Journal of Cell and Animal Biology Vol. 5(3), pp. 47-52, March 2011
Available online at http://www.academicjournals.org/JCAB
ISSN 1996-0867 ©2011 Academic Journals

Full Length Research Paper

Polymorphism of *BoLA-DRB 3.2* gene in Iranian native cattle by using PCR-RFLP method

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Accepted 11 March, 2011

Enhancing disease resistance in animal production can be achieved by genetic improvement programs. The best characterized genetic control of disease resistance and immune response in animals is that associated with the MHC. Respond to specific antigens is closely related to MHC genes and their associations might therefore provide precious answers to main questions about the host pathogen interactions. In cattle there is an association between possession of certain *BoLA-DRB3* locus (MHC class II) and resistance or susceptibility to infectious diseases. Polymorphism of exon 2 of the *BoLA-DRB3* gene was investigated by the PCR-RFLP method in 80 Iranian native cattle. In this study, 39 different alleles were recognized. As a second goal, allelic frequencies were determined in a total sample. The high frequencies of these alleles (gbb, fbd and fba) were 8.75, 8.75 and 6.25% respectively. Additionally, *BoLA-DRB3.2*11*, DRB3*2701 and DRB3*0701 were associated with resistant to BLV, whereas allele DRB3.2*01 was associated with higher stability to FMD. Finally, DRB3*2701 and DRB3*1101 alleles were also associated with higher mastitis risk. Frequencies of alleles mediating resistance and susceptibility to leukemia and FMD were revealed.

Key words: Iranian native cattle, MHC, *BoLA-DRB3*, PCR-RFLP.

INTRODUCTION

For a long time now, livestock breeders have given main emphasis on improvement of production traits with little or no attention for improvement of disease resistance traits (Kumar et al., 2008). Bovine lymphocyte antigen (*BoLA*) genes, also called bovine major histocompatibility complex (MHC) have received attention because of their association with host immunity (Takeshima et al., 2002). The *BoLA* genes are located on the short arm of bovine chromosome 23 and have been divided into three areas including Class I, II and III. The Class II MHC genes of cattle appear to be further divided into two distinct regions regions (Nassiry et al., 2005). The *BoLA* Class IIa region includes: *DRA*, *DRB*, *DQA* and *DQB* genes which encode the classical peptide presenting Class II molecules (DR and DQ) in cattle (Andersson and Davies, 1994; Lewin, 1996). Three *DRB* genes have been identified: DRBP1, DRB2 and DRB3. DRBP1 is evidently a pseudogene and functional expression of DRB2 has not been found whereas DRB3 is functionally expressed and highly polymorphic (Rupp et al., 2007). The *BoLA-DRB3* gene product is a binding protein involved in the formation of an antigen–antibody complex, and plays an essential role in the induction and regulation of acquired immune response (Rupp et al., 2007). The second exon of the third DRB bovine gene (*BoLA-DRB3.2*) and its orthologous genes in humans and sheep has attracted attention because of its role in the immune response, its relationship to infectious disease and its genetic variability and evolutionary history (Miretti et al., 2001).

Associations between *BoLA-DRB3* alleles or linked

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Abbreviations: *BoLA*, bovine lymphocyte antigen; **BLV,** bovine leukemia virus; **FMD,** foot-and-mouth disease.

genes and several infectious diseases have been reported (Dietz et al., 1997; Maillard et al., 1999; Sharif et al., 1999). Several studies have reported a relationship between Class II DRB variants and resistance or susceptibility to mastitis (Dietz et al., 1997; Sharif et al., 1998) with replication of bovine leukemia virus in cattle (Xu et al., 1993) and sheep (Konnai et al., 2003) and with protection induced by peptide vaccines against foot-and-mouth disease in cattle (Rupp et al., 2007; Garcia-Briones et al., 2000). Analysis of the *BoLA-DRB3* gene in cattle is of special interest at least for two reasons: a high functional importance of the gene (one of the key genes controlling the immune response of the organism to viral and bacterial infections) and a high level of polymorphism (Lewin, 1994; Mota et al., 2002). The first studies used restriction fragment length polymorphism of amplified DNA fragments (PCR-RFLP) for *BoLA-DRB3* gene assignments Van Eijk et al. (1992), (Van Eijk et al., 1992; Gelhaus et al., 1995).

