



RESEARCH ARTICLE

BoLA-DRB3.2 Gene Polymorphism in Cattle Naturally Infected with Bovine Leukemia Virus

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ABSTRACT

This study was conducted to determine and evaluate the BoLA-DRB3.2 polymorphisms in bovine leukemia virus (BLV) seropositive cows by DNA Sequence Analysis. Isolates of DNA obtained from seropositive (25) and seronegative blood samples (3) collected from cows between 2005 and 2010 were used in this study. DNA isolation was performed from blood samples. MHC analyses were performed using primers belonging to BoLA-DRB3.2 genes. DNA sequence analyses were performed. The number and diversity of haplotypes and nucleotide diversity were calculated. The phylogenetic analysis was determined that cows had 11 haplotypes, healthy individuals were associated together in the first haplotype, while in other haplotypes seropositive cows were associated among themselves and there was 1 seropositive cow between haplotype 6 and 11. Also, the variety of haplotypes (Hd) (0.847), total nucleotide variety (π) (0.033), variation rate (0.00285), and Tajima's D statistical value (0.103) were detected. When F_{ST} of individuals were evaluated, it was revealed that healthy individuals were genetically distant from positive individuals. In this study, it was observed that BLV seropositive individuals differed genetically compared to healthy animals. In conclusion, BoLA-DRB3.2 gene polymorphism can be considered as a supporting parameter in BLV diagnosis.

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INTRODUCTION

Bovine Leukemia Virus (BLV) is a viral infection, common in various parts of the world, characterized by tumor formations in lymph nodes, hematologic changes, and an increase in the number of lymphocytes in cattle older than 2 years (Shaghayegh, 2019, Chen *et al.*, 2021). The disease causes economic losses due to loss of body condition, decrease in milk yield, and weight loss. BLV is part of the *Deltaretrovirus* sub-group of the *Retroviridae* family (Ruiz *et al.*, 2018, Marawan *et al.*, 2021). Proviral DNA formed after reverse transcription of the viral genome is integrated into the DNA of the host cell in which the virus remains persisted. Therefore, animals exposed to BLV host this virus throughout their lives. Proviral DNA remains persisted by integrating into target lymphocytes and blocks the expression of viral antigens. The BLV spreads with in

the host by the circulation of proviral DNA integrated lymphocytes. For this reason, the presence of serum antibodies against the structural proteins of BLV continues in cattle infected with BLV throughout their lives (Nishimori *et al.*, 2017). Persistent lymphocytosis (PL) form has been reported due to an increase in B lymphocytes in 30-70% of BLV seropositive cattle. No clinical symptoms are observed in cattle with leukosis during the period of seropositivity and PL (Juliarena *et al.*, 2008, Marawan *et al.*, 2021).

Antigen-presenting cells such as dendritic cells, macrophages, B lymphocytes with major histocompatibility complex (MHC) Class II on their surfaces communicate with T-helper cells via CD4+. These antigen-presenting cell molecules are called tissue compatibility molecules (THC) or tissue compatibility antigens. The genes that encode these molecules are located

in the gene locus called the MHC. MHC locus is found in a genomic region co-formed by gene and gene families with significant functions in the innate and adaptive immune response. There may be *polymorphism* in the amino acid sequence of MHC proteins. MHC genes are defined in three classes (MHC Class I, II and III) in all species. MHC Class I and Class II molecules are responsible for antigen presentation and have very high polymorphism and numerous allele variations in the population. MHC genes are localized in different chromosome regions in human and animal species. It is found on the 23rd chromosome in cattle MHC genes, which also show a wide distribution by species, have different names and it is called as Bovine leukocyte antigen (BoLA) in cattle (Bailey *et al.*, 2000).

