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The Proviral Load of the Bovine Leukosis Virus is Associated with the Polymorphisms of the BoLA-DRB3 Gene in the HartonDel Valle Breed

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Abstract

Objective: To associate the proviral load (PVL) of the bovine leukosis virus found in the Harton del Valle (HV) breed with the alleles of the BoLA-DRB3 gene. **Methods:** In 100 HV animals, the PVL was evaluated using a real-time quantitative PCR and a TaqMan probe; the animals were classified according to their PVL in high proviral load (HPVL) or low proviral load (HPVL). Additionally, the animals were genotyped using the PCR-SBT method. The allele frequencies were estimated. Alleles were associated with HPVL (susceptible, S), LPVL (resistant, R) or neutral (N) using the Odds ratio (OR) statistic and the presence of common amino acids in the R and S alleles were determined. **Findings:** A total of 22 alleles were found, the *1101 was the most frequent. High-frequency alleles accumulate 71.9%. The PVL mean was 129740 copies/µg of DNA. Seven alleles were associated with PVL, alleles *1002, *1601 and *1701 with HPVL (S) and alleles *0902, *1101, *20012 and *2703 with LPVL (R). The PVL of the alleles S and R was 516966 and 75074 copies/µg of DNA respectively. The allelic frequency R and genotype RR accumulated was higher than that of the alleles and genotypes S. The amino acid Glu located in positions 70 of the DRβ peptide chain was common in the R alleles. **Application/Improvements:** The resistance to the PVL (LPVL) has particularities in each breed since the alleles according to the literature reports are not the same. These results can be used in selection programs in favor of the genes categorized here as R, and thus, decrease the infection rate of the virus.

Keywords: Colombian Creole Cattle, Enzootic Bovine Leukosis, Genetic Resistance

1. Introduction

The enzootic bovine leukosis (EBL), is an infectious disease, the causative agent is the bovine leukosis virus (VLB) (BLV) is a B-lymphotropic oncogenic *deltaretrovirus* infecting cattle that shares common biological and structural features with the human T-cell leukemia virus I and II (HTLV-I and II)[1]. In the majority of cases, infection is asymptomatic but 30% of BLV-infected animals will develop a persistent lymphocytosis and less than 5% will progress to B-cell lymphoma or leukemia in animals older than 3 years, after a long period of latency characterized by the absence of viral replication[2].

BLV tropism in the host is directed primarily to B cells, has a reverse transcriptase responsible for the synthesis of a DNA copy from viral RNA (provirus). The provirus is integrated into the DNA of host lymphoid cells[3]. Infected cells, surviving host immune response clearance, undergo a tight mRNA BLV transcriptional silencing, promoting cellular transformation and BLV persistence/replication through mitotic division[4-5].

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The disease goes unnoticed, this fact masks the reality, the BVL generates productive and reproductive losses, besides, and it is immunosuppressive and allows the infection with other pathogens[6]. Worryingly, in heavily infected herds a drop in milk production to either individual/herd levels has been observed[7-9], delayed-interval delivery is extended and the number of services per conception are increased[10], milk protein decreases and the somatic cell count is increased[11] followed by premature culling, and replacement of cows has been reported[12-13].

Following the recommendations from OIE, many countries-initiated programs to control EBL with the aim of reducing economic losses and to reduce the spread of the disease by trade of breeding animals[2]. The disease prevention and control measures are directly linked to the herd management, the early elimination of infected animals is the most common[13]. The new strategies propose classifying animals from the proviral load (PVL) in peripheral blood[14], eliminating animals with high load (HPVL) and allowing the reproduction of animals with low proviral load (LPVL), since it has been determined that the PVL is directly related to the level of infection in vivo[5], is variable among individuals[15-16] and apparently stable for extended periods of time[17].

The genes that component the major histocompatibility complex or bovine leukocyte antigen (BoLA) has been associated with resistance and susceptibility to a wide range of diseases. The most common example of the relationship between the allelic variants BoLA gene has been established by the resistance to BLV, specifically with the class II gene BoLA-DRB3[13,15,18]. Allelic variants located in the exon 2 encoding the peptide-binding cleft (PBC) of the surface molecule responsible for the presentation of peptides[13]. The alleles of the DRB3 gene that code for conserved and polymorphic amino acid (AA) grouped into "pockets" side chain positions interacting with the antigenic peptide o be presented[13,15].