To our knowledge, there are no studies on *BoLA-DRB3* genotyping in Iranian native breed in south west of this country; thus, the aim of the present study was to using PCR-RFLP to identify genotypes and allele frequency of the *BoLA-DRB3* gene from native cattle breed from Chaharmahal Va Bakhtiari province in south west of Iran.

MATERIALS AND METHODS

DNA extraction

Blood samples (approximately 8 to 10 mL) were obtained from the tail vein of 80 native cows into a vacutainer tube containing and stored in 10% of 0.5 *M* EDTA-coated vacutainer tubes (BD Vacutainer Systems, Plymouth, UK). The samples were collected from different villages and other regions of Chaharmahal Va Bakhtiari province in south west of Iran. Genomic DNA was isolated from white blood cells using DNA extraction kit (Qiagen, Germany), accordance to the protocol of the manufacturer. The total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell (2001).

Amplification of *BoLA-DRB3* Exon 2

The second exon of the *BoLA-DRB3* gene was amplified by seminested PCR. The isolated DNA was used for PCR amplification of the DRB3 gene fragment of 284 bp with the use of the following primers proposed by Van Eijk et al. (1992). Primers for first step PCR were MHC-F1: 5´-ATCCTCTCTCTGCAGCACATTTCC-3´ and MHC-R1: 5´-TTTAAATTCGCGCTCACCTCGCCGCT-3´. The forward primer of second step amplification was same as in the first stage and second reverse primer was MHC-R2: 5´-TCGCCGCTGCACAGTGAAACTCTC -3´. Reactions were carried out in a final volume of 25 µL. Each 25 µL PCR reaction contained 100 µL DNA, 0.2 pM of each primer, 1X PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs and 1 unit of Taq DNA polymerase (Fermentas, Germany). The first round of PCR with primers MHC-F1 and MHC-R1 was performed under the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 59°C for 1 min and elongation at 72°C for 1 min. The program was followed by a final elongation at 72ºC for 5 min. 2 to 5 µl from the first round amplicon was used as a template for the second round PCR.

The constituents of the second round PCR mixtures were the same as those described above, except that reverse primer.

Restriction endonuclease digestion

The restriction analysis of the PCR-amplified products was performed with restriction endonucleases *Rsa*I, *Psu*I and *Hae*III (Van Eijk et al., 1992) according to the manufacturer's instructions. The digestion products were resolved in 12% polyacrylamide at 200 V for 4 to 5 h. A 100 bp DNA ladder (Fermentas, Germany) was used as a DNA size marker. The digestion fragments were visualized in a 2% agarose gel by electrophoresis and "ethidium bromide" staining (Sambrook and Russell, 2001). The *BoLA-DRB3.2* nomenclature, described by Van Eijk et al. (1992) was followed to identify the different allele types obtained in the present study from the different restriction enzyme patterns (Behl et al., 2007).

Statistical analysis

The frequencies of alleles were estimated by direct counting. 95% confidence intervals for the observed genotype frequencies were calculated. Correspondence between the observed and expected distributions of genotypes was checked by the $\chi 2$ test. Computer analysis of the data was made using the software packages POPGENE 3.1 and STATISTICA 6.0.

