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ANIMAL GENETICS

Distribution of *BoLA-DRB3* Allelic Frequencies and Identification of a New Allele in the Iranian Cattle Breed Sistani (*Bos indicus*)[1]

A. Mohammadi[a], M. R. Nassiry[a], J. Mosafer[a], M. R. Mohammadabadi[b,c], and G. E. Sulimova[c]

[a] *Animal Science Department, Ferdowsi University of Mashhad, P.O. Box: 91775–1163 Mashhad, Iran; e-mail: amohamadi57@yahoo.com*

[b] *Department of Animal Sciences, Kerman Shahid Bahonar University, Kerman, Iran*

[c] *Vavilov Institute of General Genetics, Russian Academy of Science, Moscow, 119991 Russia; e-mail: sge@vigg.ru*

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Abstract—The distribution of the frequencies of *BoLA-DRB3* gene alleles in the Iranian cattle breed Sistani was studied by the PCR-RFLP ("hemi-nested") assay using restriction endonucleases *Rsa*I, *Hae*III and *Bst*YI. In the examined cattle breed (65 animals) 32 alleles have been identified one of which being described for the first time (6.15% frequency). The nucleotide sequence of the polymorphic region of exon 2 of this allele has been determined and submitted in the GenBank database under accession number DQ486519. The submitted sequence has maximum homology (92%) with the previously described sequence *DRB3*-mRNA from *Bos indicus* (AccN X79346) and differs from it by 24 nucleotide substitutions which result in 16 amino acid substitutions. The peptide (on the basis of the reconstructed amino acid sequence) has 89% identity to the sequence encoded by the BIDRBF 188 locus (*Bos indicus*). The results obtained permit the sequence described by us to be considered as a new allele of the *BoLA-DRB3* gene (*DRB3.2*X*). The total frequency of the main six alleles (*DRB3.2*8, *10, *11*, **20*, **34* and **X*) occurring with a frequency of over 5% is about 60% in Iranian Sistani cattle. Fifteen alleles have <1% frequency. The highest frequency was observed for *DRB3.2*8* allele (21.54%) like in other previously described breeds of *Bos indicus* (up to 23.07%). The Iranian breed Sistani has a high level of similarity by the spectrum of *BoLA-DRB3* alleles and their frequencies to other *Bos indicus* breeds and significantly differs by these criteria from the *Bos Taurus* breeds. The Iranian Sistani herd under study includes alleles associated with to resistance to leukemia (*DRB3.2*11* and **23*) and to different forms of mastitis (*DRB3.2*2*, **7*, **11*, **23* and **24*) although their frequencies are low (from 0.77 to 5.37%). On the whole, a high level of diversity of *BoLA-DRB3* gene alleles and the availability of alleles associated with resistance to different diseases makes this breed of interest for breeding practice.

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The bovine leukocyte antigen (BoLA) system is the major histocompatibility complex (MHC) of cattle. The genes located in the MHC class II region encode glycoproteins that are composed of α- and β-chains and are expressed on the surface of antigen-presenting cells [1]. These molecules bind foreign antigen and present them to specifically stimulate helper T-lymphocytes. The extensive structural polymorphism of the class II molecules is considered to be responsible for differences among individuals in immune response to infectious agents. It is predominantly confined to distinct structural elements lining the antigen-binding cleft [2], which is encoded, by the first domain exon (second exon) of the α- and β-chains genes. The polymorphism of the proteins genetically corresponds to a large number of alleles. Allele polymorphism of the *BoLA-DRB3* gene is maintained due to the polymorphism of exon 2 encoding the β1 domain. High polymorphism of the region is necessary for binding a broad spectrum of foreign antigens.

The use of molecular genetic methods of studying MHC allowed being characterized allele polymorphism for locus *BoLA-DRB3*. To date 54 alleles have been identified by restriction enzyme digestion of a 284-bp PCR product of *DRB3* exon 2 and 103 alleles have been identified by PCR sequence-based typing (SBT) [http://www.projects.roslin.ac.uk/bola/drb3pcr.html]. Until now, allelic polymorphism for this gene has been studied in the Holstein-Friesian breed (30 alleles detected) [3], Holstein breed (22 alleles) [4, 5], Black Pied cattle (21–22 alleles) [6, 7], Argentine Creole cattle (21 alleles) [8], Iranian Holstein [9], Ayrshire (18 alleles) [7] and other different breeds and related species. Associations between *BoLA-DRB3* alleles or linked genes and several infectious diseases have been reported [3–5, 10, 11].

