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Association of BoLA-DRB3 Alleles with the Progression of Bovine Leukosis in the Lucerna Breed

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ABSTRACT

The bovine leukosis virus causes enzootic bovine leukosis (BLV) of the *Retroviridae* family and is the most significant neoplastic disease in cattle, leading to substantial economic losses globally. This study aimed to associate the progression of bovine leukosis with *BoLA-DRB3* alleles in the Lucerna breed. A total of 104 animals were tested for the presence of BLV by nested PCR, the development of persistent lymphocytosis (PL) by peripheral blood smear, antibody titers (AT) to BLV by ELISA, and proviral load (PVL) by qPCR. Animals were genotyped for the *BoLA-DRB3* gene by PCR-SBT. Allele frequencies of the *DRB3* gene were estimated and associated using Fisher's exact test and odds ratio. About 93.2% of the animals were virus positive, and 18.3% developed PL. Fifty percent of the animals had elevated AT with a value of 88.7 $\log_2$ and 43.3% had elevated PVL with 326871 copies/10^5 cells. In total, 17 *BoLA-DRB3* alleles were found; the *BoLA-DRB3*011:01* allele (12.1%) was the most frequent. Two alleles showed an association with susceptibility to viral infection (*BoLA-DRB3*15:01* and **23:01*), and only the *BoLA-DRB3*38:01* allele was considered resistant. The *BoLA-DRB3*11:01* and **15:01* alleles were not associated with LP, and neither allele was associated with high LP. Low AT was found in the *BoLA-DRB3*13:01* and **20:01:02* alleles. The *BoLA-DRB3*15:01* and **16:01* alleles were associated with high AT. Low PVL was associated with the *BoLA-DRB3*11:01* and **23:01* alleles. *BoLA-DRB3*15:01* allele was associated with high PVL. In conclusion, the Lucerne breed has a slow progression of enzootic bovine leukosis.

Keywords: autochthonous breeds; genetic resistance; persistent lymphocytosis; proviral load

INTRODUCTION

Enzootic bovine leukosis is a malignant B-cell lymphoma of cattle caused by the *bovine leukemia virus* (BLV), an oncogenic member of the *Retroviridae* family and the *Deltaretrovirus* genus (Nakatsuchi *et al.*, 2022a). BLV is a single-stranded RNA virus that contains the genetic information for the structural proteins and enzymes in the genes gag, pol, and env (Gutiérrez *et al.*, 2020). BLV also encodes non-structural proteins in a region called pX, which are transcribed through alternative splicing in the regulatory genes tax, rex, R3, and G4 (Gutiérrez *et al.*, 2020). These non-structural proteins are fundamentally important in the interaction of the virus with the host cell, modulating the expression of viral and cellular genes, viral replication, and pathogenesis (Gutiérrez *et al.*, 2020).

The prevalence of BLV infection is high in most milk-producing countries, also including the American continent, where the spread of BLV infection in these countries is possibly a consequence of underestimation of the effects of infection without the implementation of the necessary health policies, becoming one of the most relevant neoplastic diseases in bovines (Gutiérrez *et al.*, 2020). Approximately 30% of animals infected with BLV develop a condition called persistent lymphocytosis (PL) and the animal has a B lymphocyte count twice the normal range for its age, breed, and physiological state (Hernández *et al.*, 2018). Less than 5% of BLV-positive animals develop B-cell leukemia/lymphoma after a long period of viral latency, while around 65-70% of positive animals remain asymptomatic (Chieh-Wen *et al.*, 2020). Animals infected with BLV have lower milk production (Norby *et al.*, 2016), higher incidence of mastitis and other infectious diseases (Nakada *et al.*, 2023), and lower reproductive efficiency (Ruiz *et al.*, 2018), which is reflected in the increased production costs (Kuczewski *et al.*, 2019).

Being a retrovirus, once BLV infects its target cell and integrates its genome, any secretion, and excretion that contains lymphocytes is considered a fomite of viral transmission (Kuczewski *et al.*, 2019). Thus, direct contact between animals, blood-sucking insects, and the iatrogenic route associated with poor management

practices are the main horizontal transmission routes (Kohara *et al.,* 2018). Likewise, perinatal infection, colostrum, and milk are the proven routes of vertical infection (Watanuki *et al.,* 2019). The amount of retroviral genome integrated into the host genome is known as the BLV proviral load (PVL) and is strongly related to disease progression (Hernández *et al.,* 2018; Nakatsuchi *et al.,* 2022a; Nakada *et al.,* 2023). When PVL exceeds 10,000 copies/105 cells in the blood, BLV can be detected in nasal secretions, saliva, colostrum, and milk (Watanuki *et al.,* 2019) and is correlated with the development of PL and the number of antibodies with viruses (anti-BLV) (Nakatsuchi *et al.,* 2022a,b) indicating that PVL is an excellent marker of disease progression and is considered a relevant variable in disease eradication plans in the face of the absence of the effective vaccines against the virus (Kuczewski *et al.,* 2021).