RESULTS

284 bp seminested PCR amplification resulted in DNA bands of the expected size as shown in Figure 1. After digestion with restriction endonuclease *Rsa*I, *Psu*I and *Hae*III genotypes of *BoLA-DRB3* gene in Iranian native cattle were determined. The restriction pattern analyses used the patterns described by Van Eijk et al. (1992) of which we detected 18 of the 25 *Rsa*I patterns (a, b, c, e, f, g, h, i, j, k, l, m, n, o, p, r, s, t), 8 of the 9 *Hae*III patterns (a, b, c, d, e, f, g, i) and detected all 5 *Psu*I patterns (a, b, c, d, e). The distribution and frequencies of restriction patterns in Iranian native cattle breed are showed in Table 1. Analysis of the *BoLA-DRB3.2* allele fingerprints of 80 native cattle in the present study resulted in the identification of 39 *BoLA-DRB 3.2* alleles. These genotypes are aaa, ecc, faa, fba, fbb, fbd, gaa, gab, gba, gbb, gea, haa, iba, jab, jac, jad, jba, jbb, jbd, lba, lbe, lbf, maa, mba, nba, nbb, nbf, oaa, oab, oba, obb, obf, pcc, pec, red, sbf, sab, tbd and tcc. The restriction patterns of each enzyme showed in Table 2. Of these, 24 alleles were similar to those reported in earlier studies (Van Eijk et al., 1992; Maillard et al., 1999; Nassiry et al., 2005). The remaining 15 alleles (DRB3.2 *fbb, *fbd, *gab, *jbf etc) had not been reported in studies carried out previously.

The new alleles comprised only 38.46% of the total number of the observed alleles in Iranian native cattle. 3 alleles (gbb, fbd and fba) of 39 alleles have a high frequency

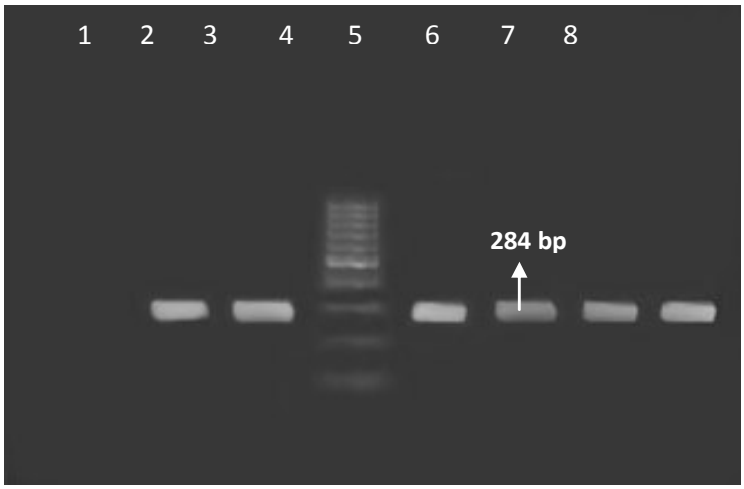

Figure 1. Identification of *BoLA-DRB 3.2* gene by seminested-PCR amplification. Line 1: negative control, Line 2: positive control, Lines 3, 5, 6, 7, 8: are positive samples. Line 4: 100 bp DNA ladder (Fermentas).

Table 1. Distribution and frequencies of restriction patterns in Iranian native cattle breed with using PCR-RFLP method.

***Rsa*I**	***Psu*I**	***Hae*III**
a (4)	a (57)	a (41)
b (1)	b (76)	b (51)
c (2)	c (9)	c (16)
e (7)	d (3)	d (21)
f (20)	e (15)	e (10)
g (12)		f (18)
h (13)		g (2)
i (4)		i (1)
j (27)		
k (2)		
l (14)		
m (5)		
n (14)		
o (13)		
p (9)		
r (2)		
s (5)		
t (6)		
Total = 160	Total = 160	Total = 160

with 8.75, 8.75 and 6.25% respectively.

DISCUSSION

The *BoLA-DRB3.2* locus in the native breed of cattle is highly polymorphic and the functional roles of the *BoLA-DRB3.2* gene alleles are different (Ruzinaa et al., 2010). The results of this study showed that the *BoLA-DRB3* locus was highly polymorphic in native cattle of Chaharmahal Va Bakhtiari province in south west of Iran. Combinations of these polymorphic restriction sites give rise to the restriction patterns reported for each enzyme, namely 25, 5 and 9 for *Rsa*I, *Psu*I and *Hae*III respectively (Van Eijk et al., 1992; Gelhaus et al., 1995; Russell et al., 1997). DNA fragments whose restriction patterns present subtle size differences resulted in overlapping DNA bands after electrophoresis, which hampered the identification of 18 *Rsa*I, 5 *Psu*I and 8 *Hae*III restriction patterns in this work. As an example, it was assumed that *Rsa*I restriction patterns in 22 different fragments (band size in 3 to 284 bp), 7 different fragments for *Psu*I (band size in 3 to 284 bp) and 13 different fragments for *Hae*III (band size in 4 to 219 bp) were identified. This present study were determined 19, 7 and 13 fragments in 18, 5 and 8 restriction patterns for *Rsa*I, *Psu*I and *Hae*III respectively. By contrast, in the present study, 23.75% of the alleles were accounted for by the 3 alleles (DRB3.2*10, DRB3.2*New and DRB3.2*Nassiri). The alleles showed in Table 3 are different and new. The remaining alleles were present at lower frequencies (Table 3). Using the $\chi 2$ test of significance, it could be collectively observed that there was a difference ($P>0.05$) between the frequencies of the *BoLA*-DRB3.2 alleles in the native cattle breed and the other reported cattle breeds.

