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RESEARCH ARTICLE

Association between Genes BoLA-DRB3.2*8 and BoLA-DRB3.2*12 with Resistance and BoLA-DRB3.2*16 with Susceptibility to Infection by Bovine Leukemia Virus

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ABSTRACT

The Bovine Leukemia Virus (BLV) is a retrovirus that affects the immune system of cattle as their target cells are B lymphocytes. Some polymorphisms at the BoLA-DRB 3.2 gene have been associated with resistance/susceptibility to diseases. The objective of this research was to determine the polymorphisms at the BoLA-DRB 3.2 gene and associate them with resistance (R), neutrality (N) or susceptibility (S) to BLV in a Holstein cow population.500 blood samples were taken. Nested PCR was performed for detecting BLV virus and PCR-RFLP was performed to identify alleles of gene BoLA-DRB 3.2. Susceptibility was determined using odds ratio (OR) and P value. According to their genotype, cows were classified in homozygous (R/R, N/N, or S/S) and heterozygous (R/N, R/S, N/S). BLV molecular prevalence was 44%. The most frequent allele was BoLA-DRB3.2*22 (16.8%), alleles associated with resistance to BLV were BoLA-DRB3.2*8 (OR=1.489; P<0.10) and BoLA-DRB3.2*12 (OR=3.897; P<0.10) and allele BoLA-DRB3.2*16 (OR=0.710; P<0.10) was associated with susceptibility. Allele BoLA-DRB3.2*8 had the highest allelic frequency for negative cows (0.19). 63.7% of cows with genotype RN and 70% of cows with genotype RR were resistant to infection by BLV. Alleles R and S have a dominant effect on allele N (P<0.05). The use of reliable diagnostic techniques in conjunction with identification of resistant or susceptible animals can monitor the progress of the disease in dairy herds. Alleles BoLA-DRB3.2*8 and *12 were positively related to the disease and therefore cows have low risk of infection, unlike allele BoLA-DRB3.2*16 which was negatively related and animals have high risk for the disease.

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INTRODUCTION

Enzootic Bovine Leukemia (EBL) is a viral disease characterized by affecting the immune system of cattle as their target cells are B lymphocytes. Bovine Leukemia Virus (BLV) is the etiologic agent of EBL, a retrovirus which belongs to family *Retroviridae* subfamily *Orthoretrovirinae*, genus *Deltaretrovirus* (Wu *et al.*, 2003; Rodriguez *et al.*, 2011). Bovine Leukocyte Antigen (BoLA) is a surface glycoprotein encoded by a gene cluster which controls antigen presentation, susceptibility to infectious diseases depends on this glycoprotein (Tizard, 2013). BoLA genes are very interesting because they are associated with resistance or susceptibility to a lot of diseases as brucellosis, mastitis or bovine leukemia virus affecting production, growth and immune response of animals (Sharif *et al.*, 1998; Hernández, 2014). BoLA-DRB3.2 which is the second exon of loci DRB-3 of BoLA type II, is responsible for domain β1 which is directly related to foreign antigens presentation and is characterized by being highly polymorphic, which is very important in immune responses against pathogens. Genetic organization of gene BoLA-DRB3.2 has been established through various techniques such as PCR, cloning and sequencing, besides characterization of various breeds has allowed to identify more than 100 different alleles (Behl *et al.*, 2012). In Holstein cattle it was found that BoLA-DRB3.2*11 alleles confer resistance to Persistent Lymphocytosis (PL) (Mirsky *et al.*, 1998) and the low proviral load, in this case the

infected animals disrupted the transmission of BLV (Juliarena *et al*., 2016); on the other hand, BoLA-DRB3.2*16 allele has been classified as of susceptibility for lymphosarcoma (LS) and persistent lymphocytosis (PL) by BLV (Juliarena *et al.,* 2008). The objective of this research was to determine polymorphisms of gene BoLA-DRB3.2 and associate them with resistance, neutrality or susceptibility to infection by BLV in Holstein cows.

