Full Length Research Paper

Study on the association of BoLA-DRB3.2 alleles with clinical mastitis in Iranian Holstein and Sarabi (Iranian native) cattle

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Seventy-five Iranian Holstein cattle which comprised of two different groups (49 susceptible cattle to clinical mastitis and 26 resistant cattle to clinical mastitis) as well as forty-five Sarabi breed cattle were studied randomly for polymorphism in BoLA-DRB3.2 gene. Genotyping by single strand conformation polymorphism- polymerase chain reaction (SSCP-PCR) and then sequencing revealed the presence of previously reported 13 alleles of BoLA-DRB3.2 gene across both studied breeds. However, 3 new alleles were identified for the studied gene in Sarabi breed in addition to the 13 alleles. Cloning and sequencing of the three newly found alleles in this study confirmed their novelty (NCBI Genebank accession numbers, EU362974, EU372975 and EU372976). Multi-primer target polymerase chain reaction (MPT-PCR) in this study failed to accurately differentiate the resistant and susceptible cattle to clinical mastitis. A combination of single strand conformation polymorphism (SSCP) and heteroduplex analysis (HA) in a non-denaturing gel, successfully detected the resistant genotype to clinical mastitis (DRB3.2*16). To the best of our knowledge, this is a pioneer study on the BoLA-DRB3.2 gene across an Iranian native breed (Sarabi). The results demonstrated that the BoLA-DRB3.2 locus is highly polymorphic in the Sarabi cattle.

Key words: BoLA-DRB3.2, multi-primer target polymerase chain reaction, single strand conformation polymorphism-polymerase chain reaction, clinical mastitis, Iranian Holstein, sarabi cattle.

INTRODUCTION

The class II genes of the major histocompatibility complex (MHC) encode cell-surface glycoproteins that bind exogenous peptides within the cell and present them to CD4- positive T helper cells (Banchereau and Steinman,

1998). Several MHC class I and class II genes are extremely polymorphic in many species. This polymorphism is maintained by some form of balancing selection (Hedrick and Kim, 1998). The immunological importance of the MHC genes and their possible role in disease resistance has been a major impetus for research on the MHC system in cattle denoted BoLA. 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 pseudo-gene and functional expression of DRB2 has not been found whereas DRB3 is functionally expressed and highly polymorphic (Davies et al., 1997). In total, 103 alleles of BoLA-DRB3 exon 2 have been identified by sequence based typing (Takeshima et al., 2001). Based on the association between BoLA-DRB3 alleles and various

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Abbreviations: SSCP-PCR, single strand conformation polymorphism- polymerase chain reaction; **MPT-PCR,** multi-primer target polymerase chain reaction; **HA,** heteroduplex analysis; **MHC,** histocompatibility complex; **SCS,** somatic cell score; **IgG2,** immunoglobulin G subclass 2; **IgM,** Immunoglobulin M; **EDTA,** ethylenediaminetetraacetic acid; **PHS,** polish heath sheep; **PLS** polish lowland sheep; **SCC,** somatic cell count.

immunological traits, it seems potentially important to carry out a study on the polymorphism at this locus. The DRB3.2 alleles potentially affect many traits related to immunity, milk somatic cell score (SCS) and mastitis incidence (Dietz et al., 1997a). Indirect associations between DRB3.2 alleles and immune function including immunoglobulin G subclass 2 (IgG2) and immunoglobulin M (IgM) concentration (Dietz et al., 1997b) and complement levels and mononuclear cell numbers have been previously reported (Dietz et al., 1997b).