The study of the genetic diversity of native breeds is necessary for conservation of genetic resource in live-

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stock. However most of such studies have been done on European cattle breeds and very little information is available concerning the genetic polymorphism cattle breeds native Iran. Iranian Sistani cattle are a heavy built breed and used as dual-purpose cattle breed in Eastern Iran. This black-in-color breed, that is also native to Pakistan and Afghanistan, is a genetic resource that shows special features of adaptation to rustic environments. Such characteristic has become a biotype of great interest for the meat production industry within the last few years. One of the most distinctive features of Sistani cattle is its great capability to resist diseases which makes it a potential reservoir of germplasm useful for future crosses. Therefore, it is important to know the genotypic characteristics of some of the loci from its bovine MHC, because definite alleles of the MHC genes are associated with the resistance and susceptibility to diseases. The aim of this work was to study DNA-polymorphism of the *BoLA-DRB3* gene in the Iranian Sistani native cattle (*Bos indicus*).

MATERIALS AND METHODS

Blood Samples ($n = 65$) were collected from the Iranian Sistani herd in Zehak Research Station located at Zabol (south-east of Iran). Whole blood (100 µl) was used as source for DNA, which was extracted by a modified guanidine isothiocyanate—silica gel method [12].

Hemi-nested PCR was used for the amplification of the second exon (284 bp) of the *BoLA-DRB3* gene, essentially as described by van Eijk et al. [13]. Briefly, the first PCR stage was performed in a final volume of 20 µl containing 40 ng of template DNA, 10 pmol of each primer (HL030: 5'-ATCCTCTCTCTGCAGCACATTTCC-3' and HL031: 5'-TTTAATTCGCGCTCACCTCGCCGCT-3'), PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.25 mM of dNTPs, and 1 U of *Taq* DNA polymerase. Amplification was started from DNA denaturation at 94°C for 3 min. followed by 10 cycles with denaturation (94°C for 25 s), annealing (60°C for 30 s) and elongation (72°C for 30 s) and a final extention at 72°C for 5 min. Subsequently, 2 µl of the first-stage PCR product was used as template DNA for the second-stage PCR in a final volume of 20 µl containing 10 pmol of each primer (HL030: 5'-ATCCTCTCTCTGCAGCACATTTCC-3' and HL032: 5'-TCGCCGCTGCACAGTGAAACTCTC-3'), 1U *Taq* DNA polymerase, and the remaining components in the concentrations stated above. The initial denaturation (94°C for 2 min), was followed by 25 cycles of denaturation (94°C for 40 s), and annealing-extention (65°C for 30 s) and a final extention (72°C for 5 min). Contamination and self-priming controls were included in each PCR round and 5 µl of the last PCR product were electrophoresed on 1.5% agarose gels in order to check the quality and specificity of DNA fragment amplification.

To examine the nucleotide sequence variability at the *DRB3.2* locus, three restriction enzymes, *Rsa*I, *Hae*III, and *Bst*YI were chosen based on their ability to cut DNA in this exon [13]. Aliquots (10 µl) of the second-stage PCR product (284 bp) were digested with the restriction enzymes according to manufacturer's instructions. The resulting DNA fragments were separated on 8% PAGE gels, using *Msp*I-digested pUC19 and DNA ladder 50-bp as molecular size markers. After ethidium bromide staining, the gels were photographed under UV light with a GAS 9000 Gel Documentation System (UVItech, UK) and the relative migration of the DNA bands was estimated.

The restriction patterns obtained were compared with previously described restriction maps (http://www.projects.roslin.ac.uk/bola/drb3pcr.html). The nomenclature for the alleles of *BoLA-DRB3* defined by the PCR-RFLP method is indicated by the format locus.exon.allele, (*DRB3.2*3001*) or by showing the number of allele in up to two digit format (*DRB3.2*3001* = *34). Allelic frequencies were computed using PopGene software version 3.1.

Those amplicons produced new pattern of RFLP were sequenced using Applied Biosystems Company's 3730XL Capillary DNA sequencer machine. The obtained sequence of new pattern was analyzed using the program Chromas version 1.45 (Conor McCarthy, Australia). Afterwards, they were compared to the *DRB3* alleles published by the *BoLA* Nomenclature Committee using the program BLAST Nucleotide-nucleotide from National Center for Biotechnology Information (http://www.ncbi.nih.gov) and MegAlign version 5.06 (DNASTAR Inc.).

RESULTS AND DISCUSSION

DNA polymorphism of the bovine lymphocyte antigen (*BoLA-DRB3*) gene was to study in the Iranian cattle breed Sistani. In the herd studied, 31 *BoLA-DRB3.2* alleles out of the 54 previously reported were detected (table). Besides that we detected a new RFLP pattern combination (*lda – Rsa*I/*Bst*YI/*Hae*III) which has not been reported before.