MATERIALS AND METHODS

Animals and samples: 500 cows from 17 herds were used, which are located in 7 municipalities of the department of Antioquia: Bello, Medellin, Belmira, Entrerrios, San Pedro de los Milagros, La Union, Rionegro. Sampling for approval by the ethics committee of the National University of Colombia was obtained (CEMED-007, May 14, 2012). Sampling was done by puncturing middle coccygeal vein with vacutainer vacuum system (DBvacutainers) and EDTA. To obtain DNA of leukocytes *salting out* technique proposed by Miller *et al* (1988), was performed. DNA was resuspended in 1X TE pH 8.0 buffer (Tris HCl 1 M and 0.5 M EDTA) and then stored at 4°C until analysis.

Molecular prevalence of BLV: A region of the viral *env* gene (gp51) was amplified by nested PCR to obtain a fragment of 444 base-pair in positive cows, used primers were described by Beier *et al.* (2001). The first reaction was performed in a final volume of 25 µl with 150 ng of DNA, 3.0 µl of 10 mM of each primer *env*5023 and *env*5608, 0.4 mM of dNTPs, 1X of PCR Tampon (ThermoScientific®), 3 mM $MgCl_2$, and 1U of Taq DNA Polymerase. In the second PCR reaction was used, as template DNA, 5 µl of the first amplification PCR product, with same concentrations of other reagents and primers *env*5099 and *env*5521 in a final volume of 30 µl. Reactions for both PCR were identical, and were performed in a T3 thermocycler (Biometra®) with the following protocol: 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, to finish with a final extension at 72°C for 5 minutes. The product of the second reaction was checked on a gel 2% in a Gel Doc (BioRad®).

Amplification of alleles of gene BoLA-DRB3.2: Semi-nested PCR with a modified protocol proposed by van Eijk *et al.* (1992) was conducted to determine alleles of exon two of gene BoLA-DRB3. Protocol modifications were proposed by Zambrano *et al.* (2011).

Digestion with restriction enzymes to determine BoLA-DRB3.2 alleles: The PCR product obtained in the second round was digested with RsaI, BstYI (New England BioLabs®) and HaeIII (Fermentas®) enzymes separately (Zambrano *et al*., 2011).

Statistical analysis: To determine the association of each BoLA-DRB3.2 allele with susceptibility or resistance to BLV infection the odds ratio (OR) was determined by logistic regression, Fisher's exact test was used to determine if OR was statistically significant (P<0.10). If OR>1 and P<0.10 allele is associated with resistance (R) since animals have low risk of being positive for infection with BLV; if OR<1 and P<0.10 allele is associated with susceptibility since animals are at high risk of being positive for infection with BLV; if OR>1 or OR<1 and P>0.10 alleles were classified as neutral (N). OR was determined for genotypes NN, NR, NS, RR, RS, and SS to establish dominance effects of alleles R or S. If OR>1 and P<0.05 there is dominance effect of allele R; if OR<1 and P<0.05 there is dominance effect of allele S; If OR>1 or OR<1 and P>0.05 there is no dominance effect of the R or S alleles. Statistical analysis was performed in program SAS version 9.2 for Windows (SAS Institute Inc, Cary NC, USA).

RESULTS

Classification of alleles BoLA-DRB3.2 gene as R, S, or N to infection with BLV: PCR molecular test determined the presence of provirus and its molecular prevalence for Holstein cows which belong to high milk production area in the department of Antioquia was 44%. In determining the alleles of BoLA-DRB3.2 in the population of 500 tested cattle, 36 alleles that differ in frequency were found. Alleles with frequencies below 1% were grouped into the classification "Other" and 21 alleles were placed in this group (BoLA-DRB3.2*1, *13, *14, *17, *18, *19, *20, *21, *25, *26, *32, *48, *49, *6, *kba, *abb, *dbb, *gbb, *ibb, *ibe, *kaa) with an overall frequency of 6.9%. The most frequent alleles were: BoLA-DRB 3.2*8 (16.3%), BoLA-DRB 3.2*16 (12.8%) and BoLA-DRB 3.2*22 (16.8%).

BoLA-DRB3.2 alleles were classified according to their OR and Fisher´s exact test in three categories: S, N, and R. A significant association between absence of infection with BLV and BoLA-DRB3.2*8 alleles (OR=1.489, P<0.10) and BoLA-DRB3.2*12 (OR=3.897, P<0.10) was found, so both were classified as alleles associated with resistance (R); furthermore, a significant association between the presence of BLV genome and BoLA-DRB3.2*16 allele (OR=0.710, P<0.10), which was classified as associated with susceptibility (S), was found. Other alleles were classified as neutral (N) (P>0.10) (Table 1).

