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HORIZONTAL TRANSMISSION DYNAMICS OF BOVINE LEUKEMIA VIRUS (BLV) AND NEGATIVE EFFECT ON REPRODUCTIVE PERFORMANCE IN NATURALLY INFECTED HOLSTEIN HEIFERS

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ABSTRACT

BLV causes one of the most relevant viral diseases of dairy cattle although the most of the infected animals are asymptomatic. There are controversies regarding reproductive losses on infected herds. Our purpose was to evaluate the horizontal transmission of the virus in a group of Holstein heifers confined in a rearing system and to determine the impact of infection on reproductive performance. Three hundred eighty-nine animals were sampled and seroconversion was determined by ELISA. The occurrence of estrous behavior, number of services/pregnancy, conception rate, percentage of pregnant animals by artificial insemination and by bull service during two breeding periods (June-July and November-December 2012) were recorded. Herd BLV initial seroprevalence was 45% and annual conception rate was 39.8% (CI 30.5%-49%). During the 18 month time course two seroconversion peaks were detected. The first one happened during the admission period, most likely due to quarantine and intensive health management of the animals. The second peak occurred during the first gestational period. At the end of the study, 73.6% of the heifers were BLV seropositive and there was reduction of 27% in the herd conception rate in the second breeding period (p=0.005). In conclusion, BLV infection and reproductive performance were negatively associated and high seroconversion was observed during the gestational period.

Keywords: Rearing system. Reproductive failure. Infectious disease.

INTRODUCTION

Enzootic bovine leukosis is an infectious disease caused by Bovine Leukemia Virus (BLV). It is worldwide distributed having a great importance, especially in dairy systems (LUCHTER, 2004). Nowadays, 21 countries and Western Australia have successfully eradicated BLV from their herds (BARTLETT et al., 2014). In South America, this disease is widely spread in bovines. In Uruguay, the prevalence can reach 77% in dairy cattle (FURTADO et al., 2013). In order to develop effective BLV control strategies, quantitative information about virus transmission on the field is needed, as well as details about duration of infectious period, probability of transmission by exposure, and veterinary practices that are associated with higher transmission, among other indicators. Although there are some data in the literature related to these aspects, information is somewhat contradictory and insufficient to design an efficient control system (BARTLETT et al., 2014; MONTI et al., 2007).

BLV can be transmitted both vertically and horizontally by infected lymphocytes. Horizontal transmission occurs mainly iatrogenically, when animals are manipulated without proper hygienic care during veterinary procedures such as dehorning, tattooing, vaccination, castration and rectal tact. Transmission by hematophage insects has also been reported, especially by insects from *Tabanidae* family. Although individually these sources of infection could not be significant, when analyzed together they become relevant to transmission of BLV (BARTLETT et al., 2014).

After the infection, approximately 40% of the animals develop persistent lymphocytosis in the following years and 5% malignant lymphoma, causing direct heard losses. Some investigations show that subclinical infection with BLV directly impact milk production and longevity of the cow (BARTLETT et al., 2014). VanLeeuwen et al. (2010) reported that BLV seropositive cows had a conception rate 7% lower when compared with seronegative cows. However, other authors did not observe any association between BLV seropositive animals and dairy yield, milk composition, or reproductive performance (KALE et al., 2007; TIWARI et al., 2007). The purpose of this study was to describe seroconversion of BLV associated with management practices and to determine the impact of infection on reproductive performance of Holstein heifers.

MATERIAL AND METHODS

Herd and health management

The experiment was carried out in a rearing farm located in Southern Uruguay that rears Holstein calves from different dairy farmers of the area. Six to ten months old animals were admitted and remain in the field for about 18 months until returning to their original farm with approximately 7 months of pregnancy. The establishment had no sanitary requirements regarding the entry of animals with positive serology for BLV.

At admission, calves were identified with a tag containing the farmer data and the number assigned to the animal. Then, they were weighted, dewormed and subjected to a prescribed health plan based primarily on antiparasitic drugs and vaccination against *Bacillus anthracis*, *Clostridium* spp., keratoconjunctivitis (*Moraxella bovis*) and brucellosis. Reproductive management consisted of estrous synchronization with two doses of a prostaglandin analogue, artificial insemination (AI) (up to three per animal) and service with bulls after each period of AI, followed by estrus recording twice a day and pregnancy diagnosis. This process took place twice, from June to July and from November to December of 2012. In order to avoid iatrogenic transmission of viruses, multidose syringes, needles, dehorning equipment, surgical equipment, gloves or any material that made direct contact with blood were soaked in disinfectant between each animal (Despadac–Calier S. A.-Spain).