The nucleotide sequence of the polymorphic region of exon 2 of this allele was determined and submitted in the GenBank database under accession number DQ486519. The submitted sequence had maximum homology (92%) with the previously described sequence DRB3-mRNA from *Bos indicus* (AccN X79346, [14]) and differred from it by 24 nucleotide substitutions which resulted in 16 amino acid substitutions (figure). The sequence of the new pattern also differed from complete CDs of *Bos taurus* mRNA for MHC class II DRB3 with accession number Z36542 [15]. The peptide (on the basis of the reconstructed amino acid sequence) had 89% identity to the sequence encoded by the BIDRBF 188 locus (*Bos indicus*). The results obtained permit the sequence described by us to be considered as a new allele of the *BoLA-DRB3* gene (*DRB3.2*X*).

Frequencies of *BoLA-DRB3* alleles in Iranian Sistani cattle

Allele sequence	*DRB3* PCR-RFLP	*Rsa*I/*Bst*YI/*Hae*III pattern	Frequency, %
*DRB3*0501,DRB3*0503*	01	aaa	1.54
*DRB3*1301*	02	bba	0.77
*DRB3*1001, DRB3*1002*	03	bbb	0.77
No sequence	04	caa	0.77
*DRB3*2201, DRB3*2202*	06	daa	0.77
*DRB3*0201*	07	ecc	0.77
*DRB3*1201*	08	faa	21.54
*DRB3*0301, DRB3*0302*	09	fda	0.77
*DRB3*1601, DRB3*1602*	10	fba	9.98
*DRB3*0901, DRB3*0902, DRB3*1202*	11	gea	5.38
*DRB3*20011, DRB3*20012, DRB3*2002, DRB3*2003*	15	iba	3.08
*DRB3*2601*	19	sbb	0.77
*DRB3*2301, DRB3*2901, DRB3*3601*	20	lbb	9.98
*DRB3*0801*	21	lbe	0.77
*DRB3*2701, DRB3*2702, DRB3*2703, DRB3*2705, DRB3*2706, DRB3*2707*	23	nba	1.54
*DRB3*0101, DRB3*0102*	24	nbb	2.31
No sequence	25	oaa	0.77
*DRB3*0601*	26	oab	1.54
*DRB3*14011, DRB3*14012, DRB3*3101*	27	obf	0.77
*DRB3*4101*	29	pcc	4.62
*DRB3*2801*	31	ibf	0.77
*DRB3*3001, DRB3*3002*	34	lab	6.93
*DRB3*2101*	35	cbb	1.54
DRB05	36	lba	0.77
No sequence	39	tba	0.77
No sequence	40	uba	1.54
*DRB3*25011*	44	kbi	3.85
*DRB3*3501*	46	vba	2.31
*DRB3*1703*	47	waa	0.77
*DRB3*3901*	48	wba	0.77
*DRB3*4201*	51	gaa	4.62
New allele	X	lda	6.15

Note: DNA-patterns, numbers of alleles and their nucleotide sequences are presented according to the data published on the website http://www.projectys.roslin.ac.uk/bola/drb3pcr.html. Standard errors of the allele frequencies do not exceed 5%.

(a)

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          81
X79346    CGAGTGTCAT TTCTTCAACG GGACCGAGCG GGTGCGGTTG CTGGACAGAC ACTTCTATAA TGGAGAAGAG TACGTGCGCT 160
DQ486519  --------.. .......G.. .......... .........C .........T .....C.... .......... .T........  72

          161                                                  Hae III                          Bst YI
X79346    TCGACAGCGA CTGGGGCGAG TACCGGGCGG TGACCGAGCT GGGGCGGCCG TCCGCCGAGC ACTGGAACAG CCAGAAGGAG 240
DQ486519  .......... .......... .T........ .......... .....____. GA.......T .......... .....___T 152

