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ORIGINAL ARTICLE

Analysis of Relationship between Bovine Lymphocyte Antigen DRB3.2 Alleles, Somatic Cell Count and milk Traits in Iranian Holstein Population

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Summary

The major histocompatibility complex (MHC) is a gene complex closely linked to the vertebrate immune system due to its importance in antigen recognition and immune response to pathogens. To improve our understanding of the MHC and disease resistance in dairy cattle, we gathered 5119 test day records of somatic cell count (SCC) and performance traits of 262 Holstein dairy cows to determine whether the DRB region of the MHC contains alleles that are associated with elevated SCC, milk yield, protein and fat percent of milk. To this purpose, genotyping of animals for DRB3 gene was investigated by polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) assay. A two-step PCR was carried out so as to amplify a 284 base-pair fragment of exon 2 of the target gene. Second PCR products were treated with three restriction endonuclease enzymes *RsaI, BstYI* and *HaeIII*. Twenty-eight BoLA-DRB3 alleles were identified including one novel allele (*40). The results in general are in good accordance with allele frequencies of Holstein cattle populations reported by previous studies. Analyses of associations were modeled based on repeated measurement ANOVA and generalized logistic linear methods for production traits and SCC data, respectively. The results of this study showed a significant relationship between the elevated SCC reflecting an increased probability of occurrence to subclinical mastitis and DRB3.2 allele *8 ($p < 0.03$). The results also revealed significant positive relationships of alleles*22 ($p < 0.01$) and allele*11 ($p < 0.05$) with milk fat percent as well as of alleles*24 ($p < 0.03$) and *22 ($p < 0.05$) with protein percent. The present study failed to find any association between milk yield and tested alleles. Because of the lack of consistency among results of similar studies, we suggest further investigations to determine the precise nature of these associations with the high polymorphic bovine MHC region to be performed based on haplotypes.

Introduction

For many years, breeding goals for dairy cattle had focused mainly on increasing the productivity and had ignored health traits such as disease resistance. Higher yielding cows tend to have higher health costs. For example, mastitis is the most prevalent production disease in dairy herds world-wide and is

responsible for several effects on production. Milk production losses, drugs, discarded milk, veterinarian service, labour, milk quality impairment and culling of cows are the economic damage of mastitis. Prospects for the development of an effective vaccine are limited by the variety of microorganisms causing mastitis and by a lack of information on the genetic factors that influence disease resistance. It is evident that resistance to infectious diseases is genetically determined and nowadays, the interest in selection for resistance to health problems in the dairy industry as well as the selection for improvement of the health of livestock for consumers are internationally of increasing importance (Stear *et al.* 2001). But unfortunately, health traits usually have low heritabilities and limited amounts of data, which hamper the potential for genetic improvement by traditional selection methods. Consequently, there has been considerable interest in defining genetic and immunological markers that could be used to select for improved disease resistance (Park *et al.* 2004).

The major histocompatibility complex (MHC) consists of a group of closely linked genes that constitute the most important genetic component of the mammalian immune system. The MHC has attracted considerable attention because of its relationship with infectious disease, genetic variability and evolutionary history. For dairy cattle, this complex, referred to as the bovine lymphocyte antigen (BoLA) complex, is organized in three distinct classes (class I, II and III), and each one is divided into regions and subregions, containing genes and pseudogenes (Andersson & Davis 1994). As a result of their role in antigen presentation, MHC classes I and II genes have been examined for associations with various autoimmune and infectious diseases as well as production traits. Class I associations include production traits (Batra *et al.* 1989), tick resistance (Stear *et al.* 1989a), nematode egg worm counts (Stear *et al.* 1988b), resistance to persistent lymphocytosis caused by bovine leukemia virus (Stear *et al.* 1988a), chronic posterior spinal paresis (Park *et al.* 1993), ketosis (Mejdell *et al.* 1994), resistance to dermatophilosis (Maillard *et al.* 1996) as well as mastitis (Solbu *et al.* 1982; Oddgeirsson *et al.* 1988; Mejdell *et al.* 1994). The class II BoLA-DR region is composed of one *DRA* locus and at least three *DRB* loci (Amills *et al.* 1998). Of the class II genes, the DRB3 locus has been the most widely studied of the BoLA complex genes because it is extremely polymorphic, with over 103 different alleles identified (Takeshima *et al.* 2002). Alleles of the second exon of DRB3 (*DRB3.2*) gene potentially affect many traits related to immunity in cattle. Dietz *et al.* (1997a,b) investigated the association of *DRB3.2* alleles of BoLA and 20 immunological variables using a gene substitution model. They reported significant associations for 13 leucocyte functions, including IgG2 concentration, IgM and complement levels, and mononuclear cell numbers, suggesting that *DRB3.2* alleles are related to factors involved in immune function. There are also several studies in the literature in which the genetic association of the BoLA-DRB3 gene with performance traits and resistance to mastitis has been investigated (Kelm *et al.* 1997; Starkenburg *et al.* 1997; Sharif *et al.* 1998a,b; Gilliespie *et al.* 1999; Nascimento *et al.* 2006; Kulberg *et al.* 2007; Rupp *et al.* 2007).

