Short Paper

Association between bovine lactoferrin gene variant and somatic cell count in milk based on *EcoR*I restriction site

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Summary

Mastitis is one of the most serious and costly diseases affecting dairy cattle production. In the present study, effects of a lactoferrin gene polymorphism (intron 6) on milk somatic cell count (SCC) and subclinical mastitis was investigated in 121 Holstein dairy cattle. Two alleles of A and B and two genotypes of AA and AB were found in an *EcoR*I recognized single nucleotide polymorphism in intron 6 of lactoferin gene with the frequencies of 85.12, 14.88, 70.25 and 29.75%, respectively. No homozygous BB cows were detected in the studied population. Marker-traits association analysis showed that *EcoR*I marker site in lactoferrin gene was significantly related to SCC ($P \leq 0.01$) with AB as a desirable genotype. The obtained results in the present study indicated that selection of the AB genotypes for *EcoR*I recognized single nucleotide polymorphism might contribute to a reduction of SCC in Holstein dairy cattle.

Key words: Lactoferrin, Somatic cell count, Polymorphism

Introduction

Mastitis is a common and the most costly disease in dairy cattle. It is an inflammatory reaction of the mammary gland characterized by an influx of leukocytes (primarily polymorphonuclear neutrophils) from the blood into the milk as the cow's immune system responds to bacterial infection, therefore, the concentration of somatic cells in milk increases (Rupp and Boichard, 2003; Kulig *et al*., 2010). In addition, mastitis is the most frequently cited reason for culling and use of antibiotics in lactating dairy cattle (Norberg, 2005). Mastitis may occur in both clinical and sub-clinical forms. The first one is easily recognized due to visible changes in the udder (swelling, tenderness, hardness) and milk characteristics (flakes, clots). Sub-clinical infections are characterized by no visible signs of disease in both the udder and milk, although concentration of somatic cells in milk increases, milk production decreases and additionally milk composition may be changed. Moreover, high somatic cell concentration in milk is undesirable because it reduces the shelf-life of dairy products. The measure of somatic cell count (SCC) in milk reflects the incidence of both clinical and sub-clinical infections but monitoring SCC is particularly useful in diagnosis of sub-clinical mastitis when milk appears to be normal (Jones, 1998; Petrovsky and Stefanov, 2006; Kulig *et al*., 2010). The use of breeding as a measure to combat mastitis in dairy cattle has been the subject of considerable research effort over the past 20 years or so. Two major modes of studies are used for the genes underlying traits associated with mastitis resistance: the polygenic and major gene approaches (Detilleux, 2009). Identification of genetic markers associated with SCC might be helpful in improving cows' health by implementing appropriate cattle-breeding programmes. Moreover, it might improve milk production traits as a healthy udder since it is the most important factor responsible for milk yield and quality (Jones, 1998). There are some promising mastitis-associated candidate genes which play a particularly important role in the immunological functions of the mammary gland. One of these candidates is the gene encoding lactoferrin (LTF), which was mapped to bovine chromosome 22, contains 17 exons and spreads out on about 34.5 k base pairs (kbp) of genomic DNA (Schwerin *et al*., 1994; Martin-Burriel *et al*., 1997). Wojdak Maksymice *et al*. (2006) reported a significant association between LTF genotypes and SCC. On account of the facts mentioned above, it is reasonable to investigate any associations between LTF polymorphism and somatic cell count.

Materials and Methods

The analysis was carried out on 121 Holstein cows sampled from a farm located in the Mashhad region in the northeastern part of Iran and all animals were kept in identical environmental conditions. The cows were milked twice a day using a pipeline milking machine.