In a separate study of 137 pre-parturient Holsteins, it was found that allele BoLA-DRB3.2*16 of the DRB3.2 locus increased SCS (Kelm et al., 1997). In the same study, BoLA-DRB3.2*8 allele was associated with greater clinical mastitis and alleles *11 and *23 were associated with decreased clinical mastitis. Also, using n = 1100 Holstein cows, a significant association between allele BoLA-DRB3.2*16 locus and increased risk of acutely elevated SCS was reported (Dietz et al., 1997a). Two DRB3 types have been identified as being associated with increased risk (DRB3.2*23) and decreased risk (DRB3.2*16) of mastitis in Canadian Holsteins (Sharif et al., 1998). Also, quantitative trait locus (QTL) mapping studies have found QTLs for mastitis-associated traits, such as somatic cell count (SCC) and SCS on BTA23 (Ashwell et al., 1997; Holmberg and Andersson-Eklund, 2004), which makes the BoLA genes interesting candidate genes for association studies with mastitis. Sarabi breed (Iranian native) cattle have unique features like adaptability to extreme climatic conditions and better resistance capabilities to withstand environmental stress and mastitis disease (Sarab Agricultual Research Centre). A study of polymorphism of the DRB3 locus in Sarabi breed is of particular interest to look for certain sarabi-specific alleles that might be related to higher degree of resistance to region diseases. The present study was designed to characterize the different allelic variants of the BoLA-DRB3 locus in the Sarabi breed. In this study genetic variability of BoLA-DRB3 locus in Iranian Holstein cattle was described.

MATERIALS AND METHODS

In the first step, multi-primer target polymerase chain reaction (MPT-PCR) technique was used to detect the presence of DRB3.2*23 and DRB3.2*16 in a single PCR reaction and was visualise by agarose gel electrophoresis as described by Ledwidge et al. (2001). In the second step, all samples were studied by single strand conformation polymorphism (SSCP) and heteroduplex bands. In order to carry out analysis, a non-denaturing composite gel for increasing accuracy of genotyping and rapid identification of new alleles was applied. The present study aimed to study the characteristics of DRB3.2 alleles and the association of BoLADRB3.2 alleles with clinical mastitis in Sarabi and Iranian Holstein cattle, respectively.

Animals

Blood samples (approximately 8 to 10 ml) were obtained from 75 Holstein breed (in across Mogan herds) that were selected in two groups for susceptibility (n = 49) and resistance (N = 26). Also, in this study, the blood samples form 45 Sarabi breed cattle (unrelated animals) were collected from regions of Sarab in East Azarbaijan state of Iran. All samples were stored in EDTA-coated vacutainer tubes (BD Vacutainer Systems, Plymouth, UK).

Clinical mastitis data

In Iranian Holstein population, Moghan herd was evaluated for occurrence of clinical mastitis. The clinical mastitis was detected by appearance of watery milk (or having an off-white color) during the pre-milking routine. Often, changes in milk characteristics were associated with more general observations such as a hot, hard quarter or sensitivity to touch. Other mastitis data were obtained from Dairy Herd Recording System, from a data set including mastitis records up to second-lactation. Cattle considered as resistant to mastitis did not show any occurrence of mastitis up to third-lactation.

DNA Extraction

Genomic DNA was isolated from total blood, drawn in Vacutainer glass tubes or in plastic Vacuette tubes with addition of EDTA as anticoagulant in each case. A modified phenol/choloroform extraction method was used (Millerr et al., 1998). The samples were stored at -20°C. The working DNA concentration was adjusted to 50 to100 ng/l.

MPT-PCR of BoLA-DRB.2

For MPT-PCR, four primers were used, that is, two outer primers amplified at exon 2 of DRB3 as control PCR and two inner primers amplified at the allele-specific site (DRB.2*23 and DRB.2*16 alleles) in individual cattle. The oligonucleotide primers, DRB3F (5'-TCC CGC ATT GGT GGG TGT), DRB3R (5'-CTC CAC ACT GGC CGT CCA C), DRB315F (5'-GGAGGG GGC GCG AGC AGT TGA ACG) and DRB327R (5'-GGG CGG GGC GTT CTC CAT TAG TGT AGC) (Ledwidge et al., 2001) were used for the PCR amplification of the DRB3 exon 2. For each reaction, 10 μl volumes using 50 ng DNA, 1 U AmpliTaq DNA polymerase, 5 pmol of each forward and reverse primers, 200 mM dNTP, 2.5 mM MgCl$_2$, 83 mM KCl and 16.6 mM Tris ± HCl were used. The samples were incubated for 10 min at 95°C to activate the DNA polymerase, followed by 30 cycles each comprising 30 s at 94°C, 30 s at 63°C and 30 s at 72°C; the last cycle was followed by an additional extension for 5 min at 72°C.