The progression of bovine leukosis understood as the probability of infection, the development of LP, the production of anti-BLV antibodies, and PVL have been related to the *DRB3* gene of bovine leukocyte antigens (BoLA) (Hernández *et al.,* 2018; Chieh-Wen *et al.,* 2020; Nakatsuchi *et al.,* 2022b). The *BoLA-DRB3* gene is very polymorphic; to date, 385 alleles have been reported (https://www.ebi.ac.uk/ipd/mhc/group/BoLA/ (access date December 12, 2023). This great diversity shows a particular distribution related to geographic region and breed (Chieh-Wen & Aida, 2022). In general, other reports consider that the *BoLA-DRB3*009:02*, **014:01:01*, and **002:01* alleles are associated with low PVL and classify the *BoLA-DRB3* alleles as resistance markers. **015:01* and **012:01* are related to high PVL and are classified as susceptibility markers (Nakatsuchi *et al.,* 2022a,b). Also, *BoLA-DRB3* alleles have been associated with resistance and/or susceptibility to different viral diseases such as herpesvirus type I and bovine papillomavirus (Longeri *et al.,* 2021; Morales *et al.,* 2020); various bacterial infections caused by *Escherichia coli*, *Staphylococci,* and *Fusobacterium* (Suprovych *et al.,* 2020); to parasites transmitted by ticks such as *Anaplasma marginale*, *Babesia bovis*, and *Babesia bigemina* (Bolaños *et al.,* 2017), but also with characteristics related to milk production and quality (Tayeng *et al.,* 2021).

The Lucerna cattle is one of the two synthetic Columbian breeds, formed in 1937 from the crossing between the Hartón del Valle, Holstein, and dairy Shorthorn breeds, in proportions of 30%, 30%, and 40%, respectively, with the need to have high-producing livestock but adapted to the environmental conditions of the tropics, where rusticity, grazing capacity, fertility, and longevity are essential (Giraldo *et al.,* 2023). In previous reports from our research group, a molecular presence of BLV of 50% in the breed was demonstrated (Hernández *et al.,* 2014) and 17 *BoLA-DRB3* alleles, H_O= 0.90; H_E= 0.92; F_{IS}= 0.03 ($p>0.05$) and without deviations from the Hardy-Weinberg equilibrium (Hernández *et al.,* 2015).

The specific effects of the disease on the Lucerna breed in terms of reduced production, reproductive effects, treatment costs, discards, and mortality, among others, are unknown. However, our research has shown that the Hartón del Valle breed can be considered a slow progressor of the disease, and the alleles of interest are *BoLA-DRB3*011:01* and **027:03* (Hernández *et al.,* 2014, 2016, 2018). Given the evidence shown, the objective of this work was to know the progression of bovine leukosis in the Lucerna breed, evaluated from BLV infection, the development of PL, the quantification of anti-BLV, and the PVL associated with the *BoLA-DRB3* alleles.

MATERIALS AND METHODS

This research was approved by the animal research ethics committee of the Universidad de Nacional de Colombia, according to a letter 03/04/2022.

Location, Animals, Sampling, and DNA Extraction

In this work, 104 adult animals (20 males and 84 females) from the El Hatico nature reserve, which is located in the municipality of El Cerrito, Valle del Cauca, Colombia, were used. According to Holdridge, it corresponds to a tropical dry forest (TDF) as it is located at 1000 meters above sea level, with an average temperature of 24 °C, 75% relative humidity, and 750 mm of annual precipitation on average (Giraldo *et al.,* 2023).

Two blood samples were taken from each animal from the coccygeal vein with the Vacutainer® system, one without anticoagulant and the other with anticoagulant (K_2EDTA 7.2 mg) and refrigerated until transported to the laboratory. The tube without anticoagulant was centrifuged at 3000 rpm for 13 minutes to separate the serum, which was stored at -80 °C until processing. The DNA extraction procedure was performed from the blood sample collected in the tube with anticoagulant, using the commercial Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and following the manufacturer's instructions. The quality and concentration of the DNA were evaluated using the Colibri spectrophotometer (Titertek Berthold, Technologies GmbH & Co.KG).

Determination of Persistent Lymphocytosis (PL)

The development of PL was evaluated using the peripheral blood plate smear method in two blood samples taken from the same animal with a difference in time of three months. Using the push technique, 20 µL of peripheral blood was spread on a slide and stained with 1% methylene blue stain, 100X DNA (AMRESCO®, Solon, OH, USA). The plates were observed under optical microscope vision (Nikon, eclipse, E200) at 100X magnification. A total of 100 white cells were counted and classified. The count of samples was considered with lymphocytosis when the percentage of lymphocytes was equal to or greater than the average plus twice the standard deviation both into sex and age group. The lymphocyte count was performed twice in different fields of the plate. An individual who presented lymphocytosis in two consecutive samples was considered PL positive (PL+). On the contrary, those animals that

did not present the conditions described above were considered negative (PL-) (Hernández *et al.*, 2016).

