71	0.00	0.85	0.69
Hemoglobin g/dL	10.01±1.61	9.54±1.52	0.00	0.52	0.45
Hematocrit (%)	31.08±4.05	29.71±4.02	0.00	0.48	0.94
MCV fL	51.21±6.81	49.04±3.52	0.71	0.13	0.40
MCHC.g/dL	32.14±2.21	32.04±1.97	0.84	0.85	0.27
RDW (%)	16.76±0.83	16.16±0.79	0.00	0.10	0.72
Metarrubricytes (%)	0.02±0.16	0.14±0.47	0.48	0.20	0.19
Leucocytes x10^3/µL	12.80±5.37	13.00±2.81	0.72	0.58	0.67
Bands/µL	3.34±21.40	-	-	-	-
Neutrophilx10^3/µL	4.57±2.25	4747.40±2132.28	0.09	0.69	0.77
Lymphocytes x10^3/µL	7.48±5.13	7691.26±2071.78	0.69	0.50	0.77
Monocytes/µL	209.80±324.10	195.17±210.28	0.95	0.79	0.58
Eosinophil/µL	549.51±653.58	387.60±393.10	0.09	0.25	0.44
Basophil /µL	-	-	-	-	-
Platelet x 10^3/µL	445±189	282±108	-	-	-
AST U/L	79.44±41.24	77.90±73.40	0.18	0.04	0.45
GGT U/L	23.74±9.20	22.32±7.32	0.02	0.66	0.10
BHB (mmol/L)	0.47±0.21	0.43±0.20	0.00	0.12	0.61
Triglycerides (mg/dL)	16.90±12.41	18.36±13.20	0.00	0.92	0.12
Cholesterol	74.75±18.43	70.66±19.51	0.03	0.58	0.47
TPP g/dL	6.54±0.67	6.45±0.78	0.00	0.79	0.70
Haptoglobin (g/dL)	11.24±21.94	2.62±2.57	0.02	0.28	0.96
Fibrinogen mg/dL	390.24±207.13	371.43±156.62	0.20	0.92	0.09

MCV, Mean corpuscular volume; MCHC, Mean corpuscular hemoglobin concentration; RDW, Red cell distribution width; AST, Aspartate aminotransferase; GGT, Gamma-glutamyl transferase; BHB, β-Hydroxybutyrylation; TPP, Total plasma protein. Differences between groups were considered when the P-value was < 0.05.

2004). The World Organization for Animal Health recommends that the serological screening of BLV in the younger population of cattle should be realized between 6 to 7 months of age. Thus, the serum prevalence showed in this study can be overestimated, due to the inclusion of the youngest heifers.

During the transition period, BLV infection did not affect RBC and WBC count in dairy heifers. ALI et al. (2019) also found no significant changes in erythrogram, leukogram, and platelet counts in seropositive cattle compared to seronegative cattle. WISNIESKI et al. (2020) reported higher lymphocyte counts in BLV+ than BLV– animals, while working with multiparous cattle from second, third, or greater parity numbers. Thus, lymphocyte counts depend on the age of animals and category, in addition, to the disease severity (BARTLETT et al., 2020). The lacking effect of BLV on hematological parameters indicate that the young heifers in our experiment could be in the early stage of BLV infection (MERLINI et al., 2016).

Regarding biochemical parameters, the effect of BLV infection was found only on the activity of the hepatic enzyme AST, with higher values in BLV+ than in BLV– dairy heifers.AST is particularly useful for diagnosing and evaluating liver and muscle disorders. Individual analysis of AST values in heifers did not reveal any animals presenting high values for AST enzyme activity, according to KANEKO et al. (2008). The difference between the groups in our study may be related to the higher metabolic demand in the BLV+ group than the BLV– group, due to the direct or indirect effect of BLV and other concomitant diseases. Similar results were reported by JULIARENA et al. (2016).

In relation to lipid metabolism, an interaction between time and group for triglycerides was detected, and the differences were observed on weeks 0 and +1. BLV+ heifers had higher values than BLV– young heifers, which indicates a possible higher rate of lipolysis in the infected group. A relationship between liver enzymes and lipid metabolism was stronger in BLV+ heifers.

Table 4 - Effect of high proviral load in the bovine leukemia virus (BLV)+ group on hematological and biochemical healthy requirements in dairy heifers during the transition period.

Variables	BLV+ (n=6)	BLV– (n=6)	Time	Group	Time × Group
Red blood cell/µL	5.71±0.80	5.85±0.67	0.54	0.67	0.94
Hemoglobin g/dL	9.47±1.18	9.52±1.28	0.21	0.80	0.46
Hematocrit (%)	28.54±3.74	28.67±5.79	0.47	0.91	0.53
MCV fL	50.19±4.43	50.38±4.14	0.00	0.81	0.76
MCHC g/dL	33.30±2.29	32.42±2.01	0.13	0.13	0.21
RDW (%)	19.31±15.55	16.89±0.92	0.00	0.68	0.32
Metarrubricytes (%)	0.02±0.16	0.10±0.37	0.82	0.23	0.49
Leucocytesx10^3/µL	22.90±9.55	13.30±4.80	0.17	0.01	0.40
Bands/µL	-	2.79±18.05	-	-	-
Neutrophilx10^3/µL	6.15±3.86	4.75±2.70	0.34	0.42	0.49
Lymphocytesx10^3/µL	15.63±8.44	7.93±3.06	0.84	0.03	0.18
Monocytes/µL	197.93±341.71	155.90±199.55	0.01	0.62	0.94
Eosinophil/µL	980.66±1255.58	452.69±474.61	0.00	0.22	0.41
Basophil/µL	-	-	-	-	-
Platelets/µL	292±118	323±118	-	-	-
AST U/L	76.02±17.66	67.83±20.04	0.13	0.28	0.13
GGT U/L	23.07±9.89	19.68±5.72	0.51	0.40	0.13
BHB (mmol/L)	0.43±0.18	0.46±0.17	0.00	0.45	0.81
Triglycerides (mg/dL)	20.35±12.69	19.29±12.6	0.00	0.47	0.46
Cholesterol	83.03±18.47	72.39±15.99	0.00	0.15	0.96
TPP g/dL	6.82±0.54	6.68±0.74	0.00	0.49	0.67
Haptoglobin (g/dL)	6.65±7.68	4.08±4.18	0.01	0.21	0.86
Fibrinogen mg/dL	319.51±197.76	371.43±192.91	0.91	0.39	0.37