Specific AA variants in the BoLA-DRB3 PBC have been associated with resistance to the development EBL[13,19-21]. Previously we reported[22-23] that Harton del Valle (HV) breed could be considered a slow progression of the disease, the infection pattern is different, it does not develop persistent lymphocytosis and maintains high antibody titers with VLB, despite having a high prevalence of the virus. However, the association of PVL with alleles of the DRB3 gene has not been carried out in HV. Therefore, the objective of this investigation was to associate the PVL found in the HV race with the alleles of the BoLA-DRB3 gene.

2. Materials and Methods

2.1 Populations Evaluated and DNA Extraction

In this research, 100 adult animals of the HV breed were used, from which DNA was extracted from blood, using the Wizard® Genomic DNA Purification Kit from Promega. Only BLV positive animals diagnosed by nested PCR were amplified by amplifying the viral *env* gene, as reported by[23].

2.2 DRB3 Genotyping

Using the PCR-SBT methodology, in a final volume of 50µl with the primers DRB3FEW (5'-CGCTCCTGTGACCAGATCTATCC-3') and DRB3REV (5'-GGTGAGCGCGGGGGTG-3')[24] at a concentration of 10 mM, 25 ng of DNA and 1X de MangoMix™ (Bioline©) a fragment of approximately 281 was amplified. The thermal amplification cycle included a denaturation at 95°C for 5 minutes, followed by 37 cycles of 95°C for 45 seconds 61°C for 45 seconds and 72°C for 2 minutes and then a final extension step of 72°C for 5 minutes. The amplicons were sequenced in the MACROGEN Company, and edited using the GENEIOUS 6.1 program (Biomatters development team, USA). The genotypes were determined using HAPLOFINDER software (www.bioinfortmatics.roslin.ac.uk/haplofinder/haplofinder.py)[25].

2.3 PVL Evaluations

This quantification was performed by quantitative real-time PCR (Q-PCR-RT). From a sample of known concentration (9.6x1010 copies/µg of DNA), a standard curve was made from 0.1 to 1x10^7 copies/µg[21-22]. The quantification of PVL was performed by amplifying a 168 bp fragment using the primers CoCoMo6-5'-MNMYCYKDRSYKSYKSAYYT CACCT-3'and CoCoMo81-5›-TACCTGMCSSCTKSCG GATAGCCGA-3', the TaqMan probe (5'-FAM-CTCAGCTCTCGGTCCNFQ-MGB-3') 10ng of DNA, 2X of iTaq™ Universal Probes Supermix, 200mM of each primer and 200mM of the FAM probe in a final volume of 10µl (21)a disease characterized by a highly extended course that often involves persistent lymphocytosis and culminates in B-cell lymphomas. BLV provirus remains integrated in cellular genomes, even in the absence of detectable BLV antibodies. Therefore, to understand the mechanism of BLV-induced leukemogenesis and carry out

the selection of BLV-infected animals, a detailed evaluation of changes in proviral load throughout the course of disease in BLV-infected cattle is required. The aim of this study was to develop a new quantitative real-time polymerase chain reaction (PCR. Each standard of the curve and each sample were run in triplicate in a CFX96 "Real-Time System" thermocycler (BIO-RAD®) with the following program: 50°C for 2 minutes for the activation of the enzyme Uracil-DNA Glycosylase, 95°C for 10 minutes for the activation of the iTaq DNA polymerase, 85 cycles of 15 seconds at 95°C and 1 minute at 60°C. The average of the cycle to which the cut-off point (CT: cycle threshold) is crossed was used to define the result. The critical classification value of animals in high load (HPVL) and a low proviral load (LPVL) was the median (1x105 copies/µg).

2.4 Statistical Analysis

The number of alleles and frequencies were caculated with the Arlequin ver 3.5 program[26]. The alleles BoLA-DRB3 were associated with HPVL or LPVL in case-control association analysis was calculated as the odds ratio (OR) with a confidence interval of 95%. OR values close to 1 were considered as neutral (N), while, OR values greater than 1 indicate that these alleles are at high risk and were considered susceptible (S) (negative association), OR values less than 1 indicate that these alleles are at low risk and were considered resistant (R) (positive association)[13]. An exact Fischer test was performed to determine significance of OR values using SAS software version 9.1[23].