The MHC class II region is located close to the centromere and contains HLA-DRA, -DRB, -DQA, -DQB, -DPA, -DPB, -DNA, -DMA, and -DOB loci. Three DRB genes have been identified: DRBP1, DRB2, and DRB3. Of these, DRB3 is the most polymorphic gene region. The BoLA-DRB3 gene product is a binding protein that participates in an antigen-antibody complex formation and has an important role in initiating and regulating the adaptive immune response. Two exons of DRB3 in cattle have attracted attention in recent years due to their role in immune response, its association with infectious diseases, its genetic diversity, and evolutionary history. Previous studies with different types of cattle breeds have reported that the highest degree of polymorphism occurs in the 2nd exon of the DRB3 gene on MHC Class II molecules. This intense structural polymorphism in Class II molecules is responsible for creating different individual immune responses to an infectious agent. A high degree of polymorphism in the DRB3.2 locus can help in determining superior haplotypes for resistance to diseases. Also, studies on MHC may contribute to the design and development of synthetic peptide-based vaccines containing one or more T-cell epitopes of pathogens. In genetic studies related to antibody production and MHC relationship in pigs, mice, chickens, and cattle, a strong relationship was observed between high or low antibody titer production and MHC in the immune response. Many studies have examined the relationship between replication of BLV in cattle and resistance or sensitivity to mastitis and variations of BoLA-DRB (Sharif *et al.*, 1998, Lo *et al.*, 2021).

There are two important reasons to focus on the BoLA-DRB 3 gene in cattle. Firstly, it is one of the key genes in the control of the organism against viral and bacterial infections and the second is that it has a high level of polymorphism (Chai *et al.*, 2019). This study was conducted in BLV seropositive cattle to examine polymorphism occurring in BoLA-DRB3.2 exons by using the DNA sequence analysis method.

MATERIALS AND METHODS

Samples: Blood samples taken from cattle brought to Selcuk University Veterinary Faculty between 2005 and 2010 confirmed seropositive (n=25) and seronegative (n=3) by ELISA for BLV (Leukosis Blocking Ab Test, IDEXX Laboratories, Inc., USA) were used for DNA isolation. The DNAs were isolated from blood samples as method by Sambrook *et al.* (1989). Isolated DNAs were stored at -80° C until analysed for polymorphism.

Quantification and qualification of samples: The concentrations of the samples were determined with NanoDrop and controlled with 1% agarose gel.

MHC analysis: Analyses were performed using the primers of BoLA-DRB3.2 genes located on the 23rd chromosome in the Class II region of bovine MHC genes. Primer names and sequences used in the study are listed in Table 1.

Amplification of BoLA-DRB3 Exon 2: Using the method reported by Van Eijk *et al.* (1992), the BoLA-DRB32 gene was amplified to obtain a 284 bp long product. Amplification was performed with primers HL030 and HL031 in the first step and with primers HL032 in the second step. The reaction mixture, which will have a final volume of 50 µl, was created from 48 µl of PCR mix (PCR buffer, 100 µM dNTP, 0.5 µM DRB3 primers and 1 unit of Taq Polymerase enzyme) and 2 µl of DNA. DNA samples were transferred to a Thermal Cycler (MJ Research PTC-200, USA) device and amplified in two steps with the Touchdown PCR protocol. PCR products were checked on a 1.5% agarose gel.

Cleaning of PCR products: PCR products (50µl) were cleaned by the manufacturer's (QIAquick PCR Purification kit) protocol. The quantity and quality of PCR products were controlled both by agar gel electrophoresis and Nanodrop ND-100 at 260/280 nm UV.

DNA sequence analysis: DNA sequence analyses were made according to the manufacturer's (Beckman Coulter DTCS Sequence kit) protocol. A total volume of 20 µl DNA sequence analysis reactions included 11 µl DTCS mix, about 100 fMol cleaned PCR product as a template, and 3.2 pMol primers (Table 1). Then, the DNA sequence analysis reactions were amplified using a thermal cycler in 30 cycles of denaturation (at 96°C for 1 min), second denaturation (at 96°C for 20 sec), annealing (at 50°C for 20 sec.), and elongation (at 60°C for 1 min.). DNA sequence analysis reaction products were first cleaned from free dNTPs, ddNTPs, enzymes, and chemicals with NaAc/Na2EDTA/Glycogen/EtOH precipitation and dissolved in 40 µl sample loading solution (SLS). The final products were then subjected to capillary electrophoresis in Beckman Coulter CEQ-8000 (Beckman Instruments Inc., USA) Genetic Analysis System using LFR-b (denaturation at 90°C for 2 min, injection at 2.0 kV for 15 seconds, separation with 57°C capillary temperature at 6 kV for 60 min). DNA sequence analysis forward and reverse readings were comparatively analyzed using the Sequencher 5.0 (Gene Code Corporation) program and contigs were obtained for each sample. With the help of the same program, they were compared with DNA sequences, and differences in DNA sequences were investigated. Possible heterozygotic DNA sequences were evaluated.