Peripheral blood samples were collected randomly from all the cows in EDTA containing tubes and DNA was purified with modified salting out method (Miller *et al*., 1988). The primer pair of Forward: 5′-GCCTCATGACA ACTCCCACAC-3′ and Reverse: 5′-CAGGTTGACACA TCGGTTGAC-3′ (Wojdak Maksy-mice *et al*., 2006) was used to amplify a fragment of 301 base pairs from intron 6 of LFT gene containing *Eco*RI restriction site. PCR reaction is prepared in 25 µl final volume containing 0.1 mM of each dNTPs, 1.0 µM of each primer, and 2.5 µl of 1 × reaction buffer, 1.5 mM $MgCl_2$, 1.0 U *Taq* DNA polymerase, and 200 ng of template DNA. PCR thermal cycling profile consists of an initial denaturing step at 95°C for 5 min, following by 30 cycles of 30 sec at 94°C, 30 sec at 60°C, 1 min at 72°C, and a final extension step of 10 min at 72°C. Presence of PCR product was tested on a 1% agarose gel. The RFLP assay of the amplified fragment was done using *Eco*RI enzyme (for 3 h, with 6.5 units/20 µl, at 37°C) and restriction fragments were analysed with 2% agarose gel electrophoresis. The *Eco*RI digestion of lactoferrin produced two patterns of bands, one containing a 301 bp fragment (allele A, no sequence recognized by the restriction enzyme), and two bands of 201 bp, and 100 bp (allele B). Allelic and genotype frequencies were determined and the Hardy-Weinberg equilibrium was verified with χ^2 test using POPGEN 1.32 software. The marker-traits associations between observed poly-morphism and milk SCC were analysed using GLM procedure of SAS program (SAS, 1989). The effects of genotypes, parity and season were treated as sources of variability. The year was divided into two seasons: autumn/winter from October to March, and summer/ spring from April to September. The raw counting of SCC was transformed to a logarithmic (log_2) scale in order to balance the distribution. The following statistical model was applied:

$$Y_{ijklm} = \mu + a_i + b_j + d_l + (ab)_{ij} + e_{ijklm}$$

where,
Y_{ijklm}: Somatic cell count (3 + log_2 SCC)
µ: Mean somatic cell count for herd (3 + log_2 SCC)
a_i: Effect of genotype
b_j: Effect of lactation number
d_l: Effect of season
$(ab)_{ij}$: Interaction effect between genotype and lactation number
e_{ijklm}: Error

Results

Two alleles of A and B were found in exon 6 of LTF gene with the frequencies of 85.12 and 14.88%, respectively. The alleles controlled the occurrence of two genotypes AA and AB with the frequencies of 70.25 and 29.75%, respectively (Table 1). The studied population had significant deviation from Hardy-Weinberg equilibrium for LTF gene (Table 1).

There was no BB homozygous genotype observed in population. The genetic balance of the population can be affected by absence or weakness of selection for performance. For association study between LTF genotypes and milk SCC, some factors such as lactation parity and season are taken into account. The results are presented in Table 2.

Table 2: Associations between log_2 SCC and the analysed factors

S.O.V[1]	df	F-value	P-value	Significance
LTF genotype	1	30.2	0.01	**
Lactation number	5	3.15	0.008	**
Season	1	1.26	0.261	NS
Genotype × lactation	4	12.66	0.01	**

[1] Source of variation. ** P<0.01, and NS = Non-significant

The results of association study revealed significant relations between SCC with LTF genotype, lactation number, and interactions between genotype × lactation (P<0.01). Furthermore, no significant association was found between SCC and season (Table 2). The cows with AA genotype had higher SCC (transformed to a logarithmic scale) than cows with AB genotype (Table 3). Furthermore, the cows in 2nd lactation number had higher SCC than cows in other lactation numbers (Table 3).

Though, the significant association between SSC and season was not observed in present study, but the SCC was numerically higher in spring/summer than autumn/ winter seasons (Tables 2 and 3).

Table 3: Means and SD of SCC (log_2 SCC) in milk in relation to analysed factors

Effect	Mean of log_2 SCC	SD
AA genotype	15.251	2.282
AB genotype	14.314	2.195
1st lactation	15.066	1.849
2nd lactation	15.783	0.646
3rd lactation	14.286	1.905
4th lactation	14.645	0.228
5th lactation and higher	15.465	0.556
Autumn/winter	16.759	1.316
Spring/summer	16. 945	1.012
Total	15.610	3.841