Sampling

Three hundred eighty-nine animals from 29 farms were considered for the study. After arriving at the rearing farm, blood samples were taken and processed by enzyme linked immunosorbent assay (ELISA) technique. Only BLV seronegative animals were included in the study. After each round of health or reproductive management, blood samples were collected and BLV infection was determined by ELISA. These animals were monitored by 18 months, being serology of every animal tested five times: on September of 2011 (first sampling), on December of 2011 (second sampling), on March of 2012 (third sampling), on July of 2012 (fourth sampling), and on December of 2012 (fifth sampling).

Anti-gp51 antibody detection by ELISA

A commercial kit for detection of antibody to BLV gp51 in bovine sera with 98% sensitivity and 100% specificity (VMRD, cod. 5505.20, WA, USA, approved by United States Department of Agriculture-USDA) was used to determine animal serum status. Samples were processed according to manufacturer's instructions and the reading was performed at 620 nm in a visible range spectrophotometer (Thermo Fisher Scientific Inc., USA). Three weak positive controls were used per plate and the cut-off for each plate was calculated from the optical density (OD) mean of the positive controls. According to their OD, positive samples were classified into weak, moderate and strong positive (GUTIÉRREZ et al., 2012).

Data collection and statistical analysis

For each animal, age by dentition, serological status, and measures of health and reproductive parameters were collected during the 18 months. The prevalence was estimated with a confidence interval of 95% (CI 95%). To analyze the different variables, Chi2 test were performed and an alpha <0.05 was considered significant. Statistical analysis was performed using STATA v 11.2 software (StataCorp, 2009) and a Kaplan-Maier curve for overall survival time and event from this study was made using Graphpad 6.01. The variables analyze were:

Estrous behavior – Detected by visual observation of estrus signs during one hour at the morning and one hour at the evening (stands while being mounted, mucus discharge and restlessness).

Number of services/conception - total number of services offered to a group of animals in a defined period divided the number of animals that resulted pregnant.

Conception rate - percentage of pregnant animals divided the total of animals served in a defined period of time.

Pregnancy rate - number of pregnant animals by bull or AI divided the total number of animals served.

RESULTS AND DISCUSSION

The serological BLV prevalence of heifers entering the establishment was 45% (CI 95%: 40.33%-49.65%). Correcting to true prevalence, 45.9% of heifers were seropositive (CI 95%: 41.23%-50.57%). Annual seroconversion rate was 39.8% (CI 95%: 30.5%-49%) and the seroconversion rate at the end of the study (18 month) was 52.7% (CI 95%: 43.5%-61.9%). Serologic prevalence of animals departing the farm was 73.6% (Table 1). No association was found between the age (milk teeth, two teeth, ≥ four teeth) and the presence of BLV antibody.

Table 1 - Dynamics of seroconversion against BLV during the study.

	2011		2012			2013
	SET	DEC	MAR	JUL	DEC	MAR
Seronegative	214	87	79	73	68	52
Seropositive	175	29	6	5	5	13
Total	389	116	85	78	73	65
Apparent prevalence (%)	45	70,5	72,9	75	77,1	81,9
Dropout among seropositives during testing interval	-	-	-	-	-	-
Dropout among seronegatives during testing interval	-	98	3	1	0	3
Seroconversion rate (%)	-	25	7,1	6,4	6,8	20,0

The seroconversion incidence was analyzed and compared with health and reproductive management in five periods. The first and last sampling period presented higher seroconversion incidence (respectively 25% and 20%), while the second, third and fourth samplings showed low and similar seroconversions (7%, 6.4% and 6.8%, respectively). Before the first sampling (seroconversion incidence 25%), the animals undertook several clinical procedures in quarantine. Heifers were tagged, dehorned, dewormed and tested for tuberculosis. Animals were also vaccinated against the main endemic diseases and injected with vitamin supplements. In the second sampling (seroconversion incidence of 7%), animals were submitted to a vaccine booster against *Clostridium* spp. and to blood collection for brucellosis. In the third (seroconversion incidence of 6.4%) and fourth (seroconversion incidence of 6.8%) samplings, procedures consisted of estrous synchronization, artificial insemination, pregnancy diagnosis by rectal examination and ultrasound, and vaccination against leptospirosis. In the fifth and final sampling (seroconversion incidence of 20%), no procedures were applied, as animals were close to departure; at this point animals were 7 to 8 months pregnant. To visualize the dynamics of seroconversion during the assay, survival analysis is shown in the Figure 1. The seroconversion probability at the end of the study was 50.94%.