In earlier reports, we described the variability of the BoLA-DRB3.2 gene in native Sarabi cattle and reported six novel alleles at this locus (Pashmi *et al.* 2006). In this study, we investigated the frequency of alleles and evaluated the association of DRB3.2 variants with production traits and somatic cell counts (SCC) of milk as an indicator of occurrence probability of subclinical mastitis in the Iranian Holstein population.

Material and methods

Data structure

Blood samples from 262 Holstein cows from 10 herds of dairy farms participating in the recording system of National Animal Breeding Center were used for DRB3.2 genotyping. For our analyses, 5119 test day records of milk SCC, milk yield, fat and protein percents collected over a 5-year period from 2001 to 2006 were taken directly from the national breeding value evaluation for the Holstein population. The number of test day records for each animal varied between 8 and 27.

BoLA-DRB3.2 genotyping

The genomic DNA was extracted from samples by the phenol–chloroform procedure with minor modifications. A two-step polymerase chain reaction (PCR) was carried out so as to amplify a 284 base-pair fragment of BoLA-DRB3.2 gene according to the procedure suggested by Van Eijk *et al.* (1992) with minor modifications. The total volume of PCR reaction 1 was 25 μl containing: 1× 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 PCR product was used for the second PCR reaction. The

total volume and concentration of reaction 2 was the same as mentioned above with the exception of the primers (HL030 and HL032). The full protocol of BoLA-DRB3 typing including information of alleles, and interpretation of banding patterns could be found in DRB3 project online (http://www.projects.roslin.ac.uk/bola/drb3pcr.html) at Roslin institute homepage.

The 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 mix including digestion buffer, enzyme and ddH_2O was 25 μl. Five microlitres of restriction enzyme-digested samples was 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 used for scoring digestion patterns.

Statistical analysis

A repeated gene substitution MIXED model (SAS Inst., Inc., Cary, NC, USA) was used to evaluate the fixed effects of herd, parity, season and year of the recorded data, BoLA alleles and random permanent effects of each animal as well as random effects of sires on SCC and production traits in the Holstein data set.

The mixed repeatability model was

$$Y_{ijklmnop} = \mu + P_i + S_j + H_k + T_l + \sum\nolimits_m b_m \mathrm{BoLA}_{ijklmno} + \mathrm{Sire}_n + R_{ijklmno} + e_{ijklmnop}$$

where, $Y_{ijklmnop}$ is the dependent variable of production traits (milk yield, fat, or protein percents), μ *is* the population mean of the analysed traits, P_i is the fixed effect of the parity i (six subclasses), S_j is the fixed effect of season j (four subclasses), H_k is the fixed effect of the herd k (10 subclasses), T_l is the fixed effect of year l (five subclasses), b_m is the regression coefficient on the number of copies of the nth BoLA allele, BoLA_m is the number of copies of BoLA allele presented in the animal m, $R_{ijklmno}$ is the random permanent animal effect with mean 0 and variance s^2_δ,which is equal to the covariance between repeated measurement within animals, Sire_n is the random effect of nth sire (out of 121 sires), assumed being distributed normally and independently with variance $\sigma^2_\delta$and finally $e_{ijklmnop}$ is the random residual effect with mean 0 and variance σ^2.