Distribution of BoLA-DRB3.2*8, *12, *16 alleles in seven municipalities of Antioquia: The municipality of San Pedro presented the highest percentage of resistant alleles (60%) compared to the other municipalities, since in this municipality 27.4% of BoLA-DRB3.2*8 alleles and 33.3% of BoLA-DRB3.2*12 alleles were classified as resistant. The municipalities of La Union and Rionegro did not present the resistance allele BoLA-DRB3.2*12. Resistance allele BoLA-DRB3.2*8 had the lowest appearance in the municipality of Belmira (4.3%); on the other hand, municipalities of Medellin and Entrerrios had the highest percentage (18.8%) for susceptibility allele BoLA-DRB3.2*16, followed by the municipality of San Pedro (17.2%). Municipality of La Union had the lowest percentage of allele BoLA-DRB3.2*16 of susceptibility (8.6%) (Table 2).

Table 1: Classification of alleles BoLA-DRB3.2 gene as R, S, or N to infection with BLV

Allele PCR-RFLP	Total Freq	Freq *env-*	Freq *env+*	OR[1]	CI (95%)		Fisher	Class[2]
*2	0.014	0.011	0.018	0.585	0.201	1.697	0.2352	N
*3	0.059	0.066	0.050	1.311	0.762	2.254	0.2077	N
* 8	0.164	0.190	0.130	1.463	1.036	2.065	0.0251 **	R
* 10	0.021	0.020	0.023	0.857	0.361	2.037	0.4487	N
* 11	0.078	0.084	0.071	1.182	0.738	1.891	0.2923	N
* 12	0.012	0.018	0.005	3.896	0.849	17.837	0.0526 *	R
* 15	0.017	0.012	0.023	0.546	0.206	1.445	0.1639	N
* 16	0.128	0.109	0.153	0.71	0.491	1.026	0.0514 *	S
* 22	0.167	0.164	0.171	0.958	0.688	1.329	0.4357	N
* 23	0.087	0.080	0.096	0.835	0.539	1.295	0.2546	N
* 24	0.095	0.094	0.096	0.983	0.644	1.502	0.5142	N
* 28	0.028	0.021	0.037	0.585	0.274	1.284	0.1178	N
* 36	0.025	0.028	0.021	1.386	0.606	3.165	0.2897	N
* 37	0.024	0.025	0.023	1.091	0.48	2.48	0.5051	N
* 51	0.012	0.011	0.014	0.779	0.25	2.433	0.4427	N
Other [3]	0.069	0.068	0.071					
Total Alleles	1.000	562	438					
Total cows	500	281	219					

Negative to BLV= *env-*, Positive to BLV= *env+*; Frequency = Freq; [1]OR = Odds Ratio. If OR>1 there is association with *env-*, if OR<1 there is association with *env+*; [2]Classification of alleles: S = Susceptible, N = Neutral, R = Resistant, according to OR and Fischer Test.S = (OR <1, P <0.10), N = (OR> 1 or OR <1, P> 0.10), R = (OR> 1, P<0.10); [3]Alleles with frequency less than 1%: BoLA-DRB3.2*1 (0.6%), *13 (0.4%), *14 (0.6%), *17 (0.3%), *18 (0.3%), *19 (0.1%), *20 (0.5%), *21 (0.5%), *25 (0.1%), *26 (0.9%), *32 (0.1%), *48 (0.1%), *49 (0.3 %), *6 (0.2%), *kba (0.1%), *abb (0.1%), *dbb (0,.%) *gbb (0.8%), *ibb (0.3%), *ibe (0.2%), *kaa (0.3%).