Statistical analysis: BoLA DRB3.2 gene region forward and reverse readings were aligned using the Bioedit (Hall, 1999) package program. Using the DnaSP program, the number of haplotypes, haplotype diversity, and nucleotide diversity were calculated (Rozas *et al.*, 2003). Circular Neighbor-Joining Tree (NJ) was obtained with the MEGA v6.1 software program (Tamura *et al.*, 2013). *F_{ST}* values

between positive and healthy individuals were created using the Arlequin v3.5 program (Excoffier and Lischer, 2010). By using these values, Neighbor-Joining Tree (NJ) was made with the Phylipand Tree View program.

Table 1: Primers for BoLA-DRB3.2 gene

Lokus	Primer sequences	PCR (bp)	Reference
HL030	ATCCTCTCTCTGCAGCACATTTC	284	Van Eijk et al. (1992)
HL031	TTTAAATTCGCGCTCACCTCGCCGCT		
HL031	TCGCCGCTGCACAGTAAACTCTC		

Table 2: Haplotype data created after sequence analysis of Bovine Major Histocompatibility System (BoLA) DRB3 exon 2 gene region.

Haplotype	Healthy	Positive
Hap 1	3	-
Hap 2	-	10
Hap 3	-	4
Hap 4	-	2
Hap 5	-	3
Hap 6	-	1
Hap 7	-	1
Hap 8	-	1
Hap 9	-	1
Hap 10	-	1
Hap 11	-	1

Table 3: Distance matrix of Bovine Major Histocompatibility System (BoLA) DRB3 exon 2 gene regions from seropositive and healthy animals according to Wright's *F* statistics (H: Positive, S: Healthy)

	S
H	0,069***

*, $P < 0.05$ (%) **; $P < 0.01$ (%) ***; $P < 0.001$ (%0.01).

Ethical approval: All procedures and animal care complied with the guidelines of the Selcuk University Veterinary Faculty Ethics Committee (Ethical approval number 2015/21 on 26/02/2015).

RESULTS

BoLA-DRB 3.2. gene amplification results: Amplification was performed with primers HL030 and HL031 in the first step and with primers HL030 and HL032 in the second step (Fig. 1).

DNA sequence analysis results: In a total of 25 seropositive and 3 healthy individuals, the phylogenetic analyses, conducted after the series analysis with the region in question, determined that the individuals had 11 haplotypes and healthy individuals were associated together in the first haplotype, while the seropositive individuals were associated among themselves in the other haplotypes, and one seropositive individual was placed between haplotype 6 and 11 (Table 2). In the analysis carried out separately, haplotype diversity (H_d) was found to be 0.847. The total variety of nucleotides (π) was 0.033; the variation rate was 0.00285; Tajima's D statistical value was 0.103 (Nei and Kumar, 2000; Rozas *et al.*, 2003).

F_{ST} values matrix and significance values (Arlequin v3.5) among breeds are given in Table 3 and the tree is shown in Fig. 3.

Using the F_{ST} of the individuals evaluated in the study and the tree in which the populations were evaluated as a total, it was observed that healthy individuals were genetically distant from seropositive individuals (Fig. 2) (Nei and Kumar, 2000; Excoffier and Lischer, 2010).