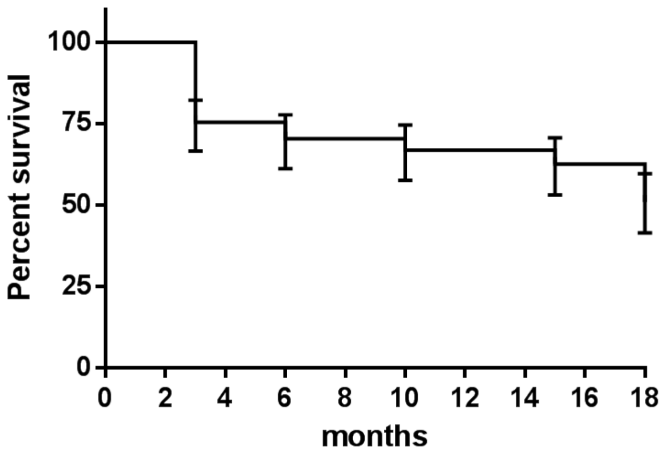

Figure 1 - Seroconversion analysis of heifers throughout the study period. Confidence intervals 95% are shown.

Different parameters of reproductive efficiency were evaluated in two periods (June-July and November-December). Analyzing the data from both periods (n= 251 heifers), the presence of antibodies against BLV did not influence the heat behavior or the number of services required per animal. Additionally, there was no relationship between seropositivity degree (weak, moderate or strong) and the number of services required or occurrence of pregnancy. Analysis of the 251 heifers inseminated in both breeding periods also did not show association between pregnancy and BLV serum status.

When it was compared total conception rate of each period, we did not find differences between June-July (80.5%, 145/180) and November-December (76.1%, 54/71) rates. When animals were grouped by serological status, conception rate of seronegative animals (77.50%) was not statistically different from seropositive animals (81.43%) in the first period of artificial insemination (June-July); however, in the second reproductive period (November-December), conception rate was significantly different between seronegative (93.33%) and seropositive (66.67%) animals (p=0.006) (Table 2).

Table 2 - BLV serology in each reproductive period by pregnancy diagnostic.

	June-July period		November-December period	
BLV serology	Empty heifers**	Pregnant heifers	Empty heifers**	Pregnant heifers
Negative	9/40 (22.50%)	31/40 (77.50%)	2/30 (6.67%)	28/30 (93.33%)*
Positive	26/140 (18.57%)	114/140 (81.43%)	26/78 (33.33%)	52/78 (66.67%)*

* p=0.006; ** Empty = non pregnant.

Later, when the animals were categorized as weak, moderate and strong seropositive, no association was found between conception rate and the level of antibodies in the animals (Table 3).

Table 3 - Grade of BLV seropositivity in each reproductive period by pregnancy diagnostic.

	June-July period		November-December period	
BLV serology	Empty heifer**	Pregnant heifer	Empty heifer**	pregnant heifer
Weak positive	1/16 (6.25%)	15/16 (93.75%)	2/6 (33.33%)	4/6 (66.67%)
Moderate positive	5/27 (18.52%)	22/27 (81.48%)	8/18 (44.44%)	10/18 (55.56%)
Strong positive	20/97 (20.62%)	77/97 (79.38%)	16/54 (29.63%)	38/54 (70.37%)

p>0.05; ** Empty = non pregnant.

Analysis of the bulls used for natural mating revealed that 57% were seropositive for BLV. However, no significant differences were found between the overall percentage of pregnant animals by bulls or AI. Moreover, the number of services needed in both periods together did not vary significantly between seropositive and seronegative animals. In the June-July breeding period, seronegative and seropositive heifers required 1.65 and 1.64 services respectively, to achieve a pregnancy, while in the November-December the amount of services needed to achieve a pregnancy was 1.46 and 2.35 for seronegative and seropositive animals respectively. Even though an increase was detected in seropositive animals, the difference was not significant.