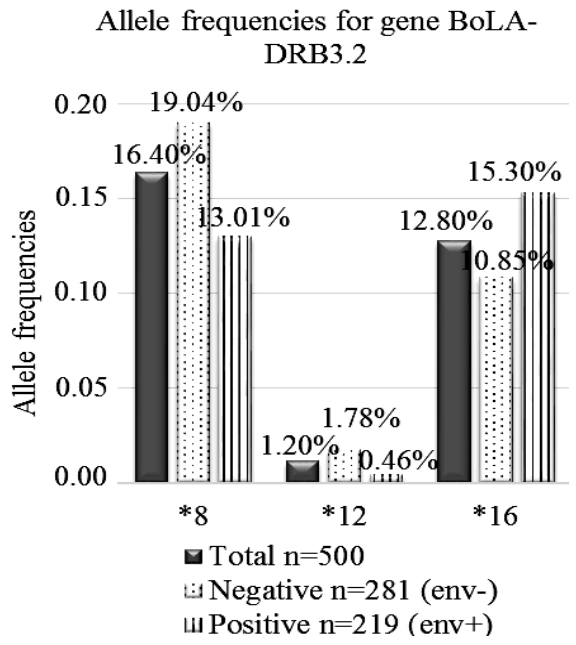

Fig. 1: Allele frequencies for gene BoLA-DRB3.2*8, *12 of resistance and BoLA-DRB3.2*16 of susceptibility in the total population analyzed (gray bar), in negative cows for infection with BLV (dot bar) and positive cows for infection with BLV (striped bar).

Table 2: Distribution of BoLA-DRB3.2*8, *12, *16 alleles in seven municipalities of Antioquia

Municipality		Allele			
		* 8 R	* 12 R	* 16 S	Total alleles
Medellin	# Alleles	10	2	24	112
	% Total	6.1	16.7	18.8	
Belmira	# Alleles	7	1	13	82
	% Total	4.3	8.3	10.2	
Bello	# Alleles	38	2	18	196
	% Total	23.2	16.7	14.1	
Entrerrios	# Alleles	42	3	24	246
	% Total	25.6	25.0	18.8	
La Union	# Alleles	13	0	11	64
	% Total	7.9	0.0	8.6	
Rionegro	# Alleles	9	0	16	64
	% Total	5.5	0.0	12.5	
San Pedro	# Alleles	4. 5	4	22	236
	% Total	27.4	33.3	17.2	
Total alleles		164	12	128	1000

Allele frequencies for gene BoLA-DRB3.2*8, *12 of resistance and BoLA-DRB3.2*16 of susceptibility: Figure 1 shows allele frequencies of alleles classified as resistant (BoLA-DRB3.2*8, *12) and allele classified as susceptible (BoLA-DRB3.2*16) in the total population and in BLV infection positive and negative bovines. Allele BoLA-DRB3.2*8 classified as R had the highest allelic frequency for negative cows (0.19), while allele *12 also of resistance presented a frequency of (0.18) on positive animals, this allele was only found in 1.20% of the total population. Allele BoLA-DRB3.2*16 classified as of susceptibility showed the highest rate (0.15) in BLV positive cows.

NN, NR, NS, RR, RS, and SS genotypes for gene BoLA-DRB3.2: Once determined alleles as R, N, or S, the six possible genotypes of cattle as RR, RN, RS, NN, NS, and SS were determined. 1% of Holstein cows was classified as SS in this category 2 out of 6 animals presented proviral genome.4% of cows were classified as RR and 14 out of 20 were negative for BLV. In NS category 55 animals out of 96 presented BLV, while in NR category 77 out of 116 were negative for the virus. The highest percentage of genotypes was for NN cows with 48%, followed by NR animals with 23%. The municipalities of Belmira, La Union, and Rionegro did not present RR genotype, while the municipality of San Pedro did not present SS genotype. Municipalities of Bello and Entrerrios had the highest number of NR animals that were genotyped BLV negative; on the other hand, the city of Medellin had the highest number of genotype NS animals that were positive to BLV infection. (Table 3).

OR for NN, NR, NS, RR, RS, and SS genotypes of gene BoLA-DRB3.2: OR was determined for genotypes NN, NR, NS, RR, RS, and SS of gene BoLA-DRB3.2 (Table 4) to explain R or S possible dominance effects on N. OR for NR genotype was higher than 1 (P<0.05) so allele R apparently has a dominant effect on allele N as only 39 out of 116 cows were positive for BLV; moreover, OR for NS genotype was under 1 (P<0.05), that is, allele S may exert a dominant effect on allele N. 55 positive cows with

NS genotype were found infected by BLV from a total of 96 cows. The other genotypes had no significant OR. (Table 4).