SSCP-PCR of BoLA-DRB3.2

Amplification for SSCP and heteroduplex analysis (HA) followed the same protocol (Ledwidge et al., 2001). SSCP and HA were conformed by polyacrylamide gel electrophoresis (10% acrylamide, 2% N',N' methylene-bis-acrylamide, 5% glycerol and 0.25% low melting agarose; 14 cm · 25 cm · 0.5 mm) TBE buffer at room temperature (10-20°C) and constant 8 W (200 V) for 14-19 h. Prior to loading, 5 μl of radiolabelled 'HOT' second round PCR product was mixed with 20 μl of SSCP loading dye [98% (w/v) deionized formamide, 0.025% (w/v) xylene cyanol, 0.025% (w/v) bromophenol blue and 0.02 M EDTA], denatured for 5 min at 95°C and snap cooled. SSCP and heteroduplex bands were visualized after staining by fast and sensitive silver method (Bassam et al., 1991).

Cloning procedure of eluted PCR products

Samples that revealed the possibility of identifying novel alleles, or

whose genotyping from the sequenced PCR product was not reliable, were cloned by pTZ57R/T vector using the InsTAclone™ PCR Cloning Kit and transformed into competent JM109 *Escherichia coli* cells (Hanahan, 1983). Clones containing BoLA-DRB3 alleles were sequenced by Sinagene Company.

Statistical analysis

A mixed linear model was used to evaluate the effect of BoLA DRB3.2 alleles on the resistance and susceptibility to mastitis. Logistic regression was applied to the gene substitution models for repeated binary measures of clinical mastitis (PROC GENMOD, repeated option; SAS Institute). Therefore, the binary nature of udder health data was taken into account, as was within cattle correlation for repeated mastitis occurrence across lactations.

Models for clinical mastitis

The logistic regression model for clinical mastitis was as follows:

$$Y_{ij} = X_{ij}\beta_{ij} + \Sigma_k \alpha_k BoLA_k + e_{ij}$$

Where, Y_{ij} is the log $(P/1 - P)$, P is the probability that cattle i is affected by at least one case of clinical mastitis during parity j; $BoLA_k$ is the fixed effect of number of copies (0, 1, or 2) of BoLA allele k (k = *16 and *23); α_k is the regression coefficient on the number of copies of the jth BoLA allele; Σ_j is the summation from 1 to j; X_{ij} is the vector of environmental effects; β_{ij} are regression coefficients on the vector of fixed effects X and e_{ij} is the random error term. Effects included in X (P ≤ 0.10) were calving season, calving year, parity, herd and sire. An unstructured correlation matrix for responses was fitted to model the correlation of the responses within cattle.

