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Genotyping of Lactoferrin and *CXCR1* Genes in Guilan Native Cows and Its Association with Milk Somatic Cell Score

Research Article

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ABSTRACT

Lactoferrin and *CXCR1* genes are involved in immune responses related to mastitis infection. In this study, the polymorphism and association of lactoferrin (LF) and *CXCR1* genes with milk somatic cell counts, as an indicator for mastitis detection, were investigated in Guilan native cow (Taleshi breed) using DNA blood samples of 100 cows from three different geographical zones (west, center, and east of Guilan province). The LF gene with a 301 bp fragment and CXCR1 with a fragment of 311 bp were amplified through PCR by using their specific primers. Then LF polymerase chain reaction (PCR) product was digested by *Eco*RI enzyme due to a single nucleotide polymorphism (SNP) at the related position (T>C) in intron 6 of LF and CXCR1 PCR product by *Bae*GI enzyme due to G > C SNP at position +735. Two alleles and three genotypes were observed for both genes in the studied populations. The observed genotypic frequencies of AA, AB, and BB were 52, 39, and 9% for LF and 67, 12, and 20% for CXCR1 locus, respectively. Three genotypes of LF locus were under Hardy-Weinberg equilibrium (P>0.05) but it was not for CXCR1 locus. The mean of somatic cell counts was 138×10^3/mL, much lower than the reported data of pure-bred and cross-bred cattle. Although there was no significant association (P<0.05%) between LF genotypes and somatic cell score (SCS), there was a tendency for association (P<0.1). The CXCR1c.+735 genotype had a significant association (P<0.05) with SCS. Sampling from different regions did not show a significant effect on SCS. The fix effects including lactation month, age, and lactation number had also no significant effect on SCS of the studied native cow.

KEY WORDS *CXCR1* gene, lactoferrin gene, milk somatic cells, native cow, PCR-RFLP.

INTRODUCTION

There are at least six native cow populations in Iran including Guilan native cow (Taleshi), Sarabi, Golpayegani, Sistani, Dashtyari and Najdi belonging to indicine (*Bos indicus*) breeds (Tavakolian, 2000). Native cow on the south coast of the Caspian sea has the largest population in Iran. There are 383149 native cows in Guilan province of Iran that constitute 70% of the total population of the province cows. The rest of the cows are about 25 thousand pure-bred and 40 thousand cross-bred cows. Due to limitations of forage crop production in Guilan province and genetic resistance of native cow, still, there is an increasing interest in keeping native cow with the lowest probability of mastitis incidence. Guilan native or Taleshi cow is a humped and horned cow with skin color variation. They graze on the pastures and forests. There are big differences in weight and height between male and female animals.

Tavakolian, (2000) reported that its milk yield ranges from 1350 to 1650 kgs per lactation; the average of adult body weight 410 kg in males and 320 kg in females and birth weight from 12 to 28.5 kgs.

Mastitis is the most important disease in dairy cattle that reduce milk and protein production and conception rate and increase parturition interval (Rekik *et al.* 2008). Milk's somatic cell count (SCC) is a reliable mean of early mastitis detection (De Haas *et al.* 2008; Green *et al.* 2004). Milk's SCC may consider as a marker to mastitis resistance in breeding programs (Shem *et al.* 2002). Zhang *et al.* (2007) showed a high genetic correlation between SCC and incidence of mastitis (0.6 to 0.8) which can be used as an accurate indirect mastitis marker.

Lactoferrin (LF) is part of the innate defense that effectively influences cow's immune system. LF is a bio-active molecule that is mainly expressed in mammary epithelial, neutrophil cells and milk (Pfaffl *et al.* 2003). Its biological functions are regulation of iron homeostasis, cellular growth, and differentiation, host defense against microbial infections, anti-inflammatory activity, and protection against cancer development and metastasis (Ward *et al.* 2005).

Many studies showed that clinical mastitis (Chaneton *et al.* 2008; Swanson *et al.* 2009) and subclinical mastitis (Hirvonen *et al.* 1999; Hagiwara *et al.* 2003) increases LF expression significantly.

There are some reports on the relationship between milk's SCC, performance and production traits with lactoferrin in pure-bred (Daly *et al.* 2006; Zhao *et al.* 2008; O'Halloran *et al.* 2010; Hemati Doust *et al.* 2013) and cross-bred cattle population (Wojdak Maksymiec *et al.* 2006), but there was no report on a native cow. Seyfert *et al.* (1994) reported a mutation in organic base 201 that replace Cytosine to Thymine to create a restriction site by *Eco*RI, result in two fragments of 201 and 100 bp and three genotypes for LF.