The allele frequencies accumulated of the alleles considered R, S, and N was estimated, as well as the accumulated genotypic frequencies, according to the classification of their alleles in N/N, N/R, N/S, R/R, R/S, and S/S.

3. Results and Discussion

In this investigation, 22 alleles were found in the BoLA-DRB3 gene, of which *1101 was the most frequent (16.88%). In this same breed, the number of alleles reported was 24 alleles[27], 27 alleles[23] and 37[28] alleles; additionally, these reports establish that the allele *1101 is the most frequent, which agrees with the present work. In Table 1, the allele frequencies calculated can be observed.

The alleles *20012, *0902, *1701, *1002, *2703, *1501 and *25011 showed allelic frequencies greater than 5% (Table 1), the remaining 14 alleles represent 28.13% of the accumulated frequencies.

In others autochthonous breads the number of alleles found was 22 in the Japanese Negro[29], 28 in Hanwoo[30], 35 in Yacumeno[27] and 71 in the native of the Philippines[31], while, in the breed Holstein's study of this gene has been wider with 18 alleles in Holstein from Argentina[32], 39 in Brazil[33], 37 in Holstein Irani[34], 17 in Holstein of Japan[29] and in Holstein different South American countries from 20 to 33 alleles[35]. The above shows the high genetic variation that this gene presents.

The PVL mean was 129740 copies/µg of DNA, the highest value was found in the animals carrying the allele *1002 (1112378 copies/µg) and the lowest in the carriers of the allele *2703 (733 copies/µg). The critical PVL value to consider the allele as HPVL or LPVL was the median, estimated at 6533 copies/µg of DNA. In that order of ideas, 11 alleles were considered HPVL of which only three (*1002, *1601 and *1701) were significantly associated with HPVL and were considered as susceptible (S). The PVL of the alleles associated with HPVL was 516966 copies/µg of DNA. While, 11 alleles were considered LPVL of which four (*0902, *1101, *20012 and *2703) were significantly associated with LPVL and were considered as resistant (R) and the PVL of these was from 75074 copies/µg of DNA (Table 1).

The cumulative frequency of the alleles associated with LPVL was 47.5%, followed by the frequency of the neutral alleles (35%) and by alleles associated with HPVL (17.5%). Alleles *0902 and *20012 associated with LPVL had HPVL; in contrast, none of the alleles associated with HPVL presented LPVL. The genotype frequencies according to the category of each allele and its PVL are presented in Table 2.

As in this report, the allele *0902 has-been associated with LPVL in Holstein[19,36], Argentine Holstein[13,32,37] and Japanese Black[36,38]. Whereas, in the other alleles associated here with LPVL, *1101 has-been associated with the susceptibility to the development of persistent lymphocytosis[19] but also, with the low infection rate of *Babesia bigemina* as well as the allele *20012[39]. On the other hand, the allele *2703 has-been associated with a high infection rate of *Babesia bigemina*[39]. This is the first association report of alleles *20012 and *2703 with LPVL. The alleles associated with HPVL reported in the literature include the alleles *0101, *1001 and *1101 *1201, *1501, *1503 and *1601[13,15,19,36-37], of which none of them presented here the same classification. In particular, the allele *1701 here associated with HPVL, was associated with LPVL by[37].

Table 1. Allelic frequencies, proviral load (PVL), OR value, confidence interval, Fischer P-value and classification of alleles found in race HV