We found a high percentage of BLV seropositive animals (45%) at the beginning of the experiment, which was unexpected, considering the age of these animals (6 to 10 months old). In Argentina, Gutiérrez et al. (2011) found a seroprevalence of 11% and 17% in animals of 9 and 18 months of age, respectively, in similar production systems to those of Uruguay. Since vertical transmission of the virus is estimated up to 15% (HOPKINS et al., 1997), and iatrogenic transmission has been suggested as the main route of transmission (by veterinary instruments with infected blood), such high prevalence found in young animals should be deeply investigated. Heifers at this age have not yet been subject to many veterinary management practices that favor viral transmission, suggesting that other ways of horizontal transmission might be playing a bigger role here. Furthermore, Gutiérrez et al. (2011) failed to reduce transmission by applying sanitary management measures aimed at preventing the spread among animals, suggesting that other pathways of viral transmission might be participating in natural conditions. Another explanation for this high percentage of infected young animals could be that vertical transmission has been underestimated.

Iatrogenic practices are considered one of the main routes of transmission of BLV (HOPKINS et al., 1997). This study was performed in the biggest rearing farm in Uruguay, where veterinarians and farmers share a genuine interest in following safe practices, but, still, the annual seroconversion found in the examined group of heifers was 39.8%. The sanitary measures employed at the rearing farm consist of disinfecting the surgical instruments by dipping them in commercial antiseptic solution (Despadac–Calier S. A.-Spain) after each use.

In this way, they try to inactivate the virus and prevent spread from animal to animal. While some commercial disinfectants are recommended to inactivate viral particles, it is questionable their effectiveness at inactivating virions present inside cells, as it is the case of BLV, whose provirus is integrated into the cellular genome. Furthermore, the accumulation of organic matter in the disinfectant most likely decreases the efficiency of the product. Gutiérrez et al. (2011), in similar conditions, found a transmission rate of 24% during 27 months, not observing significant changes in prevalence after 3 years in a closed herd in Argentina. They suggest that the BLV cycle of transmission cannot be efficiently broken with an intervention based only on preventing the iatrogenic blood contact, and that other way (s) of transmission play a key role under natural conditions. Their findings corroborate our results, suggesting that the measures taken to avoid iatrogenic transmission are not being totally effective, at least in these conditions. On the other hand, a study in Virginia (United States) achieved indeed a 27% reduction in prevalence in two years by introducing some sanitary measures: changing needles and gloves for every animal, disinfecting tattoo equipment, using an electric dehorner, milk replacer, and heating the colostrum prior to administration in dairy herds (SPRECHER et al., 1991). Despite this great reduction, it is important to highlight that this result may be an overestimate, since seroprevalence was diagnosed by agar gel immunodiffusion, which it is less sensitive technique of all available and recommended by the OIE (OIE, 2012; TRONO et al., 2001). Replacing needles instead of disinfecting them could be a more effective option to prevent transmission, however, it is not practical to implement in production systems such as those of Argentina and Uruguay, where there are herds with large numbers of animals. In fact, more research should be made demonstrating the efficacy of disinfection of needles and instruments by immersion and, if necessary, to develop a practical but effective guideline to inactivate BLV.

Iatrogenic transmission is not the only route of BLV infection. Arthropods are known to be very effective transmission vectors (RODRIGUEZ et al., 2011). Several studies have shown that horn flies (*Haematobia irritans*) and horse flies (*Tabanidae* family) are capable of carrying the virus from one animal and transmit it to others. Ooshiro et al. (2013) recently demonstrated that vector control in properties is an effective strategy to control the infection with BLV. In our study, animals were treated regularly with drugs to control both flies, so we believe that this transmission did not play an important role in the results. Still, as this was a field study, BLV transmission by vectors cannot be completely ruled out as a potential source of transmission.

Finally, some authors have suggested that direct contact between animals can play a critical role in viral transmission. Sargeant et al. (1997) demonstrated a significant decrease in prevalence after three years of complete physical separation between negative and positive animals. Gutiérrez et al. (2011) suggested an alternative design strategy based on the selective separation of animals according to their proviral load in peripheral blood. If direct contact between animals is a crucial way of transmission, surpassing iatrogenic, then the strategy used by some countries, where BLV control is based on identification and differential management of seropositive and seronegative animals (segregation of animals), is probably a more effective control measure.