The SCC in milk constitutes a good diagnostic tool that allows the early detection of either subclinical or acute form of mastitis (Green *et al*. 2004), and is therefore a valuable component of monitoring programs (Schukken *et al*. 2003). For the present study, the continuously distributed trait SCC was transformed in a binary response variable, which was set to 0 (healthy udder) if SCC < 300 000 and 1 (clinical or subclinical mastitis) if SCC > 300 000. The threshold value 300 000 cells/ml was suggested by Klastrup (1975). Other thresholds (such as 100 000 or 500 000) are advocated by other authors (e.g. Schukken et al. 2003).

The binary response variable was analysed using a logistic model using a link function $g(\mu_i)$ linking the expected value to the linear predictor η_i. The logit link function is defined by $log\left[\frac{\pi_i}{1-\pi i}\right] = \eta_i$. The data were analysed with the GLIMMIX macro (Littell *et al.* 1999) using a repeated ANOVA with the following generalized linear model:

$$\eta_{ijklmno} = \mu + P_i + S_j + H_k + T_l + \sum\nolimits_m b_m \mathrm{BoLA}_{ijklmno} + \mathrm{Sire}_n + R_{ijklmno}$$

where $\eta_{ijklmno}$ is the probability of occurrence of subclinical mastitis, μ is the overall mean effect, and the other model components are the same as in the previous model used for production traits. The compound symmetric covariance matrix structure was applied for repeated observations of subclinical mastitis and was fitted based on the Akaike information criterion (Akaike 1974). Model parameters were estimated by residual maximal likelihood. The Wald F-statistics (Wald 1943) was used to determine the significance of fixed effects on the probability of occurrence of subclinical mastitis (SS Type III – test).

Results and discussion

Data statistics and allele frequencies

The mean values and standard deviations for the milk yield, fat and protein percent were 28.64 (9.94), 3.45 (0.76) and 3.13 (0.31), respectively. These values are typical for Holstein cows in the studied population and allow a comparison of the size of the BoLA effects. Totally, 25% of SCC records were higher than 300 000 cells/ml. The geometric population mean of the number of SCC, also called 'bulk milk SCC value' as an indicator of udder

health status in studied population, was calculated as 250 000 cells/ml. In the European countries, equivalent SCC values in 2002 were 200 000, 132 000, 221 000 cells/ml for the Netherlands, Finland and Belgium, respectively (Piepers *et al.* 2007). For Uruguay ,this value was reported to be around 450 000–500 000 cells/ml, indicating about 50% prevalence of subclinical mastitis in the studied population (Gianneechini *et al.* 2002).

The distribution of frequencies of BoLA-DRB3.2 alleles is summarized in Table 1. From 28 DRB3.2 alleles, one allele (BoLA-DRB3.2 *40) was a novel allele not observed previously (Figure 1). To verify and confirm whether this pattern is, in fact, a new allele type, further DNA sequence analysis of these alleles needs to be carried out.

The seven most frequently observed alleles in this study (BoLA-DRB3.2 *8, *11, *16, *22, *23, *24 and *51) accounted for 75.38% of the alleles in the experimental population. The overall most common allele was allele *24 having 17.94% frequency. A total of 28 alternative DRB3.2 alleles appeared, frequencies ranged from 0.37 to 17.94%.

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

DRB3.2[1]	Alleles no.	Frequency (%)
03	12	2.29
06	7	1.34
07	1	0.19
08	76	14.5
09	2	0.38
10	16	3.05
11	64	12.21
12	7	1.34
13	12	2.29
14	7	1.34
15	13	2.48
16	48	9.16
20	5	0.95
21	4	0.77
22	35	6.68
23	48	9.16
24	94	17.94
25	6	1.15
26	3	0.57
27	6	1.15
28	5	0.95
32	3	0.57
36	2	0.38
37	10	1.91
40[2]	2	0.38
51	30	5.73
iaa	1	0.19
ibb	5	0.95

[1]Allelic pattern is based on the nomenclature suggested by Van Eijk *et al.* (1992).

[2]Novel allele type not observed previously.