[1]MCV, Meancorpuscular volume; MCHC, Mean corpuscular hemoglobin concentration; RDW, Red cell distribution width; AST, Aspartate aminotransferase; GGT, Gamma-glutamyl transferase; BHB, β-Hydroxybutyrylation; TPP, Total plasma protein. Differences between groups were considered when the P-value was < 0.05.

Total protein values were similar between non-infected and infected dairy heifers. BIRGUEL JR et al. (2001) also did not find different protein dynamics among serum negative and serum positive heifers manifesting with or without lymphocytosis. However, fibrinogen concentrations were higher at weeks 0 and +1 in BLV+ heifers than in BLV– young heifers, in addition, the infected group also presented with four times more chances to present with inflammation based on the individual analysis of haptoglobin. Acute phase proteins like fibrinogen and haptoglobin are unspecific and could be increased due to the direct or indirect effect of BLV infection (ECKERSALL & BELL, 2010). BLV+ heifers from our study were classified as asymptomatic BLV+, but current studies have been reporting high incidence of infectious diseases in serum positive animals due to immunosuppression caused by BLV infection (NORBY et al., 2016; FERNANDES et al., 2009; JULIARENA et al., 2007).

The ddPCR is a modern, highly sensitive, specific, quantitative tool that can be used to investigate BLV genetic material (DE BRUN et al., 2022; HUTCHINSON et al., 2020). This tool allowed us to trace the median proviral load to equally distribute serum positive animals for BLV into HPL and LPL animals. In our study, the proviral load was analyzed using ddPCR at the beginning (week –3) and end (week +3) of the experiment to ensure the maintenance of infectious status in serum positive dairy heifers during the transition period. Ideally, weekly analysis of proviral load should be performed, however, this technique is very expensive, thus limiting the design of our study.

Both JULIARENA et al. (2007) and FARIAS et al. (2018) used the same cut-off value for proviral load to classify serum positive cattle manifesting persistent lymphocytosis. They used a cut-off of 100 copies/µg of DNA to classify serum positive cattle in the LPL subgroup, whereas the

HPL animals had a cut-off of >1,000copies/µg of DNA. If we consider these cut-offs, all heifers included in our experiment presented with LPL, probably due to the animal's young age and possible recent infection.

The most BLV+ heifers from our study had persistent lymphocytosis and LPL during the transition period based on the cut-off values of SCHALM (2010) and JULIARENA et al. (2007). Thus, HPL is not restricted to animals with LPL (JULIARENA et al., 2016).

BLV+ heifers classified with HPL in our study had a higher number of leukocytes and lymphocytes than their count pairs. In contrast, animals classified with LPL had a similar hematologic profile compared with their count pair. Thus, for future studies, BLV serology for heifers should be carefully analyzed, and proviral load could be used as complementary information for the understanding of BLV physiopathology. Some authors reported the importance of the proviral load in the epidemiology of infection transmission, and the potentialuse of this tool in the BLV control program. Animals with HPL have an increased risk of transmission and should be the first to be eliminated from the herd (RUGGIERO & BARTLETT, 2019).

Our study was designed to have BLV serum negative count pairs as a control group, possibly working with heifers. This may have affected the main results of our study such as the LPL of the subjects. Leukocyte and lymphocyte counts depend on the proviral load. In addition, serum positive heifers had a pro-inflammatory profile during the transition period, probably associated with infectious disease in the post-partum period.

CONCLUSION

BLV infected heifers have a proinflammatory profile. Traditional leukocytosis and lymphocytosis were observed only in the HPL subgroups, indicating that compromising the immune response by BLV depends on virus replication.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Conceptualization, Viviani Gomes and Fabrício Torres; Data curation, Viviani Gomes, Jean Silva Ramos and Fabrício Torres; Formal analysis, Jean Silva Ramos; Investigation, Fabrício Torres, Camila Costa Baccili, María L. de Brun Méndez, and Rodrigo Puentes; Methodology, Fabrício Torres, Camila Costa Baccili, María L. de Brun Méndez, Rodrigo Puentesand Viviani Gomes; Project administration, Viviani Gomes and Fabrício Torres; Supervision, Viviani Gomes; Writing – original draft, Viviani Gomes and Larissa Miranda Padilha; Writing – review &editing, Viviani Gomes, María L. de Brun Méndez, and Rodrigo Puentesand Larissa Miranda Padilha.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

This study was conducted and approved by the Ethics Committee for Animal Use of the School of Veterinary Medicine and Animal Science (Universidade de São Paulo) (Protocol number 2188221018).

DATA AVAILABILITY STATEMENT

The authors deposited the data in the Zenodo repository: doi: https://zenodo.org/records/10146114.

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