DISCUSSION

Nested PCR detected that 44% of Holstein cows of specialized dairy of Antioquia were positive BLV, this number is higher than the one found by Ortega *et al*., (2016) who found a serological prevalence of BLV in Colombia 42.7%. These results are compared since there are no other records of molecular prevalence nor to the region or the country. In other South American countries molecular prevalence of BLV is: Chile 27.9%, Perú 42.3%, Paraguay 54.7% and Argentina 77.4% (Polat *et al*., 2016). The 7 most frequent alleles for gene BoLA-DRB3.2 in evaluated Holstein cattle were BoLA-DRB3.2*11, *16, *22, *23, *24, *3, *8, with a cumulative frequency of 77.8%, these same alleles were reported by Juliarena *et al.* (2008) and Sharif *et al.* (1998) as the most frequent in Holstein breed, the latter reported a cumulative frequency of 82.4%. Five of the alleles found in this study are among the most frequent 10 reported by Zambrano *et al.* (2011, 2014) for a herd of Holstein breed of Antioquia (BoLA-DRB3.2*23, *22, *24, *16, *33, *8, *39, *37, *27, *18). Major allele frequencies of this study were found for alleles BoLA-DRB3.2*22 (16.8%), *8 (16.3%) and *16 (12.8%), these results are comparable with those found by Juliarena *et al.* (2008) where it was found that allele BoLA-DRB3.2*16 (14.7%) and allele BoLA-DRB3.2*22 (11%) had the highest allele frequencies, whilst allele BoLA-DRB3.2*8 presented a frequency of 7%. Alleles BoLA-DRB3.2*8 and *16 with frequencies of 26.6% and 9.6% respectively, are among the most frequent in the study made by Nassiry *et al.* (2005) and allele frequency of BoLA-DRB3.2*8 was the highest in the study, however Bo-Young *et al.* (2015) reported the highest frequency for allele BoLA-DRB3.2*16 (0.332), followed by allele BoLA-DRB3.2*8 (0.193) in Holstein cattle, also they identified a new allele (BoLA-DRB3.2*7601) for this breed. Miyasaka *et al.* (2011) found that allele BoLA-DRB3.2*16 was the most common in three of Japanese Holstein herds tested, our study indicates that susceptibility allele BoLA-DRB3.2*16 is one of the most common in dairy herds of Antioquia (12.8%), which may be related to the level of BLV infection.

In this study, allele BoLA-DRB3.2*8 (OR=1.489, P<0.10) and allele BoLA-DRB3.2*12 (OR=3.897, P<0.10) showed a significant association between absence of virus and its presence; according to their OR and P value both were classified as resistance alleles to BLV infection. Juliarena *et al.* (2008) also found that allele BoLA-DRB3.2*12 (OR=3.46, P <0.05) was associated with high proviral load resistance, while allele BoLA-DRB3.2*8 (OR=0.47, P <0.05) was classified as neutral and allele BoLA-DRB3.2*16 (OR=0.710, P<0.10) was classified as susceptible, as in the present study.

Mirsky *et al.* (1998) related allele BoLA-DRB3.2*11 with resistance to Persistent Lymphocytosis as tested cattle had fewer BLV infected B cells, moreover Miyasaka *et al.* (2011) also associated this allele with low proviral load, like allele BoLA-DRB3.2*22. In this study, allele *11 is one of the most common (7.8%) and its OR= 1,182 which would classify it as a resistance allele, however, there is not statistically significant difference (P>0.10) for which it was classified as neutral, but increasing sample size in research, this allele could be classified as of resistance. Juliarena *et al*. (2016) identified that allele BoLA-DRB3.2*11 interferes with the transmission of the virus, infected cows had this allele did not have the ability to infect the BLV negative cows in a herd of 100 animals. The virus is transmitted by contact between animals infected and uninfected sharing confined spaces (Mekata *et al*., 2015), through colostrum or milk. In addition, the BLV releases "noninfectious particles" through infected cells in milk (Yamada *et al*., 2013).

Other studies have classified alleles BoLA-DRB3.2*11, *23, and *28 with resistance to Leukemia in Russian Black Pied (Sulimova *et al.*, 1995) and Persistent Lymphocytosis in Holando Argentino (Panei *et al.*, 2009), Alleles BoLA-DRB3.2*24, *22 and *51 have been associated with resistance to Persistent Lymphocytosis in Iranian Holstein (Nikbakht *et al*., 2016). Alleles BoLA-DRB3.2*11 and *22 have been associated whit a low proviral load (Miyasaka *et al*., 2013). Data that are not comparable with those found in this study.