RESULTS AND DISCUSSION

The four MPT-PCR primers were used for both Holstein and Sarabi breed cattle and exon 2 fragment of 395 bp was amplified in all samples which served as an internal control for the PCR reaction. However, the 151 bp fragment which is supposedly present when the animal carries the resistant allele that is, DRB3.2*16 (Ledwidge et al., 2001) was observed in both mastitis- susceptible and resistant Holstein cattle. Therefore, the association between the DRB3.2*16 allele and resistance to mastitis was not supported by the findings of this study. The 151 bp fragment was not observed in Sarabi breed cattle despite their high mastitis-resistance. This was ascribed to probable differences in genetic diversity between Holstein and Sarabi cattle. Therefore, resistance to mastitis should be controlled by different alleles in Sarabi breed cattle. Furthermore, the 196 bp fragment associated with DRB3.2*23 allele known as susceptibility allele (Ledwidge et al., 2001) was not amplified by MPT-PCR. A linear model was used to test the association between amplification of BoLADRB3.2 alleles by MPT-PCR and the occurrence of clinical mastitis. The reference BoLAD RB3.2* 23 allele and *16 allele were not associated significantly (P > 0.01) with susceptibility and resistance to clinical mastitis. SSCP technique was applied for genotyping and identifying DRB3.2 alleles. SSCP detects single-base sequence changes by abnormal electrophoresis migration of one or both single strands on a non-denaturing polyacrylamide gel. DNA strands fold differently if they differ by a single base and it is believed that mutation-induced changes of tertiary structure of the DNA result in a different mobility. Mutations are identified as new bands on autoradiograms of silver-stained bands, or of fluorescent PCR products detected by DNA sequencing. In the present study we used the PCR-SSCP method and silver staining to identify poly-morphism in exon of 2 BoLA-DRB3.2 genes in cattle of two breeds (Holstein and Sarabi). SSCP analysis showed 13 and 16 different patterns near the 800-900 bp double stranded DNA regions for Holstein and Sarabi cattle, respectively. Among these, 13 were previously reported. Cloning and sequencing of the PCR product related to the three extra patterns observed in Sarabi cattle revealed the presence of three novel alleles of BoLAD RB3.2 gene in Sarabi cattle (NCBI Genebank accession numbers, EU362974, EU372975 and EU372976). Almost 95% of the cattle studied were heterozygous for the DRB3.2 locus. In contrast to MPT-PCR method, the SSCP-PCR has proved to be an accurate and efficient method for genotyping a cattle population as the 151 bp fragment which represents the DRB3.2*16 allele and associated with resistance (Sharif et al., 1998) was observed (Figure 1). However, there was no indication of its association with resistance to mastitis as it was also observed among the mastitis-susceptible group. This finding was in contrast with Sharif et al. (1998) that reported associations of two alleles of BoLA-DRB3 with lowered somatic cell score (SCS) and occurrence of disease (BoLA-DRB3·2*16 and *23, respectively). Therefore, SSCP can be used to efficiently screen large populations for the presence of specific alleles. This method has the potential to be modified to identify most other BoLA-DRB3 alleles. Gruszczyńska et al. (2005) applied single strand conformation polymorphism in exon 2 of the Ovar-DRB1 gene in two Polish breeds of sheep and total of 33 SSCP patterns identified, 27 in polish heath sheep (PHS) and 13 in polish lowland sheep (PLS). They concluded that PCR-RFLP method when used alone does not supply the complete information on gene poly-morphism, while PCR-SSCP approach allows the identifi-cation of more patterns. Therefore, the use of PCR-SSCP simultaneously with PCR-RFLP is recommended. Sharif et al. (1999) in another study evaluated potential relation-ships between BoLA-DRB3 alleles with production traits, namely 305-day milk, milk fat and milk protein yield, in a population of Canadian dairy cattle (Holstein, n = 835 and Jersey, n = 66) over the course of two lactations. No significant associations were detected between BoLA alleles and production traits in Jerseys. In Holsteins, alleles *16 and *23 also did not show asso-ciations with production. Rupp et al. (2007) found that alleles DRB3.2*3 and *24 were associated with higher AMIR (antibody-mediated immune response) but lower CMIR (cell-mediated
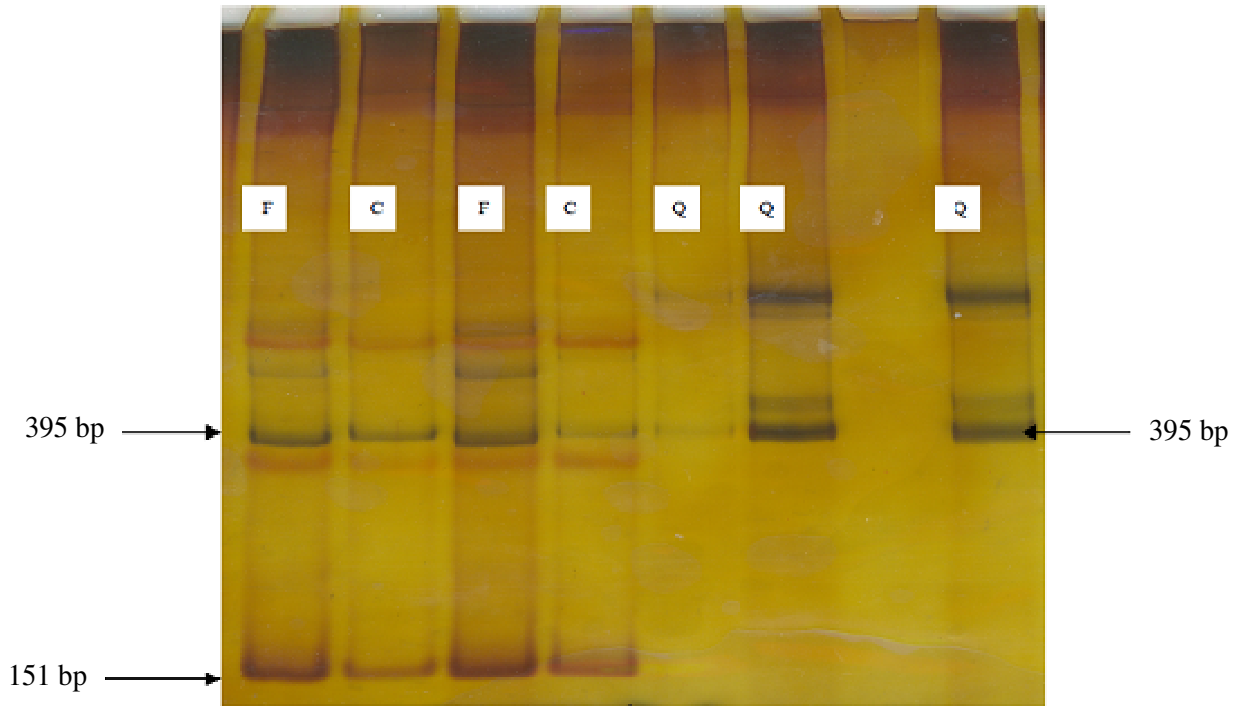
Figure 1. SSCP patterns of BoLA-DRB3.2. Patterns F and C show the presence of DRB3.2*16 allele while pattern Q shows the presence of DRB3.2*23 allele.