Fig. 1: Agarose gel image of BoLA-DRB3.2 gene amplicons after PCR purification of 8 samples from BLV seropositive cattle (M: Marker, S1-S8: Samples)



Fig. 2: Circular Neighbor-Joining Tree (NJ) (MEGA v6.1) that defines the genetic distance between seropositive and healthy individuals for Bovine Major Histocompatibility System (BoLA) DRB3 exon 2 gene region following sequence analysis. H: seropositive individuals, S: Healthy individuals

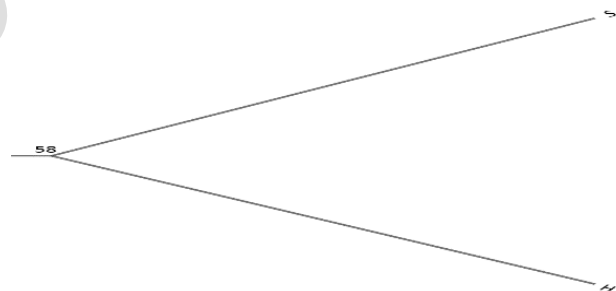


Fig. 3: NJ tree for F_{ST} distances of individuals (cladogram presentation of Phylip and TreeView program, H: seropositive individuals, Q: Healthy individuals)

DISCUSSION

The DRB3 gene region is highly polymorphic and includes a large number of different alleles, as shown by many studies (Peters *et al.*, 2018; Ahmed *et al.*, 2020, Suprovych *et al.*, 2020, Lo *et al.*, 2021).

This study indicates that the BoLA-DRB3.2 gene region could be used to identify animals resistant or susceptible to BLV according to polymorphisms of the gene region and the variety of haplotypes. Peters *et al.* (2018) evaluated the genetic diversity of DRB3 exon 2 (BoLA-DRB3.2) in African, American, and Asian cattle

breeds, a high number of breed-specific original haplotypes were identified in 174 cattle. In the same study, it was determined that the highest number of breed-specific haplotypes was 10 and they were found in all three cattle breeds studied (Brangus, Sokoto Gudali, and Dajal). It was also stated that the lowest number of haplotypes was 4 and they were found in the Holstein and Sahiwal cattle breeds.

In the present study, haplotype diversity (H_d) (0.847), total nucleotide diversity (π) (0.033), variation rate (0.00285), and Tajima's D statistical value (0.103) were calculated (Nei and Kumar, 2000; Rozas *et al.*, 2003). Nucleotide and haplotype diversity are the two most commonly used parameters in assessing the level of genetic diversity of populations or genes found in a population (Adefenwa *et al.*, 2013). A population's exposure to pathogens and their ability to respond immunologically are mainly dependent on the level of genetic diversity of MHC loci (Carignano *et al.*, 2017). Therefore, it is considered that high haplotype and nucleotide diversity of the DRB3.2 gene region will benefit the determination of resistance to diseases, a positive trait in livestock. Peters *et al.* (2018), accordingly with our results in the present study, reported high nucleotide and haplotype diversity. The negative Tajima D value (Tajima, 1989) indicates that rare alleles are in high number in the population studied and also points out that there are more variations than expected in the normal population. Therefore, in the samples studied here, the variation in BoLA-DRB3.2 was preserved. In this study, a high ratio of nucleotide diversity was linked to the high polymorphism rate of BoLA-DRB3 exon 2 regions, while positive Tajima's D statistical value was thought to be due to a small number of samples (Tajima, 1989).

The importance of DRB3.2 alleles as a potential marker for determining mastitis resistance or susceptibility in cattle was pointed by researchers (Kelm *et al.*, 1997, Yoshida *et al.*, 2009). The F_{ST} of the seropositive individuals in this circular tree were close to each other. A study by Juliarena *et al.* (2008) examined the relationship between BoLA-DRB 3.2 gene alleles and BLV infections. In particular, cows carrying alleles *0902 or *1701 were reported to have the ability to produce neutralizing antibodies, an indicator of sensitivity to viruses, against 3 bovine viruses and had no significant differences compared to other animals of the population. Xu *et al.* (1993) detected, in the amino acid chain of the BoLA-DRB 3.2 gene region, a significant difference between healthy animals and animals with PL.