Seroconversion against BLV throughout the study period could be linked to specific veterinary practices or physiological stage s of the animals. During the 18 months, two peaks were detected in seroconversion. The first occurred at the beginning of the experiment with a seroconversion incidence of 25% of seropositive animals and coincided with the period of greatest health management of the heifers, which could have transferred infected blood between animals. Horizontal transmission of the virus by vaccinations, dehorning, rectal examination and surgeries has been described as one of the main routes of transmission of bovine leukosis (HOPKINS et al., 1997; MAMMERICKX et al., 1987). It should be noted that, in the case of BLV, there is a window period of 2 to 8 weeks after infection, during which antibodies are not detected (TOMA et al., 1990). Therefore, some animals could have entered the rearing system already infected, but presented a false negative result in the first ELISA screening, but were positive in the first sampling, increasing the number of seroconversions during this period. The other peak of seroconversion (20%) was observed in the fifth sampling, when the majority of the herd was pregnant at this point. During the gestational period, a state of natural immune modulation is established. A gradual decrease in some immunologic functions marks this period, reaching its minimum expression immediately before parturition (LACETERA et al., 2005; WAGTER et al., 1996). It has been found for example that prostaglandin E2, a hormone produced during pregnancy in cattle, suppresses T cell proliferation and inhibits the production of IL-12 by macrophages and of Th1 cytokines (e.g. IL-2 and IFN gamma) (KABEYA et al., 2001). It has also been shown that treatment with corticosteroids and prolactin in combination with insulin stimulates the expression of BLV in cell lines (NIERMANN and BUEHRING, 1997). Although these are *in vitro* experiments, this could be an indicator of how is the infection in the context of pregnancy. At this point, reproductive management of heifers (concentration of animals for heat detection twice a day, artificial insemination, pregnancy diagnosis and service with bulls) had been made. Thus, these procedures could too be related to the increased transmission of the virus during this period. Bull servicing also cannot be dismissed as possible viral transmission route, since 57% of the bulls used were positive to BLV. Because BLV can potentially be excreted in the semen (DUS SANTOS et al., 2007), Erskine et al. (2012) proposed that the use of artificial insemination in heifers could reduce the prevalence of BLV, compared with using natural service. During the others sampling periods, only small rates of seroconversion were observed, coinciding with periods of lesser veterinary handling of the heifers.

Significant differences in conception rate between seropositive and seronegative animals were found in the second breeding period (November-December), during which conception rate was 27% lower in seropositive animals. VanLeeuwen et al. (2010) found that conception rate of seropositive animals was 7% lower than in seronegative animals, but that difference was not significant. Kale et al. (2007) found no statistical differences not only in reproductive performance but also in the number of inseminations necessary for pregnancy in seronegative and seropositive animals. Our results, showed a higher number of services required to achieve pregnancy during the period of November-December (1.46 and 2.35 in seronegative and seropositive animals respectively), and also it was also not significant. Nonetheless, even if the positive heifers didn’t need more services to become pregnant, they did not presented full term pregnancies, exhibiting a higher rate of abortions ($p=0.006$). Gutiérrez et al. (2012) suggested that the level of p24 antibody titers reflects the viral load in infected animals. Even though we used an ELISA that detects antibodies against gp51, we tried to establish a relationship between production losses and antibody levels, comparing antibody titers (weak, moderate or strong) and reproductive rates of seropositive animals. However, differences in reproductive rates by level of seropositivity were not significant.

It is still unclear why a significant difference in conception rate between seropositive and seronegative animals was found in the breeding period of November-December, but not in the June-July period. Nutritional and management factor could explain the differences in the conception rates. To verify this, diet and clinical practices employed during each period were compared and cross-referenced against conception rates, but there were no differences in treatments applied. Another explanation could be that seasonal factors influenced viral load in seropositive animals, which, in turn, influenced the conception rate. In this study, the differences in conception rate between BLV seropositive and seronegative animals were precisely during the spring and summer (November - December). During the warmer seasons, the presence of hematophagous insects is notoriously higher. Reinfection of positives animals could occur, increasing viral loads (booster effect). This hypothesis has not been demonstrated and it should be tested in future field experiments.