immune response) whereas allele *22 was associated with lower AMIR but higher CMIR. Additionally, BoLA DRB3.2*3 and *11 were associated with lower SCC, whereas alleles *22 and *23 were associated with higher SCC. The results of associations between BoLA DRB3.2 and production traits were, in some cases, antagonistic in that BoLA DRB3.2 alleles *11 and *23, which are associated with increased production traits, were associated with lower and higher SCC, respectively. Collectively, these findings advocate the use of alleles *3, *23 and *22 as reference points for more detailed mechanistic studies. Duangjinda et al. (2009) in the study on Holstein x Zebu found that DRB3*1 and *52 were the most associated with the occurrence of clinical mastitis, whereas *15, *51 and *22 were associated with resistance in crossbred populations. Nassiry et al. (2005) analyzed the frequency BoLA-DRB3 alleles in Iranian Holstein cattle. They found significant distinctions between Iranian Holstein cattle and other studied cattle breeds. The alleles associated with the resistance to mastitis and to bovine leukemia virus infection BoLA-DRB3.2*11 and *23 are detected with the frequencies 10.4 and 4.4%, respectively. Consequently they concluded that it is possible to find alleles in Iranian Holstein cows, which are associated with resistance to various diseases. These do not imply that genetic selection for mastitis resistance should be based on BoLA alleles, but that information on a variety of genes may aid in identification and selection for improved health.

Conclusion

The results of this study demonstrated that BoLA-DRB3.2 locus in the Sarabi breed cattle is highly polymorphic. More study and identification of all BoLA-DRB3.2 alleles in Sarabi breed could be useful for genetic programs in order to improve the genetic potential of cattle breeds such as Sarabi breed has high resistance to diseases, especially mastitis. Moreover, these findings did not support the association of BoLA-DRB3.2*16 and *23 alleles with resistance and susceptibility of Holstein and Saberi cattle to clinical mastitis. In contrast to SSCP, MPT-PCR technique was found inefficient in screening large populations for the presence of specific alleles.

ACKNOWLEDGMENT

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