In the innate immune system response, Interleukin 8 is a chemokine produced by macrophages and some other cells such as endothelial cells which are bound to receptors CXCR1 and CXCR2. Youngerman *et al.* (2004) introduce novel single nucleotide polymorphisms and haplotypes within the bovine *CXCR2* gene in Holsteins and Jerseys cows. Galvao *et al.* (2011) examined single nucleotide polymorphisms (SNP) on *CXCR1* gene (CXCR1c.+735G>C) by using *Bae*GI enzyme in Holstein cows. Zhang *et al.* (2012) while hypothesized the CXCR1 as a candidate gene for bovine mastitis, they studied the association of bovine CXCR1 polymorphism with SCS in Chinese Holsteins. All of these reports are related to high productive breeds.

Thus, in the present study, we investigated the PCR-RFLP based polymorphism of LF and *CXCR1* genes and its association with SCS for Guilan native cows in the three different regions of Guilan province.

MATERIALS AND METHODS

Hundred whole blood samples were collected from the jugular vein using venoject tubes in ethylene diamine tetra acetic acid (EDTA). All these samples were collected from those Guilan native cows registered by the National Animal Breeding Center of Iran. The studied native cows were from an extensive region, form the west (Masoleh) to the east (Lahijan) of the province which was divided into three separate regions including Fuman (30 cows), Astane-ye Ashrafiyeh and Lahijan (34 cows) and Rasht (36 cows). Genomic DNA was extracted using the modified salting out method (Miller *et al.* 1988). The primers sequences used for amplifications of both genes were presented in Table 1.

RFLP-Genotyping was performed using two restriction enzymes: *Eco*RI for LF and *Bae*GI for CXCR1. *Eco*RI cut the PCR product due to an SNP at the related position (T>C) in intron 6 of LF and *Bae*GI due to G > C SNP at position +735. *Eco*RI digestion in a total volume of 20 µL was performed with 2 µg of PCR product, 0.5 µL buffer 10X and 2 µL enzyme; then samples were incubated at 37 ˚C for 3 h. *Bae*GI digestion was done with 2 µg of PCR product, 2 µL buffer 10X and 0.1 µL enzyme in a total volume of 15 µL, then samples were incubated at 37 ˚C for 17 h. The digestion products were electrophoresed 60 min at 90 volts, and digested fragments size was recognized by pUC Mix Marker, 8 DNA markers. Population gene and genotype parameters such as observed alleles, effective alleles, Hardy-Weinberg equilibrium, gene frequency, heterozygosity, and Shannon's index were analyzed by using POPGENE (version 1.32). Milk somatic cell count was recorded using DeLaval cell counter DCC (USA). Log transformation (Log_2) of SCC was done to make distributions less skewed. In order to study association between SCS and the studied genotypes, statistical analysis was done by generalized linear model (GLM) procedure of SAS software using following statistical model (SAS, 2004).

$$y_{ilklmn} = \mu + a_i + ln_j + lm_k + g_m + r_n + e_{ijklmn}$$

Where:
y_{ilklmn}: Log_2 (SCC/1000) or SCS.
μ: mean of SCS.
a_i: effect of cow age.
ln_j: effect of lactation number.
lm_k: effect of lactation month.
g_m: effect of genotype.
r_n: effect of sampling region.
e_{ijklmn}: error.

Table 1 The primers sequences for amplifications of LF and *CXCR1* genes

Gene		Primer sequence	Restriction enzyme		Reference
LF	Forward	5′ GCCTCATGACAACTCCCACAC 3′	*Eco*RI	5' -G AATTC- 3' 3' -CTTAA G- 5'	Wojdak Maksymiec *et al.* (2006)
	Revers	5′ CAGGTTGACACATCGGTTGAC 3′			
CXCR1	Forward	5′ CTTCCGTGAGGCCTATCAAC 3′	*Bae*GI	5' -GGC C- 3' 3' -C CGG- 5'	Galvao *et al.* (2011)
	Revers	5′ AGGTCTCAGCAATCACATGG 3′			

The fixed effects of this model are cow age, lactation number, lactation month and sampling regions.