Discussion

Mastitis is one of the most important diseases in dairy

Table 1: Allelic and genotype frequencies of LTF in Holstein cattle

Allele	Observed No.	Frequency (%)	Genotype	Observed No.	Frequency (%)
A	103	85.12	AA	85	70.25
			AB	36	29.75
B	18	14.88	BB	0	0
Total	121	100		121	100

a: Observed, and b: Expected

cattle which are occurring in mammary gland by pathogen infection. Antibiotics treatments resist bacteria and pose an increasing problem. Animal welfare aspects and the risk of antibiotic residues in dairy products are other important reasons for reducing the frequency of mastitis. Thus, genetic selection for decreased mastitis susceptibility will be of great interest (Norberg, 2005). Identification genetic markers associated with mastitis susceptibility, resistance or both would allow producers to decrease costs associated with mastitis by improving herd health through animal selection. Lactoferrin can be considered as a candidate gene affecting mastitis resistance in dairy cattle populations. The lactoferrin gene polymorphism occurs in both coding and regulatory regions as well as in introns (Seyfert and Kuhn, 1994; Martin-Burriel *et al*., 1997; Li *et al*., 2004). Li *et al*. (2004) found polymorphisms in exons 4, 8, 9, 11, 15 and intron 4 of lactoferrin gene. A mutation occurring in exon 4 led to amino acid substitution (isoleucine to valine), while other mutations were silent. In this study, we investigated the association between the lactoferrin gene polymorphism occurring in intron 6 and susceptibility to mastitis. Two genotypes of AA and AB were found in the present study with the frequencies of 70.25 and 29.75%, respectively. No BB genotype was detected in our study. The absence of BB genotype in our study could be due to small sample size. Seyfert and Kuhn (1994) found two alleles of A and B and three genotypes of AA, AB, and BB in the LTF locus using *EcoR*I-RFLP with genotypic frequencies of 35.8, 3.54 and 60.66%, respectively. Moreover, Wojdak Maksymice *et al*. (2006) found two alleles of A and B and three genotypes of AA, AB and BB with frequencies of 67.74, 32.56, 37.9, 2.42 and 59.68%, respectively. The allelic frequencies in our study were nearly close to that reported by Sender *et al*. (2010). They showed frequencies of 79.23 and 20.77% for A and B alleles, respectively. In agreement with these results, the BB genotype showed the lowest frequency (4.8%) in their study. In the present study, the observed genotypes were significantly associated with SCC and cows with AB genotype showed higher resistance to increase of somatic cell count and subclinical mastitis than AA genotype (Table 3). In agreement with our results, Wojdak Maksymice *et al*. (2006) reported a significant association between LTF genotypes and SCC, but homozygous AA animals had a lower SCC than heterozygotes AB animals. Statistically significant association between SCC and lactation number was found in the present study. The highest and the lowest SCC were found in the milk of cows in the second and the third lactation number, respectively.

In contrast, previous study showed that lactational means of SCC tended to increase with advancing order of lactation and age of calving within each parity in a linear relationship. In general, as cows become older, a greater percentage of them have higher SCC. Higher SCC scores in older cows were not caused by age but by increased rate of udder infections (Laevens *et al*., 1997; Amin *et al*., 2000). Associations between milk SCC and lactation number (age), herd, breed and lactation stage (days elapsed from calving) were previously published in some studies (Laevens *et al*., 1997; Busato *et al*., 2000; Wojdak Maksymice *et al*., 2006).

According to our results, there was no significant association between SCC and season. This is similar to the findings of Wojdak Maksymice *et al*. (2006). The SCC is generally lowest during the winter and highest during the summer (Dohoo and Meek, 1982), which coincides with an increased incidence of clinical mastitis during the summer months (Smith *et al*., 1985). Smith *et al*. (1985) showed that the rate of infection with environmental pathogens was highest during the summer, and coincided with the highest number of coliforms in bedding. They suggested that the stress of high temperatures and humidity could increase susceptibility to infection as well as increase the number of pathogens to which the cows were exposed. In our study, though no significant association was found between SSC and season, the SCC was numerically higher in spring/summer season than in autumn/winter.

The results obtained in the present study suggest that lactoferrin locus might be a promising candidate gene that can influence udder health. However, it would be better to include a larger sample size in the future studies and, if possible, to add information about clinical mastitis. This would increase the efficiency of selection for udder health, and therefore for milk quality.

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