Evaluation of the Presence of BLV by Nested PCR

Across a nested PCR, a highly conserved region of the viral *env* gene was amplified. In the first reaction, 2X of MangoMix™ super mix (Bioline™, London, UK) 25 ng of DNA, 10 mM of each primer env5032 5′-TCTGTGCCAAGTTCCCAGATA-3′ and env5608r 5′-AACAACAACCTCTGGGAAGGGT-3′ (Nakada *et al.*, 2023; Úsuga-Monroy *et al.*, 2023). In the second reaction, 1 µl of the amplified product from the first reaction was used as template DNA. The same concentrations of the other reagents and the internal primers were env5099 5′-CCCACAAGGGCGGCGCCGGTTT-3′, and env5521r 5′-GCGAGGCCGGGTCCAGAGCTGG-3′ (Nakada *et al.*, 2023; Úsuga-Monroy *et al.*, 2023).

The thermal profile of the first reaction included an initial denaturation at 94 °C for 5 minutes, followed by 40 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes. The thermocycler conditions for the second reaction were the same, except that the annealing temperature was increased to 72 °C. Amplifications were carried out in a T100 Thermal Cycler (Bio-Rad™, Berkeley, CA, USA). The amplifications were observed in 1.2% agarose gels stained with GelRed™ (Biotium, Fremont, CA, USA) in a SUB-CELL® GT chamber (Bio-Rad™, Berkeley, CA, USA) and visualized with ultraviolet light in the positive animals. To BLV (env+), a fragment of 444bp was observed, which was not present in uninfected animals (env-).

DNA from an animal previously tested positive for BLV by ELISA, nested PCR and real-time quantitative PCR (qPCR) was used as a positive control in the PCR reactions. A clinically healthy animal was also used as a negative control. Both samples belong to the Animal Genetics Laboratory of the Universidad Nacional de Colombia – sede Palmira. To ensure that DNA samples were free of PCR inhibitors and avoid false negatives, each sample was tested by amplification of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene using 5mM of the primers GAPDH.276 F 5′-TGGAAAGGCCATCACCATCT-3′ and GAPDH.335R; 3′-CCCACTTGATGTTGGCAG-5′, 40ng of DNA and 2X MangoMix™ Super Mix.

Quantification of Antibody Titers with the Virus (AT)

To estimate the antibody titers (AT) of the animals against LBV (anti-BLV), an ELISA test was performed in two serial dilutions using the Svanova kit, SVANOVIR® BLV gp51-Ab (Svanova, Orlando, FL, USA), according to the manufacturer's instructions. The titers were expressed as the reciprocal of the last dilution with reactivity above the cut-off value. Titers were expressed as the binary logarithm of the last dilution that showed reactivity above the cut-off value. The median AT of all animals was used to categorize animals as high (AT+) and low (AT-) titers.

Quantification of Proviral Load (PVL)

To estimate the PVL, it was quantified by qPCR using the BLV-CoCoMo-qPCR-2 methodology and FAM probes (Takeshima *et al.*, 2015). Briefly, 10 ng DNA and 2X iTaq™ Universal Probes Supermix (Bio-Rad™, Berkeley, CA, USA), in a thermal cycler in real-time CFX96 Real-Time System (Bio-Rad™, Berkeley, CA, USA), using the primers CoCoMo6 (5′-MNMYCYCYKDRSYKSYKSAYYYYTCACCT-3′) and CoCoMo81 (5′-TACCTGMCSSCTKSCSCGGATA GCCGA-3′) and a FAM probe (6-carboxy-fluorescein FAM-labelled MGB probe 5′-FAM-CTCAGCTCTCTCGGTCC-NFQ-MGB-3′) (Takeshima *et al.*, 2015). The primer combination amplifies a 120 bp fragment in the viral LTRs. 50 nM of each primer and 150 nM FAM-BLV probe, 10 ng DNA, and 2X iTaq ™ Universal Probes Supermix were used. The amplification program included Uracil DNA Glycosylase (UDG) enzyme activation at 50 °C for 2 minutes, followed by iTaq ™ activation at 95 °C for 10 minutes, followed by 85 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. Reactions were performed on a CFX96 Real-Time Thermal Cycler (BIO-RAD®) (Takeshima *et al.*, 2015). The PVL expresses copy number in 10^5 white blood cells (Nakatsuchi *et al.*, 2022a). When an individual showed a quantity equal to or greater than 10,000 copies/10^5 cells, they were considered to have high PVL (PVL+). Otherwise, they were considered to have low PVL (PVL-).

BoLA-DRB3 Genotypes

The genotypes were obtained by the sequencing-based typing method (PCR-SBT) (Hernández *et al.*, 2015). Using primers DRB3FRW (5′-CGCTCCTGTGACCAGATCTATCC-3′) and DRB3REV (5′-GAGTTTCACTGTGCAGCGGCGA-3′) at a concentration of 10 mM, 25 ng of DNA, and 2X MangoMix™ super mix, a 281 bp fragment was amplified. The latter was sequenced bidirectionally in Macrogen and edited using the Geneious Prime 2023.2 program and the genotypes assigned with the ASSIGN 400 AFT program (Conexio Genomics, Fremantle, Australia).

Statistical Analysis

Descriptive statistics were performed for the PL, anti-BLV, and PVL variables. The percentage of animals that presented or did not present persistent lymphocytosis (PL), positive and negative to the BLV virus by PCR (*env*), that had high and low titers of antibodies against the virus BLV (anti-BLV) and with high and low proviral load was estimated (PVL). Allele frequencies for the *BoLA-DRB3* gene were estimated using the GenAlEx 6.5 program (Peakall *et al.*, 2012).