          241              Hae III
X79346    ATCCTGGAGC GGAGGCGGGC CGAGGTGGAC AGGGTGTGCA GACACAACTA CGGGGTCGGT GAGAGTTTCA CTGTGCAGCG 320
DQ486519  C.G......G A..A..... ..C....... .CCTG..A.. ..T..T.... ...C....-- ---------- ---------- 210
                                          Rsa I
```

(b)

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          31
X79346    FFNGTERVRL LDRHFYNGEE YVRFDSDWGE YRAVTELGRP SAEHWNSQKE ILERRRAEVD RVCRHNYGVG ESFTVQRRVE  110
DQ486519  ..S.....F. ...Y.H.... .......... .......... D..Y.....D L..EK..A.. TC.RYY...- ----------   69
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Alignment of the nucleotide sequence of exon 2 of the new pattern (accession number DQ486519) identified in this study (a) and the predicted amino acid sequences (b). Dotted lines indicate sequence identity with respect to the *B. indicus BoLA-DRB3* mRNA (accession number X79346). Alignment does not cover the downstream and upstream of reference sequence. Sites of the restriction endonucleases *Rsa*I, *Hae*III and *Bst*YI are underlined.

This is the first study on *BoLA-DRB3* gene of Iranian Sistani cattle. Thus, investigation of DNA polymorphism for *BoLA-DRB3* gene in the breed studied may be essential in practice, as well as theoretical value. Comparison between the present results with the spectrum of *BoLA-DRB3* alleles and their frequencies to other *Bos indicus* breeds previously reported in *Bos indicus* showed that they had a high level of similarity. Four alleles (*DRB3.2*47, *34, *45* and **51*) were only found in Sistani and Zebu Brahman cattle [16]. The most frequently detected *BoLA-DRB3* alleles of Kenya Boran cattle [14], Caracu [17] and Saavedreno Creole [18] was *DRB3.2*8*. In our study, the same results were observed for Sistani cows, the frequency of the allele *8 is 21.54%. Similarly, Miretti and coworkers [17] showed that *BoLA-DRB3.2*8* accounted for 23.07% of the alleles in a population of Caracu cows in Brazil. In contrast, the allele **8* had a frequency of 0.70, 2.94 and 3.45 on Brazilian dairy Gir breed [19], Curraleiro and Argentinean Creole [17], respectively. Four most frequently presented alleles in Gir cows were *BoLA-DRB3.2*16, *20, *2* and **29* and these accounted for about 51.7% of the alleles in this population [19]. In the studied Iranian cattle other four of 32 detected alleles (*BoLA-DRB3.2*8, *10, *20* and **34*) had the most frequent alleles and their cumulative frequency was 48.4%.

More significant distinctions have been found between Iranian Sistani cattle and *Bos taurus* breeds studied. Polymorphism of the *BoLA-DRB3* gene has been reported in the studies of Holstein-Friesian [4, 9]; Jersey [20]; Japanese Black, Japanese Shorthorn, and Japanese Jersey cattle [21, 22]; Russian Ayrshire and Russian Black Pied cattle breeds [6, 7]. All the breeds differed in the allele frequency profile. For example, the six most frequently detected alleles in Jersey cows were *BoLA-DRB3.2*8, *10, *15, *21, *36,* and **ibe*, accounting for approximately 74% of the alleles in the population of the herd (172 animals) [20]. The other six most frequently detected alleles (*BoLA-DRB3.2*8, *9, *21, *27, *7*, and **24*) accounted for 70% of the alleles in a population of Japanese Shorthorn cows [22]. The combined frequency of the alleles *DRB3.2*7, *8, *10, *24* and **28*, is 77% in Russian Ayrshire cattle [7]. Alleles **22, *24, *11, *16, *18, *23, *8* and **27* were the most frequent in Russian Black Pied cattle [7].On the other hand, the most frequently detected *BoLA-DRB3.2* alleles of 65 Sistani cows were *BoLA-DRB3.2*8,*10, *11, *20, *34* and **X*. The allele *DRB3.2*7DRB3.2*7* which is classed with rare alleles in Sistani cattle is prevalent (37.6%) in Russian Ayrshire cattle [7].

Associations between *BoLA-DRB3.2* alleles and several infectious diseases have been reported. Alleles *BoLA-DRB3.2*16* and **22*, which were associated with a lower risk of cystic ovarian disease in Holstein dairy cows [3], were not seen in Sistani cattle. We observed the alleles **2, *7, *11, *23* and **24* with the frequencies from 0.77 to 5.37, which reported to have a favourable effect on mastitis resistance [3, 4, 23]. The allele *DRB3.2*16* which identified as the allele decreased risk of mastitis in Canadian Holsteins [3], was not detected in our study.

The Iranian cattle breed Sistani is native to Pakistan and Afghanistan. It is a genetic resource that shows special features of adaptation to rustic environments and a great capability to resist on diseases. The alleles associated with to resistance to leukemia and to different forms of mastitis were found in the Iranian Sistani herd under study. The further analysis needs to be conducted on allelic patterns reported in this study to determine their association with some diseases. On the whole, a

high level of diversity of *BoLA-DRB3* gene alleles and the availability of alleles associated with resistance to different diseases makes this breed of interest for breeding practice.

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