RESULTS AND DISCUSSION

Genotyping of lactoferrin and *CXCR1* genes

The 301 bp PCR product of the lactoferrin gene (LF) was digested with *Eco*RI (Figure 1) restriction enzymes for PCR-RFLP analysis. The pattern of PCR-RFLP by *Eco*RI showed there are two restriction sites which make three genotypes: AA: 301 bp, BB: 201, 100 and AB: 301, 201, 100. For the *CXCR1* gene, the pattern of 311 bp PCR product digested by *Bae*GI related to an SNP located at CXCR1c.+735 also showed (Figure 2) three genotypes: GG: 311 bp, CC: 189, 122 and GC: 311, 189, 122.

Parameters of population genetic

The observed genotypes and allele frequency (%) are presented in Table 2. Higher AA genotype frequency related to the LF gene may show the priority of this genotype and undesirability of BB genotype resulted in elimination by artificial or natural selection in the native cow population. GC genotype related to the *CXCR1* gene also had a higher frequency than homozygote genotypes. It means those cows with different alleles in the *CXCR1* gene locus may have more viability than homozygous genotypes in the population. The High frequency of GC cows for CXCR1 locus may result from a natural selection which can generate disequilibrium in the population.

The χ^2 test (Table 2) showed that the genotype and allele frequencies of LF do not deviate from Hardy-Weinberg equilibrium ($\chi 2$=0.232, P>0.05). Equilibrium may happen due to lack of migration and effective selection in the native cow population with higher sustainability due to higher resistance and tolerance compared to a cross-bred or pure-bred cow. However, due to the relatively large amount of χ^2, this equilibrium was not seen for *CXCR1* gene. Allele frequencies of the LF gene for A and B were 0.715 and 0.285, respectively (Table 2). The high difference in observed allele frequencies gives higher Shannon's index (LF=0.60 and CXCR1=0.6907) as an indicator of gene diversity. There is no study for LF and *CXCR1* genes in humpback native cow (*Bos indicus*), but genetically improved cattle (*Bos taurus*) have been studied in different countries (Pawlik *et al.* 2009).

Wojdak Maksymiec *et al.* (2006) presented three genotypes of AA, BB, and AB for the LF gene with frequencies of 37.9, 2.42 and 59.68%, and allele frequency of 67.74 and 32.56% for A and B respectively, for Polish crossbred dairy cows with a various share of the Holstein-Friesian.

They observed significant deviation from the Hardy-Weinberg equilibrium (P<0.000252) and suggested that the disturbance in the genetic balance may have resulted from longtime selection for performance traits. Sender *et al.* (2010) identified all three genotypes of the LF gene with frequencies of 0.63 (AA), 0.32 (AB) and 0.05 (BB) with significant departure from the Hardy-Weinberg equilibrium for Polish Holstein cows. The low frequency of BB genotype in these studies on *Bos taurus* and our results on *Bos indicus* showed that elimination of B allele was not related to prior selection for productivity traits, as Sender *et al.* (2010) mentioned it, but it may result from natural selection related to viability traits.

Galvao *et al.* (2011) studied CXCR1c.+735G>C polymorphism and showed that the frequency of genotypes CC, GC, and GG were 22.3, 49.7, and 28.0%. The higher frequency of heterozygotes is the same as our results. Leyva-Baca *et al.* (2008) identified three novel SNP including: *CXCR1c.−344T > C*, *CXCR1c.−1768T > A*, and *CXCR1c.−1830A > G*, and a previously identified SNP (*CXCR1c.777G>C*) in the coding region. The last one is the same as CXCR1c.+735G > C which Galvao *et al.* (2011) have already fully explained. Leyva-Baca *et al.* (2008) also reported the higher frequency of heterozygotes in Holstein Cattle in Canada (CC, GC, and GG were 26.6, 54.8, and 18.60%).

Association of LF and *CXCR1* genotypes with SCS

Based on our results, the average somatic cell count was 138.61 × 10^3. Due to extensive sampling regions for this research, this report is extensible to Guilan native cow population. Relative lower SCC in the native cow population is a consequence of the better immune system, a higher number of immune-related genes, lower productivity and anatomical barriers to infectious. There is no observation on mastitis occurrence in Guilan native cow. Table 3 shows while the LF gene tended to associate significantly with SCS; CXCR1c.+735G > C had a significant association (P<0.05). Our data showed that GC and CC of CXCR1 genotypes had significantly lower SCS.