CONCLUSION

In conclusion, our results suggest that BLV may impact cattle reproductive performance, specifically decreasing conception rate of Holstein heifers. Moreover, we found a high prevalence of young seropositive animals entering the rearing system, most of which had not been previously exposed to veterinary management practices. Thus, even though iatrogenic transmission may play a part in BLV transmission, it is clearly not the only component involved. Since we detected a great number of heifers that seroconverted during the gestational period, a feasible BLV control measure would be separate seropositive and seronegative animals at least during the gestational period, preventing seroconversions and vertical transmission at once.

DINÂMICA DA TRANSMISSÃO HORIZONTAL DO VÍRUS DA LEUCOSE BOVINA (BLV) E EFEITO NEGATIVO SOBRE O DESEMPENHO REPRODUTIVO EM NOVILHAS DA RAÇA HOLANDESA NATURALMENTE INFECTADAS

RESUMO

O BLV causa uma das doenças virais mais relevantes do gado de leite, embora a maioria dos animais infectados seja assintomática. Há controvérsias a respeito da ocorrência de perdas reprodutivas em rebanhos infectados. O objetivo deste trabalho foi avaliar a transmissão horizontal do vírus em novilhas Holandesa confinadas num sistema de recria e determinar o impacto da infecção sobre o desempenho reprodutivo. Trezentos e oitenta e nove animais foram amostrados e a soroconversão foi determinada por ELISA. A ocorrência do comportamento estral, número de serviços/gestação, taxa de concepção, percentagem de animais prenhes por inseminação artificial e por serviço de touro durante dois períodos reprodutivos (Junho-Julho e Novembro-Dezembro/2012) foram registrados. A soroprevalência inicial era 45% e a taxa de concepção anual 39,8% (IC 30,5%-49%). Durante os 18 meses de ensaio, dois picos de soroconversão foram detectados. O primeiro aconteceu durante o período de entrada dos animais ao campo, provavelmente devido à quarentena e manejo sanitário intensivo dos animais. O segundo pico ocorreu durante o primeiro período gestacional. No final do estudo, 73,6% das novilhas foram soropositivas e houve uma redução de 27% na taxa de concepção no segundo período reprodutivo (p=0,005). Em conclusão, a infecção pelo BLV e o desempenho reprodutivo foram negativamente associados e foi observada uma alta soroconversão durante o período gestacional.

Palavras-chave: Sistema de recria. Falha reprodutiva. Doença infecciosa.

DINAMICA DE LA TRANSMISIÓN HORIZONTAL DEL VIRUS DE LA LEUCOSIS BOVINA (BLV) Y EFECTO NEGATIVO SOBRE EL DESEMPEÑO REPRODUCTIVO EN VAQUILLONAS HOLANDO NATURALMENTE INFECTADAS

RESUMEN

BLV causa una de las enfermedades virales más importantes en el ganado lechero pero la mayoria de los animales infectados son asintomáticos. Existe controversia sobre pérdidas reproductivas causadas por esta enfermedad en los rebaños infectados. El objetivo de este ensayo fue evaluar la transmisión horizontal del virus en vaquillonas Holstein confinadas en un sistema de cría y determinar el impacto de la infección sobre el rendimiento reproductivo. Trescientos ochenta y nueve animales fueron muestreados y la seroconversión se determinó mediante ELISA. La manifestación de celo, número de servicio/gestación, tasa de concepción, porcentaje de hembras preñadas por inseminación artificial y servicio de toro durante dos temporadas de cría (junio-julio y noviembre-diciembre/2012), fueron registrados. La seroprevalencia inicial contra BLV fue del 45% y la tasa de concepción anual fue del 39,8% (IC del 30,5%-49%). Durante los 18 meses del ensayo, se detectaron dos picos de seroconversión. El primero ocurrió durante el período de ingreso de los animales al campo, muy probablemente debido a la cuarentena y manejo intensivo de los animales. Y el segundo pico se produjo durante la gestación. Al final del ensayo, 73,6% de las vaquillonas eran seropositivos y hubo una reducción del 27% en la tasa de concepción en el segundo periodo reproductivo en los animales seropositivos a BLV (p=0,005). En conclusión, la infección con BLV y el rendimiento reproductivo se asociaron negativamente y se observó una alta seroconversión durante el período gestacional.

Palabras clave: Sistema de recria. Fallas reproductivas. Enfermedad infecciosa.

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