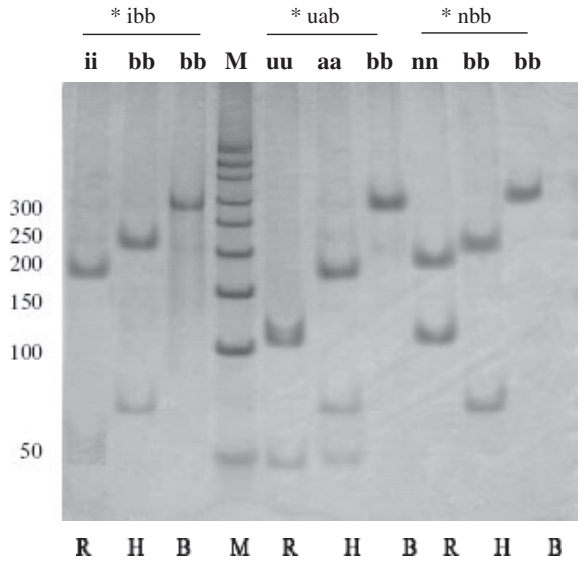

Figure 1 The patterns of restriction enzyme digestion of three BoLA-DRB3.2 alleles (*ibb, * uab and *nbb). One of them (uu aa bb lanes) is a novel allele type (BoLA-DRB3.2 *40) and was not observed previously. The second PCR products were digested with *RsaI* (R), *HaeIII* (H) and *BstYI* (B). M is 50 bp ladder.

In a similar study, Nassiry *et al.* (2005) reported 26 alleles in Iranian Holstein population, with four alleles *8, *11, *16, *24 accounting for 67% of total allele frequency. Similarly, Dietz *et al.* (1997b) and Sharif *et al.* (1998b) reported that the six most frequent alleles *8, *11, *16, *22, *23, *24 accounted for 67% and 83.5% of the total allele frequency of Holstein population in USA and Canada, respectively. The six most frequently detected alleles in the studies of Kelm *et al.* (1997) and Ledwidge *et al.* (2001) have confirmed these results. Our results were completely in accordance with the outcomes of the aforementioned investigations. A high level of polymorphism identified in this study substantiates that, despite many generations of intense selection primarily for yield, a considerable degree of polymorphism still has remained in the Holstein cattle.

Association analyses

Table 2 summarizes the association results of effects included in the statistical model. Like with the majority of previous studies, only the seven most frequent variants out of 28 detected alleles were included in the statistical model. The effects of year,

Table 2 Estimated regression coefficients with standard error (SE) and p-value for each of the 5 BoLA-DRB3.2 alleles having significant association with analysed traits

	Fat percent			Protein percent			Probability-SCM			
Allele	Estimate[1]	SE	p-value	Estimate[1]	SE	p-value	Estimate	SE	Probability[2]	p-value
*8							0.29	0.14	5.80	0.03
*11	0.12	0.03	0.05							
*22	0.17	0.7	0.01	0.07	0.03	0.05	0.37	0.20	7.65	0.06
*24				0.05	0.02	0.03				
*51							0.35	0.20	7.24	0.08

Probability-SCM is the probability of the occurrence of subclinical mastitis.

[1]Regression coefficient is equivalent to the change in the milk fat and protein percents of animals carrying one additional copy of the respective allele.

[2]The change in probability (%) of the occurrence of subclinical mastitis in animals with one copy of given allele.

season, herd and parity on each analyzed trait were significant ($p < 0.05$).

The results of association analysis by the mixed repeatability model revealed a significant positive effect of alleles *22 ($p < 0.01$) and *11 ($p < 0.05$) on milk fat percentage and of alleles *24 ($p < 0.03$) and *22 ($p < 0.05$) with increased protein percentage. These analyses did not reveal a significant association of alleles with milk yield. Significant relationships between BoLA DRB3.2 alleles and production traits have been previously documented (Table 3). In a similar study, Starkenburg *et al.* (1997) reported a positive effect of allele *24 on increased fat yield during first lactation in lines of Holstein cows selected for milk yield. However, allele *24 was not associated with other effects on yield traits across lactations. Allele *8 was associated with significantly decreased milk and protein yields and approached significance ($p < 0.10$) for decreased fat yield during third lactation. Conversely, allele *11 had positive effect on increased milk and protein yields in the selection lines during third lactation. Recently, Rupp *et al.* (2007) have also reported a positive effect of allele *11 on milk yield and composition in a Canadian dairy population. Sharif *et al.* (1998b) observed a positive effect of allele *8 on protein yield, whereas allele DRB3*22 was significantly associated with decreased protein yield. The latter

Table 3 Trait associations found in the literature for four alleles which showed significant relationships in this study