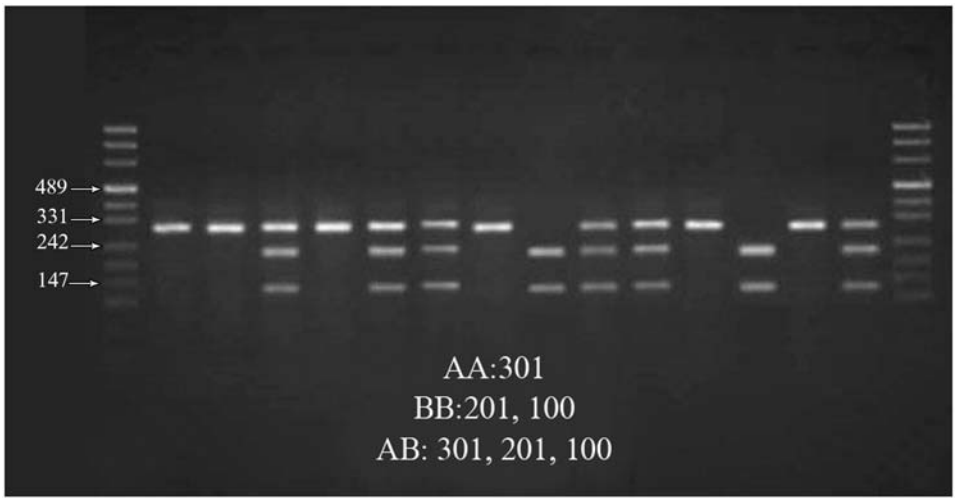

Figure 1 Polymorphism of the LF gene following digestion with *Eco*RI

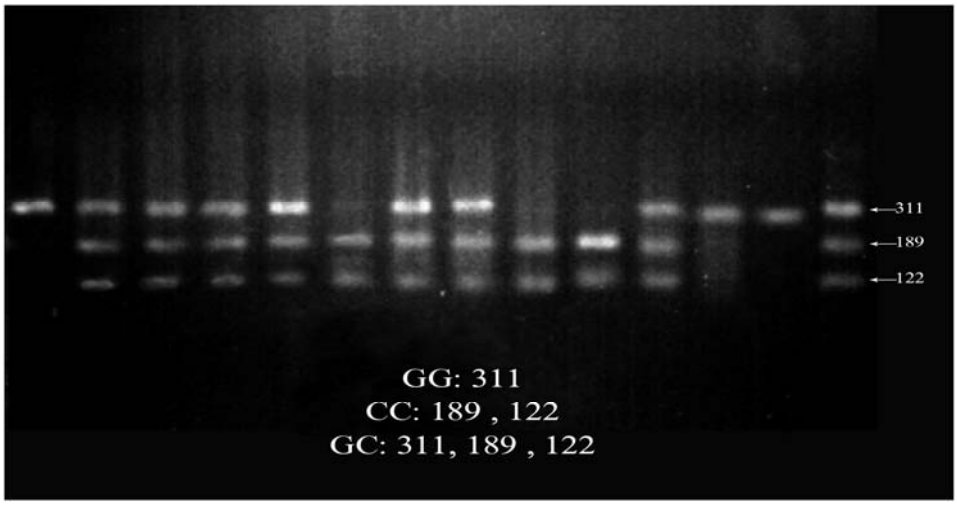

Figure 2 Polymorphism of the LF gene following digestion with *Bae*GI

Sender *et al.* (2010) also reported the same of our result for LF genotypes and a significant relationship between BB and SCS (P<0.01) in one of two studied herds. Zhao *et al.* (2008) reported an association of LF promoter polymorphism and susceptibility to mastitis. Although no significant difference was observed between heterozygotes of two groups, there were significant differences for both homozygote genotypes (P<0.01). The frequency of alleles A and B in cows with subclinical natitis (experimental group) had significant departure from Hardy-Weinberg equilibrium (P<0.01). A study on the CXCR1 chemokine receptor gene in Canadian Holstein cattle showed that the SNP *CXCR1c.−1768T > A* was associated with estimated breweding value (EBV) for SCS in the first and second lactations and overall three lactations (Leyva-Baca *et al.* 2008).

Polymorphisms in bovine immune genes and their associations with SCC in Irish Holstein cattle showed that CXCR1-777 had a tendency for association (P<0.1) with SCS in the cows of Irish dairy germplasm (Beecher *et al.* 2012). Zhang *et al.* (2012) showed that the SNP CXCR1c.+735G > C had no significant association with the SCS, milk yield, milk fat and milk protein percentage of Chinese Holsteins which is different from our finding on the native cows. Galvao *et al.* (2011) reported two genotypes (GC and CC) of well-known locus of CXCR1c.+735 were associated with the incidence rate of clinical mastitis. Therefore, this result and our finding showed that both of these genotypes (GC and CC) are related to cow immune system. This conclusion can also be confirmed by Chegini *et al.* (2018). They reported that there is considerable genetic correlation (r_g) between SCC and mastitis.

