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PCR Based RFLP Genotyping of Bovine Lymphocyte Antigen DRB3.2 in Iranian Holstein Population

[1]Morteza Pashmi, [2]Saber Qanbari, [3]Seyed Ali Ghorashi and [4]Abdolreza Salehi
[1]Department of Animal Science, Abhar Azad University, P.O. Box 22, Iran
[2]Research Institute of Physiology and Biotechnology (RIPB), University of Zanjan, Iran
[3]National Institute for Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran
[4]Department of Animal Science, Aboureyhan Campus, University of Tehran, Iran

Abstract: Major Histocompatibility Complex (MHC) class II locus DRB3 was investigated by PCR based restriction fragment length polymorphism (PCR-RFLP) assay. A total of 262 Holstein cows participating in the national recording system were sampled from 10 herds. A two-step polymerase chain reaction was carried out in order to amplify a 284 base-pair fragment of exon 2 of the target gene. Second PCR products were treated with three restriction endonucleas enzymes *RsaI*, *BstYI* and *HaeIII*. Digested fragments were analyzed by polyacrylamid gel electrophoresis. Twenty-eight BoLA-DRB3 alleles were identified. Identified alleles are: BoLA-DRB3.2 *3, *6, *7, *8, *9, *10, *11, *12, *13, *14, *15, *16, 20, *21, *22, *23, *24, *25, *26, *27, *28, *32, *36, *37, *40, *51, *iaa and *ibb. The BoLA-DRB3.2*40 allele that was observed in this study has not been reported previously. The calculated frequencies were as follows: 2.29, 1.34, 0.19, 14.5, 0.38, 3.05, 12.21, 1.34, 2.29, 1.34, 2.48, 9.16, 0.95, 0.77, 6.68, 9.16, 17.94, 1.15, 0.57, 1.15, 0.95, 0.57, 0.38, 1.91, 0.38, 5.73, 0.19 and 0.95% respectively. The six most frequently observed alleles (BoLA-DRB3.2 *8, *11, *16, *22, *23 and *24) accounted for 69.65% of the alleles in these 10 herds. The results of this study confirm the allelic distribution of six most frequent alleles in Holstein population's worldwide.

Key words: BoLA-DRB3.2, polymerase chain reaction, restriction fragment length polymorphism, Iranian Holstein Cattle

INTRODUCTION

Breeding goals for dairy cattle have focused mainly on increasing the productivity and have ignored health traits such as disease resistance. Development of molecular techniques has resulted in identification of new genetic markers which enables researchers to characterize genes responsible for host immunity and productivity traits (Van Dorp *et al*., 1999). Major histocompatibility complex (MHC) genes of cattle, known as the bovine leukocyte antigen (BoLA), have received attention because of their association with immunity, reproductive and productive traits (Schukken *et al*., 1994; Lewin, 1996; Dietz *et al*., 1997a,b; Sharif *et al*., 1998a,b; Park *et al*., 2004). The MHC is a large cluster of tightly linked genes that constitute the most important genetic component of the mammalian immune system (Klein, 1986). The MHC encodes cell surface glycoproteins that bind antigens derived from pathogens or parasites and present them to T-lymphocytes which trigger the appropriate immune response (Sommer, 2005). Three major classes of MHC genes can be distinguished. MHC class I genes play an essential role in the immune defense against intracellular pathogens. In contrast, MHC class II genes are predominantly involved in monitoring the extracellular environment by presenting peptides mainly derived from parasites to the T-cells (Klein *et al*., 1997). The class III genes encode secreted proteins, some of which are associated with the immune regulation. This complex has been mapped to chromosome 23 and spans approximately 2.5 Mb of the cattle genome (Lewin, 1996). Bovine Lymphocyte Antigen class II genes are distributed in two distinct regions, IIa and IIb. The DRA, DRB, DQA and DQB genes are located in the IIa region, while the DOB, DIB, DYA and DYB genes are located in the IIb region. The class IIb of DRB gene includes DRB1, DRB2 and DRB3. Of the class II genes, cattle express one DR gene pair (DRA and DRB3) and one or two DQ gene pairs per haplotype (Amills *et al*., 1998). In particular, the DRB3 locus and its gene product are among the best defined in cattle, functionally important and highly polymorphic. The product of the BoLA-DRB3 gene is