In this study, the characterization of the 284 bp region of BoLA-DRB3 exon 2 revealed a high rate of nucleotide diversity and haplotype diversity f2009, which can be explained by the high rate of polymorphism of this gene region. After examining the haplotype structure of the BoLA- DRB3 gene region of BLV seropositive and healthy individuals, healthy individuals were found to be in the same haplotype while seropositive animal shad 10 different haplotypes. It was determined that 6 of these 10 haplotypes were original haplotypes. Furthermore, it was also shown that the seropositive and healthy individuals were completely separated from each other in the trees created by the Neighbor-Joining and F_{ST} distances. The condition of contracting infectious diseases or having any disease at different severity could be directly related to differences in the genetic characteristics of individuals. In line with this,

extensive research has been conducted to determine the possible relationship between the probability of the emergence of infections and the genetic traits of animals (Rupp *et al.*, 2007). Recent studies have focused on the BoLA-DRB2 exon 2 gene regions since they display a high ratio of polymorphisms specific to cattle. Studies aimed to determine the frequencies of BoLA-DRB2 exon 2 alleles in specific domestic breeds in various countries and to reveal the presence of new alleles (Othman and Ahmed, 2010).

In a study examining the relationship between BLV incidence and BoLA-DRB3 gene polymorphism in Holstein and Holstein x Jersey hybrid cattle, similar results with our study such as the high rate of polymorphism and the high number of alleles and haplotypes were mentioned (Carignano *et al.*, 2017). Miyasaka *et al.* (2013) reported that haplotype differences present on the DRB3 gene in Japanese Black cattle depended on differences in BLV proviral load.

Bai *et al.* (2015), following the characterization of BoLA-DRB3 through extensive screening of cattle infected with BLV, stated that this gene region contained a highly variable genetic profile. Juliarena *et al.* (2013) determined that the *0902 alleles identified on the BoLA-DRB3 gene may be associated with a low proviral load in cattle infected with BLV, however, the data were not considered to be sufficient to extrapolate for all resistant animals in all herds.

In conclusion, this study revealed that healthy and BLV seropositive animals differed genetically. Haplotypes carried by infected individuals can be used as a guide in herd health management and animal breeding. However, it is also important that more samples should be used from different diseases to make more decisive and proper conclusions.

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Authors contribution: The work presented here was carried out in collaboration between all authors. All author's reviewed and approved the final manuscript.

REFERENCES

- Adefenwa MA, Peters SO, Agaviezor BO, *et al.*, 2013. Identification of single nucleotide polymorphisms in the agouti signaling protein (ASIP) gene in some goat breeds in tropical and temperate climates. *Mol Biol Rep* 40:4447–57.
- Ahmed RO, Bello SF and Hegarty MJ, 2020. Identification of BoLA-DRB3.2 alleles present in White Fulani and Muturu cattle breeds. *Open J Anim Sci* 10:725-34.
- Bai L, Otsuki H, Sato H, *et al.*, 2015. Identification and characterization of common B cell epitope in bovine leukemia virus via high-throughput peptide screening system in infected cattle. *Retrovirology* 12:106.
- Bailey E, Marti E, Fraser DG, *et al.*, 2000. Immunogenetics of the horse. In: *The Genetics of the Horse* (Bowling AT and Ruvinsky A, eds.) CABI Publishing, New York, USA.
- Carignano HA, Beribe MJ, Caffaro ME, *et al.*, 2017. BoLA-DRB3 gene polymorphisms influence bovine leukaemia virus infection levels in Holstein and Holstein x Jersey cross breed dairy cattle. *Anim Genet* 48:420–30.