Allele	Milk yield	Fat	Protein	Somatic cell count	Source
*8	Decreased milk yield during third lactation	Decreased fat yield during third lactation	Decreased protein yield during third lactation		Starkenburg *et al.* (1997)
			Increased protein yield		Sharif *et al.* (1998b)
				Increase in estimated breeding value for clinical mastitis	Kelm *et al.* 1997
				Increase in estimated breeding value for clinical mastitis	Dietz *et al.* (1997b)
				Higher mastitis risk	Rupp *et al.* (2007)
*11	Increased milk yield during third lactation		Increased protein yield during third lactation		Starkenburg *et al.* (1997)
				Resistance to mastitis	Kulberg *et al.* (2007)
	Higher milk yield	Higher fat yield	Higher protein yield	Lower SCC	Rupp *et al.* (2007)
*22				Decreased SCC among second-lactation cows	Dietz *et al.* (1997b)
				Increased SCC during first and thirds lactation	Starkenburg *et al.* (1997)
				increased clinical mastitis	Kulberg *et al.* (2007)
				Higher SCC	Rupp *et al.* (2007)
			Decreased protein yield		Sharif *et al.* (1998b)
*24		Increased fat yield during first lactation			Starkenburg *et al.* (1997)
				Resistance to mastitis	Kulberg *et al.* (2007)

was in contrast to the findings of our study which suggested a significant association of allele *22 with an increase in milk protein yield. There are also some studies that failed to find any relationships between DRB3.2 alleles with production traits (Lunden *et al.* 1993; Arriens *et al.* 1996).

The results of this study showed a significant relationship between the subclinical mastitis incidence based on SCC and DRB3.2 allele *8 ($p < 0.03$). As such, the logistic regression coefficient representing the effect of allele in the model was estimated as 0.29 (SE = 0.14). Back-transforming of this estimate using the inverse link function $\pi = exp(x\beta)/[1 + exp(x\beta)]$ shows the 5.8% change in probability of occurrence of subclinical mastitis in animals with one copy of given allele. A tendency to being significant was also observed for alleles *22 ($p < 0.06$) and *51 ($p < 0.08$) with this trait (Table 2). Because all estimates are numerically positive, alleles *8, *22 and *51 may have unfavourable effects on mastitis resistance. The BoLA-DRB3 gene is highly polymorphic, and several alleles have been favourably or unfavourably associated with indicators of mastitis in different studies (Table 3). So far, however, no allele has shown a consistent and significant association with mastitis throughout the studies. For example, allele *22 has been associated with both elevated somatic cell score (SCS) during first and third lactations in combined lines (Starkenburg *et al.* 1997), and decreased SCC among second-lactation cows in previous studies (Dietz *et al.* 1997b). However, two recent comprehensive studies by Kulberg *et al.* (2007) and Rupp *et al.* (2007) reported a close relationship for allele *22 with increased clinical mastitis and higher SCC in Norwegian Red and Canadian Holstein, respectively. The most consistent results were found for allele *8. Dietz *et al.* (1997b) found allele *8 to be significantly associated with an increase in acutely elevated SCC during first lactation. Starkenburg *et al.* (1997) also found allele *8 to approach a significant ($p < 0.10$) association with increasing acute SCS for second lactation only. The presence of *DRB3.2* *8 was also associated with a significant increase in estimated breeding value for clinical mastitis (Kelm *et al.* 1997). The findings of Rupp *et al.* (2007) also confirm these reports. Our results together with the reports of previous studies suggest that alleles *8 and *22 of the bovine MHC has an unfavourable effect on elevated SCC as an indicator of udder health status in Holstein cattle.

Like with the other reports, the results presented in this study are in coincidence with and in some cases differ from the results of previous studies. This inconsistency could be explained by different reasons. The contradictory reports in the case of mastitis resistance may be explained by differences between environmental conditions, traits characterized and bacterial flora (Nascimento *et al.* 2006; Kulberg *et al.* 2007). The incongruities may also result from the different states of linkage disequilibrium in the populations under study. Morris & Kaplan (2002) showed that haplotype-based tests can have greater power than unphased tests of association in the case when the disease locus has multiple disease-causing alleles. As such, using haplotype information may collapse the dimensionality of the statistical test, and thereby gain statistical power. Regarding the availability of well-developed statistical methods in haplotype-based association studies (Meuwissen *et al.* 2001), this report strongly recommends that more comprehensive, haplotype-based studies need to be performed to better understand the nature of associations in this region.

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