Corresponding Author: Morteza Pashmi, Department of Animal Science, Abhar Azad University, P.O. Box 22, Iran
Tel: +98 2425226080 Fax: +98 245226988

a beta chain of an MHC class II molecule, expressed on antigen presenting cells (APCs). Correspondingly, most research focuses on the second exon of the DRB3 gene. As a result of the role in antigen presentation, DRB3 alleles have been examined for associations with various autoimmune and infectious diseases. In cattle, the number and the significance of the MHC-disease associations and production traits increased dramatically following the development of class II typing techniques. Van Eijk *et al.* (1992) developed a PCR-RFLP class II typing technique and detected thirty different BoLA-DRB3.2 alleles based on evaluation of 168 animals representing 10 cattle breeds. Fourteen additional novel BoLA-DRB3.2 alleles were identified by Gelhaus *et al.* (1995). In the other studies, Lewin (1996) and Dietz *et al.* (1997a) identified 35 and 22 BoLA-DRB3.2 alleles in Holstein cows respectively. In a larger study involving BoLA-DRB3.2 genotyping of 1100 Holstein cows from 93 commercial dairy farms in the United States, 24 previously described alleles and five new alleles were found (Dietz *et al.*, 1997b). After that, twenty-one *BoLA-DRB3* alleles with two novels were identified in 176 individuals using sequence based polymorphism technique (Takeshima *et al.*, 2002). To date 103 *BoLA-DRB3* alleles have been identified exploiting several DNA typing techniques from various breeds of cattle, including Holstein, Red Pied, Martinique, Brahman, Japanese Black, Jersey, Hereford, Ethiopian Arsi, N'Dama, Boran, Swedish Red-and-White, Simmental and Brazilian Gir (Van Eijk *et al.*, 1992; Gilliespie *et al.*, 1999; Takeshima *et al.*, 2003; Da Mota *et al.*, 2004). In the earlier report we also identified six novel alleles in BoLA-DRB3.2 locus of Sarabi cattle not reported previously (Pashmi *et al.*, 2006). Looking through the previous studies, alleles *8, *11, *16, *22, *23, *24 and *28 have been reported as the most effective and frequently detected alleles in cattle breeds. Up to the present time there is not any comprehensive study on the allelic distribution of DRB3.2 gene within Iranian dairy farms. Corresponding to well documented association of BoLA allelism with dairy production, in this project we aimed to study of the variability of the motif and determine the frequency of DRB.3.2 alleles in the Iranian Holstein population.

MATERIALS AND METHODS

DNA isolation: Blood samples of 262 Holstein cows were born during 1995-2003 from ten herds of Tehran and suburb dairy farms, participating in the national recording system were collected and transferred to the Animal Genetics Division of NIGEB. Blood samples (approximately 5-10 mL) were taken from each cattle via the jugular vein and stored at -20°C. The genomic DNA extracted from samples by phenol-chloroform procedure with minor modifications.

DRB3.2 Gene amplification: Two-step polymerase chain reaction (PCR) was carried out in order to amplify a 284 base-pair fragment of BoLA-DRB3.2 gene according to Van Eijk *et al.* (1992) procedure with some modification. Total volume of reaction 1 was 25 μL containing: 1X PCR buffer, 200 μM dNTP mix, 2.5 mM $MgCl_2$, 0.5 μM of each primer (HL030 and HL031), 1 unit of Taq DNA polymerase and 25 ng of genomic DNA. Then 2 μL of the first PCR product was used for the second PCR reaction. Total volume and concentration of reaction 2 was the same mentioned above with the exception of the primers (HL030 and HL032).

Restriction endonuclease digestion: The PCR-amplified DNA fragments from the second PCR reaction were digested with three restriction endonuclease enzymes *RsaI*, *HaeIII* and *BstYI* (MBI Fermentas GMBH). For each restriction endonuclease digestion, 5 μL of the second PCR reaction product was used. PCR products were digested with *RsaI* and *HaeIII* separately for 3 h at 37°C and with *BstYI* for 3 h at 50°C. The total volume of each digestion was 25 μL.