An association study was performed based on Fisher's exact test and the odds ratio (OR) value comparing the allele frequencies between animals with and without LP, positive and negative for the virus (*env*), with high and low TA, and with high and low CPV.

Alleles with Fischer's p-value<0.05, OR value <1, and with a 95% confidence interval that did not include one were classified as resistance alleles (R). On the contrary, those with Fischer's p-value<0.05, OR > 1, and with a 95% confidence interval that did not include 1, were defined as susceptibility alleles (S); the other alleles were considered neutral (N) (Chieh-Wen *et al.*, 2020). The animals were genotyped according to the classification given to their alleles as N/N, N/R, N/S, R/R, R/S, and S/S, and a new Fischer and OR test was performed. Statistical analyses were performed using the Jamovi program (version 2.3, https://www.jamovi.org).

RESULTS

Overall, 93.2% of the animals were positive for the BLV virus (env+), 18.3% developed PL, 50% had high anti-BLV antibodies, and 43.3% had high PVL (Table 1). The average lymphocyte count, AT, and PVL of the animals that did not develop PL had low AT and low PVL with 45.6 ± 5.7 lymphocytes, 6.90 ± 0.73 titers (log_2), and 902 ± 1511 copies /10^5 cells (Table 1).

In the study population, 17 *BoLA-DRB3* alleles were found, ten of which had frequencies greater than 5%. The most frequent alleles were *BoLA-DRB3*011:01*, **015:01*, **007:01*, and **023:01* (Table 2). Table 3 shows the alleles associated with the progression of bovine leukosis in the Lucerna breed. Two alleles showed an association with susceptibility (S) to infection with the virus (*BoLA-DRB3*15:01* and **23:01*), and only the *BoLA-DRB3*38:01* allele was considered resistant (R) to the infection with the virus. The *BoLA-DRB3*11:01* and **15:01* alleles showed low lymphocyte counts and were associated with not developing PL (R). There was no relationship with susceptibility to PL. Low ATs were found in the *BoLA-DRB3*13:01* and **20:01:02* alleles, so they were considered resistant. On the contrary, the *BoLA-DRB3*15:01* and **16:01* alleles presented high AT values that are related to susceptibility. The low PVL was associated with the *BoLA-DRB3*11:01* and **23:01* alleles, so they are considered resistant. Only the *BoLA-DRB3*15:01* allele showed an association with high PVL in the Lucerna breed. The other alleles had no effect and are therefore considered neutral.

The differences between the percentages of BLV positivity, the development of PL, the amount of AT, and the PVL between alleles with and without association are presented in Figure 1. None of the alleles found was associated with resistance to the four characteristics under study. The *BoLA-DRB3*11:01* allele stands out and shows a protective effect against the development of PL and the maintenance of a low PVL. On the contrary, the *BoLA-DRB3*15:01* allele was related to susceptibility in three of the variables analyzed, so the animals carrying this allele were infected by the virus more frequently and had anti-BLV titers higher and maintained a higher PVL (Figure 1).

As expected, the highest accumulated allele frequency was in the category of neutral alleles (72.8%), followed by resistant (14.8%) and susceptible (12.5%) in all variables (Table 4). The accumulated frequencies for the PL and PVL variables were higher in the resistant than susceptible category, and the opposite occurred in the variables' presence of the virus BLV (env) and AT.

In general, 6.18% of the animals had two resistance alleles, 7.03% had two susceptibility alleles, the majority had two neutral alleles (55.5%), and only 1.68% presented the R/S genotype (Table 5). When performing the Fisher test and estimating the OR value, the

Table 2. Allelic frequencies in the *BoLA-DRB3* gene in the bovine Lucerna breed

BoLA-DRB3 allele	Frequency (%)
*007:01	11.50
*010:02	6.70
*011:01	12.10
*013:01	5.00
*015:01	11.60
*016:01	5.00
*020:01:01	1.70
*020:01:02	5.00
*022:01	1.70
*022:02	3.30
*023:01	10.00
*025:01:01	6.70
*027:01	3.20
*027:02	3.30
*028:02	1.70
*029:01	8.20
*038:01	3.30

Table 1. Percentage and descriptive statistics of animals that developed or did not develop persistent lymphocytosis and with high and low antibody titers and proviral load in the bovine Lucerna breed

Variables		Percentage	Mean	Standard deviation	Minimum	Maximum
PL	Yes	18.3	61.9	6.7	55.0	80.0
	No	81.7	45.6	5.7	19.0	54.0
	Average	----	48.6	8.6	19.0	80.0
AT (titers log_2)	High	50.0	8.87	0.50	7.99	10.1
	Low	50.0	6.90	0.73	4.84	7.93
	Average	----	7.88	1.17	4.84	10.1
PVL (copies /10^5 cells)	High	43.3	99677	104173	10481	326871
	Low	56.7	902	1511	0	5687
	Average	----	43641.4	84105.04	0	326871

Note: PL= persistent lymphocytosis; AT= antibody titers; PVL= proviral load.