They have also conducted studies of resistance or susceptibility to BLV in other breeds in Colombia, Hernandez *et al.* (2014) established the relationship between alleles BoLA-DRB3.2*21, *24, and *37 with resistance to BLV infection in Colombian Creole Breed and alleles BoLA-DRB3.2*6 and *42 were identified as of susceptibility.

Cattle with an allele R (RR, RN or RS) was 31% of the studied population and, out of these, RR genotype was 4% of population (12/500), of which the largest numbers of cattle were in the municipalities of San Pedro and Bello; the municipalities of Belmira, La Union and Rionegro did not present animals with this genotype.NR genotype corresponds to 23% of total, out of these the 63.37% were negative for BLV, moreover 70% of animals

Table 3: NN, NR, NS, RR, RS, and SS genotypes for gene BoLA-DRB3.2

Municipality	NN		NR		NS		RR		RS		SS		Total		Total
	env -	*env+*	*env -*	*env+*	*env-*	*env+*	*env-*	*env+*	*env-*	*env+*	*env-*	*env+*	*env-*	*env+*	
Bello	38	13	22	2	11	1	6	0	4	0	1	0	82	16	98
Belmira	15	7	4	3	3	7	0	0	0	1	0	1	22	19	41
Entrerrios	30	34	22	12	5	10	1	2	2	3	2	0	62	61	123
La Union	4	7	7	3	3	5	0	0	1	2	0	0	15	17	32
Medellin	11	13	2	5	9	12	1	1	1	0	1	0	25	31	56
Rionegro	1	11	1	4	0	10	0	0	2	2	0	1	4	28	32
San Pedro	34	24	19	10	10	10	6	3	2	0	0	0	71	47	118
Total genotypes	133	109	77	39	41	55	14	6	12	8	4	2	281	219	500
Total	242		116		96		20		20		6		500		
Percentage	48		23		19		4		4		1				

Table 4: OR for NN, NR, NS, RR, RS, and SS genotypes of gene BoLA-DRB3.2

Genotype	(*env-*/*env+*)	OR	CI (95%)		Fisher
NN	(133/109)	0.930	0.685	1.262	0.655001
NR	(77/39)	1.559	1.025	2.369	0.0280965*
NS	(41/55)	0.592	0.375	0.936	0.0103068*
RR	(14/6)	1.714	0.587	5.007	0.160893
RS	(12/8)	1.169	0.470	2.910	0.461531
SS	(4/2)	1.559	0.283	8.588	0.468074

with RR combination, was negative for BLV infection, these data are comparable with those obtained by Juliarena *et al*. (2008) work in which 77% of the animals had the combination of alleles NR and 100% RR combination, they were associated with low proviral load of BLV. 57% of cows presenting NS genotype were positive to BLV and only 33% of which presented SS genotype, this percentage is much lower than the one found by Juliarena *et al*. (2008) which was 83.33%. 33.6% of cows with NR genotype were positive for BLV and 42.7% of cows with NS genotype were negative for BLV, which supports the idea of Juliarena *et al*. (2008) that there is a "large number of genetic and epigenetic factors that are involved in infection by Bovine Leukemia" in addition to alleles R, N, or S of BoLA-DRB3.2.

Conclusions: BLV infection level was established through nested PCR molecular technique, it was 44% in dairy cattle in the department of Antioquia. Alleles of gene BoLADRB3.2 were classified as Resistant, Neutral and Susceptible to BLV; Alleles BoLA-DRB3.2*8 and *12 were positively related to the disease and therefore cows have low risk of infection, unlike allele BoLA-DRB3.2*16 which was negatively related and animals have high risk for the disease. Most frequent alleles were BoLA-DRB3.2*22 (16.8%), BoLA-DRB3.2*8 (16.3%) and allele BoLA-DRB3.2*16 (12.8%). Interaction between the two alleles of gene BoLA- DRB3.2 is not set, as 70% of animals with RR genotype were negative for BLV, but only 33% of SS cows were positive to infection, so it follows that there is a set of interactions that are not clear, but allow an animal to be negative for BLV when its genotype is SS.

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Author's contribution: CU executed the experiment and analyzed the data. CU, JJE, and AL reviewed the results and content of the manuscript.

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