A high degree of polymorphism in the *BoLA-DRB 3.2* has also been reported in Taurine and Zebu cattle (Van Eijk et al., 1992; Gelhaus et al., 1995; Dietz et al., 1997). The study of Sharif et al. in 1998 on 66 Jersey cows showed most frequently on *BoLA* alleles and they are reported the *BoLA-DRB3.2* *7, *10, *17, *21, *28 and *32

Table 2. The restriction patterns of *Rsa*I, *Psu*I and *Hae*III enzymes for *BoLA-DRB 3.2* gene in Iranian native cattle breed.

***Rsa*I**																		***Psu*I**					***Hae*III**							
a	b	c	e	f	g	h	i	j	k	l	m	n	o	p	r	s	t	a	b	c	d	e	a	b	c	d	e	f	g	i
78	111	111	141	141	141	111	180	93	156	234	111	180	284	111	111	141	143	199	284	196	197	112	167	219	167	190	167	167	164	167
54	54	93	51	54	104	69	54	78	78	50	104	64		51	90	93	141	85		85	87	87	65	65	65	65	117	65	65	113
50	50	50	50	50	39	54	50	63	50		69			50	50	50						85	52		49	29		48	55	4
39	39	30	39	39		50		50						39	30													4		
33	33													30																
30																														

Table 3. Frequencies and association of *BoLA-DRB3* alleles with diseases and comparison with patterns described by Van Eijk et al. (1992) in Iranian native cattle.

Association with disease	Frequency (percent)	Allele number	Restriction patterns	Row
FMD resistance	1.25	DRB3.2*01	aaa	1
-	1.25	DRB3.2*07	ecc	2
BLV sensitive	3.75	DRB3*1201	faa	3
-	8.75	DRB3.2*10	fba	4
-	2.5	DRB3.2*New	fbb	5
-	8.75	DRB3.2*New	fbd	6
-	2.5	DRB3*4201	gaa	7
-	3.75	DRB3.2*New	gab	8
-	5	DRB3.2*New	gba	9
-	2.5	DRB3.2*Nassiri	gbb	10
BLV resistance	1.25	DRB3.2*11	gea	11
FMD sensitive	5	DRB3.2*12	haa	12
-	1.25	DRB3.2*15	iba	13
-	2.5	DRB3.2*New	jac	14
-	2.5	DRB3.2*New	jad	15
-	3.75	DRB3.2*New	jba	16
-	6.25	DRB3.2*New	jbb	17
BLV sensitive	3.75	DRB3*1501	jbd	18
-	1.25	DRB3.2*New	jbf	19
-	2.5	DRB05	lba	20
-	1.25	DRB3.2*21	lbe	21

Table 3 Contd.

FMD sensitive	1.25	DRB3.2*18	lbf	22
-	1.25	DRB3.2*32	maa	23
BLV/mastitis sensitive	1.25	DRB3*1101	mba	24
BLV resistance/mastitis sensitive	2.5	DRB3*2701	nba	25
BLV sensitive	2.5	DRB3.2*24	nbb	26
-	1.25	DRB3*2704	nbf	27
-	2.5	DRB3.2*25	oaa	28
-	2.5	DRB3*0601	oab	29
-	2.5	DRB3.2*37	oba	30
BLV resistance	1.25	DRB3*0701	obb	31
BLV sensitive	1.25	DRB3*1401	obf	32
-	1.25	DRB3.2*29	pcc	33
-	1.25	DRB3.2*New	pec	34
-	1.25	DRB3.2*New	red	35
-	2.5	DRB3.2*New	sbf	36
-	1.25	DRB3.2*New	sab	37
-	1.25	DRB3.2*New	tbd	38
-	1.25	DRB3.2*New	tcc	39