Table 2 The observed genotype and allele frequencies and Hardy-Weinberg equilibrium test

Gene	Genotype frequency (%)			χ^2	Allele frequency (%)	
LF	AA	AB	BB	0.232	B: 28.5	A: 71.5
	52	39	9			
CXCR1	GG	GC	CC	12.64	C: 45.96	G: 54.04
	20	67	13			

Table 3 Association of *LF* and *CXCR1* genotypes with somatic cell score (SCS)

LF			CXCR1		
Genotype	Number	Somatic cell score (SCS)*	Genotype	Number	Somatic cell score (SCS)**
AB	39	6.74^{a}	GC	60	6.76^{a}
AA	52	6.24^{ab}	GG	27	5.97^{b}
BB	9	5.78^{b}	CC	13	5.95^{b}

* P < 0.1 and ** P < 0.05.

Table 4 Association of SCS' mean with age, parity and lactation months

Age*		Parturition**		Lactation months**	
Year	Somatic cell score (SCS)	Parity	Somatic cell score (SCS)	Month	Somatic cell score (SCS)
3	4.32^{b}	1	6.24^{a}	1	6.89^{abc}
4	5.90^{a}	2	6.16^{a}	2	5.39^{c}
5	6.68^{a}	3	6.36^{a}	3	5.57^{bc}
6	6.48^{a}	4	6.53^{a}	4	6.20^{abc}
7	6.42^{a}	5	6.10^{a}	5	6.78^{abc}
8	6.24^{a}	6	6.80^{a}	6	5.95^{abc}
9	6.36^{a}	7	6.01^{a}	7	6.42^{abc}
10	6.31^{a}	8	6.32^{a}	8	6.96^{abc}
11	6.62^{a}	9	7.34^{a}	9	6.45^{abc}
12	6.45^{a}	10	7.20^{a}	10	6.91^{abc}
13	6.51^{a}	11	6.36^{a}	11	7.66^{a}
14	6.90^{a}	-	-	12	7.22^{ab}
15	6.60^{a}	-	-	-	-

** P ≤ 0.05.

Therefore, it can be recommended as a criterion to improve udder health, especially when there is no record for mast.

Association of evaluated fixed effects and SCS

The fixed effects including age, parturition, the month of lactation and sampling regions were evaluated according to the statistical model. There was a significant relationship between age and SCS (P<0.1), with a correlation coefficient equal to 0.1. In the first column of Table 4, SCS increases by rising age. The number of parturition has shown no significant effect on SCS (P<0.05) with a low correlation coefficient of about 0.06. As these two factors of age and parity are commonly associated with SCS in dairy cattle (Tančin, 2013; O'Brien *et al.* 2009), therefore our results may be a specific event of a native cow. Lactation month significantly affected the SCS (P<0.05). Higher SCS in the first month shows the possibility of mastitis prevalence in the first lactation month. The correlation coefficient for the lactation month and SCS was 0.24. According to O'Brien *et al.* (2009), SCC decreased between the period 5 to 35 days in milk (DIM) and 36 to 65 DIM and increased thereafter.

Higher parturition showed rapidly increasing SCS in the mid to late lactation period.

The association of SCS' mean and sampling region was shown in Table 5.

Table 5 Relationship of sampling region and SCS' mean

Region	Somatic cell score (SCS)*
Fuman	6.56^{a}
Lahijan	6.44^{a}
Rasht	6.21^{a}

* P < 0.1.

Sampling region didn't show a significant effect on SCS which indicates the same keeping and breeding conditions in the traditional systems, the similarity of the sampling regions and the similarity of migration patterns.

CONCLUSION

Although due to low productivity, native cows are not economically profitable, they have high precious characters such as disease resistance, environmental adaptation, gene flow and fitting with low-input agricultural systems that is

suitable in organic farming systems. Due to a growing market for organically produced dairy products, milk with lower SCC was preferred for organic systems. According to our report, Guilan native cow has lower milk SCC (138.61×10^3) compared to cross-bred cattle (261.49×10^3), which is a very substantial factor for the health of udder. Moreover, lower SCS has many advantages for healthy products, dairy costs and the welfare of livestock and farmers. Moreover, alleles associated with SCS can be considered for genetic improvement of native cow or other breeding programs using native cow.

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