Allele	Frequency	PVL Mean (copies/µg of DNA)	OR	IC (95%)		Fischer P-value	Classification
*0101	1.88%	907	1.48	0.77	7.45	0.2476	N
*0501	3.75%	52571	1.88	1.07	9.45	0.2143	N
*0701	1.25%	2453	1.44	1.01	9.40	0.4984	N
*0902	11.25%	7748	0.27	0.01	0.57	0.0004	R
*1002	5.63%	1112378	4.32	2.09	6.99	0.0173	S
*1101	16.88%	5318	0.32	0.11	0.51	0.0001	R
*1104	2.50%	4391	1.53	1.05	3.96	0.1226	N
*14011	0.63%	1084	1.38	1.15	7.41	1.0000	N
*1501	5.63%	353790	1.08	0.98	2.99	1.0000	N
*1601	4.38%	266037	6.42	4.09	8.75	0.0045	S
*1701	7.50%	172482	7.41	5.01	9.89	0.0230	S
*20012	13.75%	46495	0.71	0.45	0.99	0.0031	R
*2006	0.63%	2306	1.38	1.15	7.41	1.0000	N
*2201	1.88%	96965	1.96	0.20	8.94	1.0000	N
*25011	5.63%	701540	0.59	0.13	1.90	0.5015	N
*25012	0.63%	10022	1.38	1.15	7.41	1.0000	N
*2703	5.63%	733	1.78	1.07	9.82	0.0010	R
*2802	1.88%	1580	1.48	0.77	7.45	0.2476	N
*2902	2.50%	9325	1.53	1.05	3.96	0.1226	N
*3501	0.63%	2950	1.38	1.15	7.41	1.0000	N
*3601	2.50%	1991	1.53	1.05	3.96	0.1226	N
*3901	3.13%	1207	1.88	1.07	9.45	0.2143	N

Table 2. Accumulated genotypic frequency according to the classification given to each allele and the average PVL in each genotype

Item	N/N	N/R	N/S	R/R	R/S	S/S
Frequency (%)	25	22.5	17.5	18.8	7.5	8.7
PVL Mean (copies/µg of DNA)	82872	68599	155221	15074	230170	516966

The amino acids located in positions 70 and 71 of the DRβ peptide chain (motif ER: Glu-Arg) have been associated with low proviral load[13,19], the alleles considered here as resistant have the RR motif in these same positions, whereas, the susceptibles have the amino acids Ala, Glu and Lys in position 70 and Arg in position 71.

Other genes/alleles associated with PVL are BoLA-DQA1 *0204 that associates LPVL and that allele BoLA-DQA1 *10012 that is associated with HPVL[36]. It also indicates that the DRB3-DQA1 haplotypes can be correlated with the proviral load, thus the DRB3*0902-DQA1*0204 and DRB3*1101-DQA1*10011 haplotypes were associated with LPVL and the DRB3*1601-DQA1*10012 haplotype with HPVL[36].

Through other genotyping and association methodologies, using SNP50 K BeadChip, three SNPs were

associated with LPVL, two located on chromosome 23[40], in another study using BovineSNP50 v2 BeadChip, 24 SNPs located on chromosome 23 were associated with the LPVL[41], genes close to this region include immune response, cell cytoskeletal reorganization, and modeling of the extracellular matrix[42].

Of the homozygous genotypes, N/N was the most frequent followed by R/R and S/S, while in the heterozygous genotypes; the N/R presented the highest frequency followed by N/S and R/S (Table 2). Using this same genotyping system, it has been reported that the individuals N/N and S/S are the most and least frequent respectively against the infection against the EBL[22-22,37].

The S/S individuals had 34 times more PVL than the R/R individuals, this genotype had the lowest PVL, the genotypes that have at least one allele N allele had low proviral loads, even more, when it was homozygous or with the allele A. The R/S genotype had lower proviral load than the S/S genotype. These results suggest that low maintenance of the proviral load can be improved in heterozygous individuals, also called heterozygous advantage, as has been amply demonstrated against mastitis[25,43] and EBL[13,23,36].

Animals with LPVL disseminate EBL less frequently[44]; in addition, good PVL is a good predictor of its development[23], which indicates that selection in favor of alleles associated with LPVL could help control of the EBL.

4. Conclusions

The genetic diversity of the DRB3 gene in HV is high, determined by the number of genes and their frequencies. Seven of twenty-two alleles were associated with PVL, alleles *1002, *1601 and *1701 with HPVL and alleles *0902, *1101, *20012 and *2703 with LPVL. The PVL and the R alleles were significantly lower than in the S alleles. The cumulative frequency of the S alleles and the SS genotype was lower compared to the R alleles and genotypes with at least one resistance allele. As in other investigations, the presence of common amino acids between alleles with the same classification (R or S) was evidenced. These results can be used in selection programs in favor of the genes categorized here as R, and thus, decrease the infection rate of the virus.

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