alleles in Canada (Sharif et al., 1998). In a later study on Jersey cattle by Gilliespie et al. (1999), it was observed that the most frequently isolated alleles were *BoLA-DRB3.2* *8, *10, *15, *21, *36 and *ibe (Gilliespie et al., 1999). The allele DRB3.2 *7, which was the most common allele type detected in the Jersey cows in the study carried out by Sharif et al. (1998) was not observed to be present in the Jersey herd (Gilliespie et al., 1999). Therefore, it could be observed that differences in allelic frequencies existed within the Jersey breed. Dietz et al. (1997) carried out polymorphism studies on the *BoLA-DRB3.2* locus in a population of 127 Holstein cows. They observed that *BoLA-DRB3.2* *8, *11, *16, *22, *23 and *24 were the 6 most frequently detected alleles, accounting for almost 70.3% of the total alleles (Dietz et al., 1997). In another study on Holstein animals (n = 835), Sharif et al. (1998) observed that 7 alleles *BoLA-DRB3.2* *3, *8, *11, *16, *22, *23 and *24 represented 88.7% of the total alleles (Sharif et al., 1998). In Argentine Creole cattle (n = 194), 68% of the gene frequencies were represented by 5 alleles (DRB 3.2 *15, *18, *20, *24 and *27) (Dietz et al., 1997). Approximately 70% of the alleles in the Japanese Shorthorn cattle were accounted for by 6 alleles (*BoLA-DRB3.2* *8, *9, *21, *27, *7 and *24) (Takeshima et al., 2002).

In a study carried out on 125 Saavedreno Creole dairy cattle, it was observed that the most frequently occurring alleles were *BoLA-DRB3.2* *7, *8, *11, *16, *27, *36 and *37. These alleles accounted for 70% of the total variation in the DRB3 locus (Ripoli et al., 2004). In Iranian Holstein cows (n = 250), the 4 most frequently detected alleles were *BoLA-DRB3.2* *8, *24, *11 and *16. These accounted for approximately 67% of the alleles in the herd (Mohammadi et al., 2009). In another study on Iranian Golpayegani cattle, 5 alleles (*BoLA-DRB3.2* *16, *7, *19, *28 and *11) accounted for 50% of the alleles (Nassiry et al., 2005). The *BoLA-DRB3.2 *16* allele, which was observed to be significantly associated with lower somatic cell score values, was also found to be present in the Kankrej animals, though at a low frequency of 2% (Sharif et al., 1998; Ballingall et al., 2004). The number of genotyped native cattle in the present study is not very high. Nonetheless, in spite of the smaller number of the animals that have been genotyped in the present study, the DRB3 locus showed 39 different allelic variants of the DRB3.2 locus in a total of 80 animals studied, thereby suggesting that this locus has a very high degree of polymorphism in the native cattle taken up for the study. This is the first description of genetic diversity in the DRB3 gene for native cattle breeds, in Chaharmahal Va Bakhtiari province in south west of Iran. Thus, our study has shown the distribution of the frequencies of alleles and genotypes for the *BoLA-DRB3* locus in the Iranian native cattle breeds.

The Iranian native cattle breeds are characterized by a high level of allelic diversity, uniform distribution of the frequencies of alleles and genotypes, and a high level of heterozygosity for this locus. In conclusion, the results of this study showed significant relationship between polymorphism of the *BoLA-DRB3* gene and susceptibility and resistance to disease, such as persistent lymphocytosis caused by bovine leukemia virus, FMD and clinical mastitis caused by *Staphylococcus* species in Iranian native cattle.

ACKNOWLEDGMENT

The authors gratefully acknowledge all the staff of Biotechnology Research Center of Islamic Azad University of Shahrekord Branch in Iran for their sincere support.

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