Table 3. Association of *BoLA-DRB3* alleles with the progression of bovine leukosis in the bovine Lucerna breed

Allele	*env* (%)			PL (count)			AT (titers log_2)			PVL (copies /10^5 cells)		
	env+	*env-*	OR (CI 95%)	Yes	No	OR (CI 95%)	High	Low	OR (CI 95%)	High	Low	OR (CI 95%)
*007:01	45.4	54.6	0.22 (0.09-0.53)	56.7	46.2	4.17 (1.61-10.6)	8.68	7.31	2.03 (0.83-4.87)	29061	1055	2.48 (1.02-6.01)
*10:02	48.5	51.5	0.13 (0.04-0.40)	57.0	46.4	3.98 (1.25-12.6)	8.88	7.15	0.50 (0.15-1.58)	109473	215	0.36 (0.11-1.12)
*11:01	49.9	50.1	0.13 (0.04-0.40)	58.8	46.4	0.23 [R] (0.20-0.37)	9.01	6.34	1.95 (0.78-4.47)	10481	663	0.24 [R] (0.29-0.72)
*13:01	51.3	48.7	0.32 (0.08-1.20)	0.0	45.6	7.68 (1.72-34.2)	8.17	3.04	0.02 [R] (0.01-0.76)	0.0	346	7.75 (0.06-29.5)
*15:01	1.0	99.0	3.12 [S] (1.04-4.20)	57.7	48.0	0.25 [R] (0.18-0.74)	9.33	3.13	2.22 [S] (1.97-2.53)	78399	0.0	2.11 [S] (2.04-2.28)
*16:01	55.1	44.9	0.13 (0.03-0.48)	55.0	42.9	4.94 (1.31-18.5)	8.48	3.1	2.20 [S] (1.99-3.01)	145137	739	0.51 (0.13-1.89)
*20:01:01	45.8	54.2	0.13 (0.01-1.26)	0.0	40.8	7.48 (0.77-72.4)	0.0	5.38	0.14 (0.01-1.29)	0.0	65	7.43 (0.46-119)
*20:01:02	44.9	55.1	0.13 (0.03-0.48)	65.0	47.2	1.25 (0.33-4.73)	8.63	3.15	0.32 [R] (0.12-0.76)	129555	492	3.12 (0.83-11.7)
*22:01	54.2	45.8	0.51 (0.05-4.95)	0.0	45.0	7.43 (0.46-11.1)	9.51	0.0	7.46 (0.77-72.2)	0.0	433	7.43 (0.46-119)
*22:02	53.8	46.2	0.13 (0.02-0.65)	64.0	45.4	1.96 (0.39-9.88)	8.75	7.10	1.96 (0.39-9.84)	33573	0.0	0.14 (0.02-1.29)
*23:01	1.0	99.0	3.14 [S] (1.04-4.32)	60.0	44.6	0.99 (0.32-1.58)	8.7	7.06	1.26 (0.49-3.24)	11363	1972	0.29 [R] (0.01-0.65)
*25:01:01	52.1	47.9	0.13 (0.04-0.40)	69.3	43.2	3.99 (1.25-12.6)	8.52	7.22	0.36 (0.11-1.12)	81366	3791	0.27 (0.08-0.89)
*27:01	50	50.0	0.13 (0.02-0.65)	70.0	40.7	3.88 (0.77-19.5)	8.73	6.42	1.00 (0.19-5.54)	202663	3196	0.99 (0.58-1.58)
*27:02	49.7	50.3	0.13 (0.02-0.65)	66.0	49.3	1.96 (0.39-9.88)	8.39	6.35	0.26 (0.05-1.29)	156885	10	0.25 (0.05-1.29)
*28:02	44.9	55.1	0.13 (0.01-1.26)	0.0	47.7	7.48 (0.77-72.4)	0.0	6.72	0.14 (0.01-1.29)	239015	0.0	0.14 (0.01-1.29)
*29:01	46.9	53.1	0.27 (0.09-0.70)	66.3	46.7	2.21 (0.89-8.92)	9.08	6.98	0.27 (0.10-0.73)	97874	348	1.81 (0.62-5.24)
*38:01	99.0	1.0	0.02 [R] (0.01-0.09)	62.0	53.0	0.58 (0.10-10.5)	8.52	6.20	1.96 (0.39-9.84)	276797	379	1.96 (0.39-9.86)

Note: *env*= presence of BLV by nested PCR; PL= persistent lymphocytosis; AT= antibody titers; PVL= proviral load; **BoLA-DRB3* alleles; [R]Alleles with p<0.05 and whose OR value allows them to be classified as resistant; [S]Alleles with p<0.05 and whose OR value allows it to be classified as susceptible; OR= Odds ratio; CI= confidence interval.