- Chai J, Shi X, Zheng X, *et al.*, 2019. Association of BoLA-DRB3.2 gene polymorphism with Tuberculosis susceptibility of dairy cattle in Chinese Holstein cattle. *J Tuberc* 2:1010.
- Chen YC, Chin WY, Chang CC, *et al.*, 2021. Potential Risk Factors Associated with Infection with Bovine Leukaemia Virus in Dairy and Beef Cattle in Taiwan. *Pathogens*, 10: 1553.
- Excoffier L and Lischer HEL, 2010. Arlequin suite ver 3.5: A new series of program stoper form population genetics analyses under Linux and Windows. *Mol Ecol Resour* 10:564–67.
- Hall TA, 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41:95-98.
- Juliarena MA, Lendez PA, Gutierrez SE, *et al.*, 2013. Partial molecular characterization of different proviral strains of bovine leukemia virus. *Arch Virol* 158:63–70.
- Juliarena MA, Poli M, Sala L, *et al.*, 2008. Association of BLV infection profiles with alleles of the BoLA-DRB3.2 gene. *Anim Genet* 39:432-38.
- Kelm SC, Detilleux JC, Freeman AE, *et al.*, 1997. Genetics and breeding: genetic association between parameters innate immunity and measures of mastitis in periparturient Holstein cattle. *J Dairy Sci* 80:1767–75.
- Lo CW, Takeshima SN, Okada K, *et al.*, 2021. Association of bovine leukemia virus-induced lymphoma with BoLA-DRB3 polymorphisms at DNA, amino acid, and binding pocket property levels. *Pathogens*, 10: 437.
- Marawan MA, Alouffi A, El Tokhy S, *et al.*, 2021. Bovine Leukaemia Virus: Current Epidemiological Circumstance and Future Prospective. *Viruses* 13: 2167.
- Miyasaka T, Takeshima SN, Jimba M, *et al.*, 2013. Identification of bovine leukocyte antigen class II haplotypes associated with variations in bovine leukemia virus proviral load in Japanese Black cattle. *Tissue Antigens* 81:72–82.
- Nei M and Kumar S, 2000. *Molecular Evolution and Phylogenetics*. Oxford University Press, New York.
- Nishimori A, Konnai S, Okagawa T, *et al.*, 2017. Identification of an atypical enzootic bovine leukosis in Japan by using a novel classification of bovine leukemia based on immunophenotypic analysis. *Clin Vaccine Immunol* 24: e00067-17.
- Othman OE and Ahmed S, 2010. Genetic Polymorphism of BoLA-DRB3 Exon 2 in Egyptian Buffalo. *Genes, Genomes and Genomics* 4:70-3.
- Peters SO, Hussain T, Adenaike AS, *et al.*, 2018. Genetic diversity of bovine major histocompatibility complex class II DRB3 locus in cattle breeds from Asia compared to those from Africa and America. *J Genomics* 12:88-97.
- Rozas J, Sánchez-DelBarrio JC, Messeguer X and Rozas R, 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19:2496–497.
- Ruiz V, Porta NG, Lomónaco M, *et al.*, 2018. Bovine leukemia virus infection in neonatal calves. Risk factors and control measures. *Front Vet Sci* 5:267.
- Rupp R, Hernandez A and Mallard A, 2007. Association of bovine leukocyte antigen (BoLA) DRB3.2 with immune response, mastitis, and production and type traits in Canadian holsteins. *J Dairy Sci* 90:1029–38.
- Sambrook J, Fritsch EF and Maniatis T, 1989. *Molecular cloning: A laboratory manual*. 2nd Ed., Cold Spring Harbor Laboratory Press, New York, USA.
- Shaghayegh AR, 2019. Detection and identification of enzootic bovine leukosis (EBL) in calves in Iran. *Arch Razi Inst* 74:321-25.
- Sharif S, Mallard BA, Wilkie BN, *et al.*, 1998. Associations of the bovine major histocompatibility complex DRB3 (BoLA-DRB3) alleles with occurrence of disease and milk somatic cells core in Canadian dairy cattle. *Anim Genet* 29:185-93.
- Suprovych TM, Suprovych MP, Kolinchuk RV, *et al.*, 2020. Association of BoLA-DRB3.2 alleles with fusobacteriosis in cows. *Regul Mech Biosyst* 11:249-54.
- Tajima F, 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 123:585–95.
- Tamura K, Stecher G, Peterson D, *et al.*, 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30:2725-729.
- Van Eijk MJT, Stewart-Haynes, JA and Lewin HA, 1992. Extensive polymorphism of the BoLA-DRB 3 gene distinguished by PCR-RFLP. *Anim Genet* 23:483-96.
- Xu A, Van Eijk MJ, Park C and Lewin HA, 1993. Polymorphism in BoLA DRB3 exon 2 correlates with resistance to persistent lymphocytosis caused by bovine leukemia virus. *J Immunol* 151: 6977-985.
- Yoshida T, Mukoyama H, Furuta H, *et al.*, 2009. Association of BoLA-DRB3 alleles identified by a sequence-based typing method with mastitis pathogens in Japanese Holstein cows. *Anim Sci J* 80:498-509.