Gel electrophoresis: Five micro liter of the restriction enzyme digested samples were electrophoresed in 8% polyacrylamide with TBE buffer (0.9 M Tris base, 0.09 M boric acid, 2.5 mM EDTA; pH = 8.1). Gels were run at 80 V for 4 h and stained with silver nitrate. The BoLA-DRB3.2 allelic nomenclature as described by Van Eijk *et al.* (1992) was considered to scoring digestion patterns.

RESULTS AND DISCUSSION

The 284 base-pair PCR amplified fragment of the BoLA-DRB3.2 gene was digested with *RsaI*, *HaeIII* and *BstYI* restriction enzymes. Frequency analysis of BoLA-DRB3.2 alleles of 262 Iranian Holstein cattle summarized in Table 1. A total of twenty-eight BoLA-DRB3.2 alleles were observed in the experimental population. From these alleles, one allele (BoLA-DRB3.2 *40) was the novel allele type not reported previously (Fig. 1). Although this allele was rare with a frequency of 0.38, we were interested in the observation of a new allele in the universal Holstein cattle. The six most frequent observed alleles (BoLA-DRB3.2 *8, *11, *16, *22, *23 and *24) accounted for 69.65% of the alleles in the experimental population. Similarly, Dietz *et al.* (1997b) reported that the six most frequently detected alleles (BoLA-DRB3.2 *8, *11, *16,

Table 1: Allele frequencies for BoLA-DRB3.2 of Holstein cows as identified by PCR-RFLP analysis

	Patterns[a]				
DRB3.2*	*RsaI*	*BstYI*	*HaeIII*	Alleles No.	Frequency (%)
03[b]	b	b	b	12	2.29
06[b]	d	a	a	7	1.34
07[b]	e	c	c	1	0.19
08[b]	f	a	a	76	14.5
09[b]	f	d	a	2	0.38
10[b]	f	b	a	16	3.05
11[b]	g	e	a	64	12.21
12[b]	h	a	a	7	1.34
13[b]	h	b	b	12	2.29
14[b]	h	b	a	7	1.34
15[b]	I	b	a	13	2.48
16[b]	j	b	d	48	9.16
20[b]	l	b	b	5	0.95
21[b]	l	b	e	4	0.77
22[b]	m	b	a	35	6.68
23[b]	n	b	a	48	9.16
24[b]	n	b	b	94	17.94
25[b]	o	a	a	6	1.15
26[b]	o	a	b	3	0.57
27[b]	o	b	f	6	1.15
28[b]	o	b	b	5	0.95
32	m	a	a	3	0.57
36	l	b	a	2	0.38
37	o	b	a	10	1.91
40[d]	u	b	a	2	0.38
51[c]	g	a	a	30	5.73
iaa	I	a	a	1	0.19
ibb	I	b	b	5	0.95

a) Patterns as described by Van Eijk *et al.* (1992). b) Allele type designation based on nomenclature identified by Van Eijk *et al.* (1992). c) Allele that reported by Pashmi *et al.* (2006). d) New allele type observed in Holstein cattle not reported previously

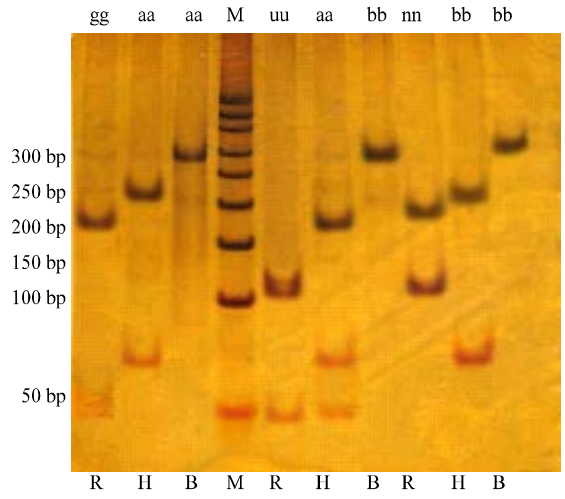

Fig. 1: The patterns of restriction enzyme digestion of three alleles were electrophoresed in 8% polyacrylamide that one of them (uu aa bb lanes) is novel alleles type (BoLA-DRB3.2 *40) not reported previously. The second PCR products were digested with *RsaI*, *HaeIII* and *BstYI*. M is 50 bp ladder and R, H and B are first letters of endonuclease enzymes