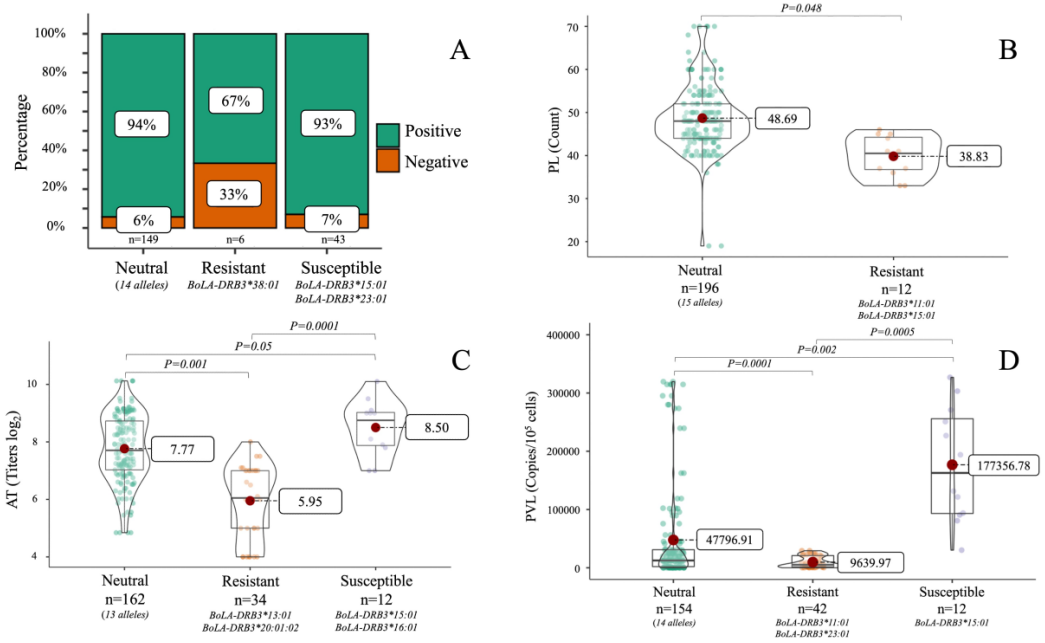

Figure 1. Differences between alleles considered neutral, resistant, and susceptible to virus positivity (A), to the development of persistent lymphocytosis (B), with anti-BLV antibody titers (C), and PVL (D). Note: PL= persistent lymphocytosis; AT= antibody titers; PVL= proviral load.

R/R genotype shows a protective effect in all variables. Likewise, the S/S genotype shows a susceptibility effect for three variables except for PL. In particular, the N/R genotype had a protective effect on all variables ($p<0.05$ and OR<0.21), while the N/S genotype showed a neutral effect, as did the N/S genotype N and R/S (Table 5).

DISCUSSION

This work is the first report of the association between the variables related to the progression of bovine leukosis and the *DRB3* gene of the BoLA complex in the Lucerna breed from Colombia. In a previous report, using molecular methods, a molecular prevalence of BLV in the Lucerna breed was found to be 50% (Hernández *et al.*, 2014). The same author presents a value of 83.3% in the Hartón del Valle breed and another study of 38.3% (Hernández *et al.*, 2016). While in 500 animals of the Holstein breed in Antioquia (Colombia), the prevalence was 44% (Úsuga-Monroy *et al.*, 2023). The most up-to-date prevalence in this regard presented a value of 62% in 92% of the herds evaluated (Corredor-Figueroa *et al.*, 2020). In all reports, the presence of BLV found was lower than in this investigation (93.2%). Likewise, it was confirmed that genotypes 1 (97%) and 3 (3%) are those circulating in the country, with the first being the most frequent (Úsuga-Monroy *et al.*, 2023). Some reasons for this variation include inappropriate handling practices, such as the repurposing of surgical equipment and palpation gloves. Also, there is a lack of disease control and eradication programs, including management practices that minimize the likelihood of horizontal and vertical transmission. Finally, genetic or breed-associated effects have been reported that may make them more or less susceptible (Úsuga-Monroy *et al.*, 2023).

Table 4. Cumulative allelic frequencies of genes associated with virus progression in the bovine Lucerna breed

Variables	Allele category		
	Neutral	Resistant	Susceptible
env (%)	75.10	3.30	21.60
PL (%)	76.30	23.70	0.00
AT (%)	73.40	10.00	16.60
PVL (%)	66.30	22.10	11.60
Average (%)	72.80	14.80	12.50

Note: *env*= presence of BLV by nested PCR; PL= persistent lymphocytosis; AT= antibody titers; PVL= proviral load.

The *BoLA-DRB3* alleles **10:01*, **11:01*, **20:06*, **27:03*, **30:01*, and **48:02* were associated with a low probability of infection and were considered resistant. On the contrary, the *BoLA-DRB3* alleles **09:02*, **16:01*, **17:01*, **20:01:02*, and **25:01:01* were related to susceptibility to infection in the Hartón del Valle breed (Hernández *et al.*, 2018). None of these alleles showed a similar association in the Lucerna breed. Several factors that may contribute to the differential performance of *DRB3* alleles may be related to the overall genetic composition of the breed or that the particular allele is in linkage disequilibrium with other *loci* that influence resistance or susceptibility to BLV. In addition, differences in expression levels of *DRB3* alleles may be due to regulatory elements such as nutritional status or co-infection with other diseases. Epistatic interactions and polygenic responses to infection may also cause differences in reported results. Finally, variations in the methodological design of the studies support these findings. The above suggests the presence of racial particularities, as reported by Hernández *et al.* (2016).

Some reports have estimated that between 30% and 70% of animals infected with BLV develop persistent lymphocytosis (Chieh-Wen & Aida, 2022). The example of reported animals with PL in the breeds is Hartón del Valle (32.7%), Lucerna (94%), and Holstein (100%) [13]. The above contrasts with our results, where only 18.3% presented PL (Hernández *et al.*, 2016). However, the difference could be explained by the different sample sizes between studies.

On the other hand, in the Hartón del Valle breed, the *BoLA-DRB3*11:01* allele is related to the absence of PL, while the *BoLA-DRB3*25:01:01* and **27* alleles were associated with susceptibility (Hernández *et al.*, 2018). In the Lucerna breed, no PL susceptibility alleles were found. Some reports have proposed that the Glu-Arg (ER) amino acids in position 70-71 of the *BoLA-DRβ* chain are associated with resistance to the development

Table 5. Percentage of bovine Lucerna breed according to the classification of their alleles and *Odds ratio* value

Variables	Genotype											
	N/N		N/R		N/S		R/R		R/S		S/S	
	%	OR (CI 95%)	%	OR (CI 95%)	%	OR (CI 95%)	%	OR (CI 95%)	%	OR (CI 95%)	%	OR (CI 95%)
env	56.7	1.01 (0.98-1.05)	8.9	0.20$^{(R)}$ (0.08-0.55)	16.7	3.12 (0.83-11.7)	0.0	0.42$^{(R)}$ (0.29-0.72)	0.0	7.45 (0.88-52.4)	17.8	2.22$^{(S)}$ (1.97-2.53)
PL	67.8	1.08 (0.88-1.11)	32.2	0.21$^{(R)}$ (0.07-0.57)	0.0	1.96 (0.39-9.84)	0.0	0.55$^{(R)}$ (0.20-0.82)	0.0	7.48 (0.77-72.4)	0.0	3.12 (1.04-4.20)
AT	50	1.00 (0.78-1.20)	13.3	0.19$^{(R)}$ (0.06-0.59)	20.0	1.96 (0.39-9.88)	3.3	0.54$^{(R)}$ (0.20-0.81)	6.7	1.05 (0.55-4.73)	6.7	2.20$^{(S)}$ (1.01-3.53)
PVL	47.6	1.10 (0.68-1.15)	23.8	0.10$^{(R)}$ (0.05-0.45)	10.7	1.25 (0.33-4.73)	21.4	0.57$^{(R)}$ (0.19-0.89)	0.0	7.75 (0.06-29.5)	3.6	1.90$^{(S)}$ (1.05-4.53)
Average	55.50%		19.60%		11.90%		6.18%		1.68%		7.03%	

Note: *env*= presence of BLV by nested PCR; PL= persistent lymphocytosis; AT= antibody titers; PVL= proviral load; ^{R}Alleles with $p<0.05$ and whose OR value allows them to be classified as resistant; ^{S}Alleles with $p<0.05$ and whose OR value allows it to be classified as susceptible; OR= Odds ratio; CI= confidence interval; N/N= Neutral/Neutral; N/R= Neutral/Resistant; N/S= Neutral/Susceptible; R/R= Resistant/Resistant; R/S= Resistant/Susceptible; S/S= Susceptible/Susceptible.

of PL (Hernández *et al.,* 2018) controversially, the two alleles associated with this characteristic here reported have the Arg-Arg motif in these positions.

The AT against BLV in the Lucerna breed (7.84 $\log_2$) is similar to those found in this study (7.88 $\log_2$) (Kohara *et al.,* 2018) but higher than those reported in the Holstein breed from Japan 6.96 $\log_2$ (Nakatsuchi *et al.,* 2022a) and from Colombia (6.05 $\log_2$) (Hernández *et al.,* 2016) and much lower than those reported in the Hartón del Valle (9.67 $\log_2$), where a strong racial effect on this characteristic is also shown. In addition, in the Hartón del Valle breed, the *BoLA-DRB3*11:01* and **27:03* alleles were positively associated with ATs, and the *BoLA-DRB3*16:01* and **17:01* alleles were negatively associated with ATs (Hernández *et al.,* 2018). The resistance alleles differed from those reported here, while only the *BoLA-DRB3*16:01* allele showed a similar association in the Lucerna breed. The average AT in animals that have resistance alleles (*BoLA-DRB3*002:01*, **009:02* or **014:01:01*) was lower than that found in susceptibility alleles (*BoLA-DRB3*012:01* or **015:01*) (6.3 $\log_2$ Vs. 7.4 $\log_2$; P= 0.00012) in the Holstein breed (Nakatsuchi *et al.,* 2022a). The same trend was found in this study, although the resistance and susceptibility alleles related to ATs were different. Contrary to what was reported by Nakatsuchi *et al.* (2022a), the cumulative frequency of susceptibility alleles was the highest (47.9%), followed by neutral (33.2%) and resistant alleles (18.9%).