*22, *23 and *24) accounted for 70.3% of the alleles in a population of Holstein cows. In another study, Sharif *et al.* (1998a) showed BoLA-DRB3.2 *8, *11, *16, *22, *23 and *24 alleles are the most frequent in a population of Holstein cows in Canada, as such their frequency were 83.5%. Our results were completely in accordance with aforementioned investigation outcomes. The six most frequently detected alleles in the study of Kelm *et al.* (1997) and Ledwidge *et al.* (2001) were similar to Dietz *et al.* (1997b) and Sharif *et al.* (1998a). In a comprehension study Dietz *et al.* (1997b) reported 29 BoLA-DRB3.2 allele based on genotyping 1100 Holstein cows in the United States. Also they pointed out that BoLA-DRB3.2 *8 and *11 alleles have frequencies of 21 and 18% respectively. Similarly Kelm *et al.* (1997) reported the frequencies of aforementioned alleles as 21 and 17%, respectively. In the present study the frequencies of BoLA-DRB3.2 *8 and *11 alleles were 14.5 and 12.21%, respectively.

Previous studies indicate that there is significant association between infectious disease of cattle and BoLA genes. Sharif *et al.* (1998b) reported that there is no significant association between BoLA-DRB3.2 *16 and *23 alleles and production traits in Holstein cattle. In contrary BoLA-DRB3.2 *8 allele was significantly associated with increased 305-days milk, fat and protein yields and BoLA-DRB3.2 *22 was associated with decreased milk and protein yield. The frequency of these alleles (BoLA-DRB3.2 *8 and *22) in our study were 14.5 and 6.68% respectively. Sharif *et al.* (2000) reported significant relationship between the presence of glutamic acid at position β74 and occurrence of mastitis caused by *Staphylococcus* sp. This motif is present in BoLA-DRB3.2 *22, *23 and *24 alleles. Presence of arginine or lysine at position 13 also showed a tendency ($p = 0.1$) towards an association with a higher risk of clinical mastitis caused by the same bacteria. This motif is present in BoLA-DRB3.2 *8 and *23 alleles (Sharif *et al.*, 2000). The frequency of BoLA-DRB3.2 *8, *22, *23 and *24 alleles in present study were 14.5, 6.68, 9.16 and 17.94% respectively. Dietz *et al.* (1997b) have identified BoLA-DRB3.2 *16 as a potential risk factor for acute intramammary infection. Also they have mentioned there is genetic relationship between BoLA-DRB3.2 alleles and several indicator traits of innate and adaptive immunity in 127 periparturient Holstein cows. In a comprehensive study Starkenburg *et al.* (1997) surveyed associations of some DRB3.2 alleles with production traits and reported substantial differences in allelic frequencies of milk selected and control lines of Holstein cows, as BoLA-DRB3.2 *24 was associated with increased fat yield during first lactation and *8 was related to decreased milk and

protein yields. Also BoLA-DRB3.2 *7 was associated with a significant increase in protein yield during first and second lactation as well as a significant association in chronically elevated SCS and acutely elevated SCS during second lactation. They also reported that the frequencies of *24, *8 and *7 in milk selected lines were 28, 9 and 5%, respectively. In contrary, the frequencies of aforementioned alleles in control lines were 7, 23 and 10%, respectively. At a glance, a positive relationship between milk selected and control lines in production related traits was observed. Interestingly the frequencies of *24, *8 and *7 alleles in the present study were 17.94, 14.5 and 0.19%, respectively. Ascending or descending trends of these allele frequencies is in coincidence with milk selected lines in Starkenburg's study (1997). An explanation for this accordance could be successive years of artificial insemination with imported elite sire's semen in dairy farms of the country.

Results of the present study substantiate that minor differences exist between Iranian, Canadian and USA Holstein population with regard to BoLADRB3.2 allelic frequency. Our results also confirm the allelic distribution of 6 most frequent alleles in Holstein population's worldwide. Studies are in progress to evaluate relationship between BoLA-DRB3.2 allele types and SCC and some production traits in Iranian Holstein cattle. The significance of this report is that it offers interesting perspectives for the incorporation of molecular genetic techniques to animal breeding in Iran.

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