In the case of the association study, no association has been found between the *BoLA-DRB3* alleles and the production of antibodies against viruses such as bovine viral diarrhea, herpes virus type I, and foot-and-mouth disease virus (Rodríguez-Habibe *et al.,* 2020). The above may suggest that establishing a selection program aimed at increasing the frequency of the alleles here related to resistance (*BoLA-DRB3*13:01* and **20:01:02*) would not affect susceptibility to other viruses of interest, even more so when the cumulative frequency of susceptibility alleles was greater than those of resistance (Table 4).

In previous works, the PVL found in the Hartón del Valle, Lucerna, and Holstein breeds were 663828, 963831, and 1182992 copies/ng of DNA, respectively, in animals classified as having high PVL, and 5723 and 10234 copies/ng of DNA in the Hartón del Valle and Lucerna breeds, respectively, in animals with low PVL (Hernández *et al.,* 2016). Although the PVL quantification units are different in these studies, it was possible to determine that 48% of the Lucerna animals had low PVL, a lower value than the one presented here (56.7%). Particularly, in the Hartón del Valle breed, the *BoLA-DRB3*11:01* allele was related to low PVL, while the *BoLA-DRB3*16:01* allele was associated with high PVL (Hernández *et al.,* 2018). Additionally, some works have shown that the alleles related to low PVL in the Holstein breed of various origins are *BoLA-DRB3*002:01*, **009:02* and **014:01:01*, while those associated with high PVL are *BoLA-DRB3*012:01* and **015:01* (Chieh-Wen *et al.,* 2020; Chieh-Wen & Aida, 2022; Nakatsuchi *et al.,* 2022b; Nakatsuchi *et al.,* 2023). The reported PVLs are 4 216, 19 206, and 14350 copies/10^5 cells in the resistance, susceptibility, and neutrality alleles, respectively (Nakatsuchi *et al.,* 2023). Regarding resistance alleles, only the *BoLA-DRB3*11:01* allele from Hartón del Valle presented a similar association; the alleles reported in Holstein were not found in the Lucerna population under study. In contrast, only *BoLA-DRB3*015:01* related to high PVL in Holstein was also related to high PVL in the Lucerna breed.

Both in Hartón del Valle (Hernández *et al.,* 2018) and in Holstein (Nakatsuchi *et al.,* 2023), the cumulative frequencies of resistant alleles (21.5% in Hartón del Valle and 16% in Holstein), susceptible (8.1% in Hartón del Valle and 22% Holstein), and neutral (70.4% in Hartón del Valle and 62% in Holstein) are different from those presented here. However, the high frequency of resistance alleles would facilitate a selective process in favor of them, which could somehow keep the spread of the virus under control since PVL has been considered the main risk factor related to its spread (Kuczewski *et al.,* 2021). The above was experimentally proven when a herd formed with heterozygous animals carrying the *BoLA-DRB3*009:02* allele did not spread the disease during the 20 months of the experiment, so low PVL in addition to the allele is considered a protective factor (Juliarena *et al.,* 2016). On the contrary, other factors that increase PVL are the lymphocyte count and the calving number per cow (Ohno *et al.,* 2015). The above could be explained in part by the positive correlation found between PVL and AT in the blood (r=0.45; p=1.4×10^{-8}) and in milk (r=0.38; P=7.3×10^{-3}) (Nakatsuchi *et al.,* 2022a). Similar results are reported in Holstein of Japan (r=0.69; p<0.05) (Konishi *et al.,* 2018).

The principal protective effect found in N/R animals compared to R/R animals (Table 5) suggests a possible overdominance effect, as suggested by other authors (Hernández *et al.,* 2014, 2018). It has been proposed that individuals who are heterozygous for major histocompatibility complex can recognize a wide spectrum of antigens, thus increasing the efficiency of these individuals compared to homozygous individuals. However, the neutrality effect found in the N/S genotype also suggests dominance between the characteristics, a hypothesis that should be addressed in future research.

Finally, our results suggest that the *BoLA-DRB3*11:01* allele may determine disease progression within the individual and control dissemination within and between herds. The high frequency of this allele is explained because it is also the most frequent in the Hartón del Valle breeds (Hernández *et al.,* 2015) and Holstein (Chieh-Wen & Aida, 2022) breeds that are the origin of the Lucerna. The possible effect of the Shorthorn breed on disease resistance and how this affects the performance of the Lucerna breed should be included in future studies. On the other hand, the *BoLA-DRB3*15:01* allele was negatively related to three of the characteristics evaluated in the Lucerna breed. This allele has also been reported as a risk factor in disease progression in several breeds (Chieh-Wen & Aida, 2022). These findings allow us to consider a possible plan for eradicating or controlling the disease based on the selective elimination and segregation of animals based on their genotype, PVL, and AT levels.

CONCLUSION

In our work, the bovine Lucerna breed showed a high prevalence of BLV, developing PL as expected, showing high antibody production, and maintaining a low proviral load. The *BoLA-DRB3* gene showed significant polymorphism within this breed. Several alleles of the *BoLA-DRB3* gene were positively correlated with several parameters associated with the onset and progression of bovine leukosis. Consequently, the Lucerna breed can be classified as a breed with slower disease progression, suggesting a degree of inherent resistance. This resistance is likely, at least in part, due to specific *BoLA DRB3* alleles.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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