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Research Article

Detection of Single Nucleotide Polymorphism in the 5'-Untranslated Region (5'UTR) of Lactoferrin Gene and Its Association with Clinical Mastitis in Cattle

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ABSTRACT

Lactoferrin (Lf), plays an important role in the innate immune system and defence mechanism of mammary gland of milk producing animals. The amount of lactoferrin increases during inflammatory process and viral infections. Many polymorphisms have been found in the bovine Lf Gene. The present study aimed to identify single-nucleotide polymorphic (SNP) site in the 5' untranslated region (5'-UTR, position +32) of the bovine Lf gene in Holstein dairy cattle and to quantify their association with clinical mastitis using Allele-specific polymerase chain reaction (PCR) method. Blood samples were collected from 89 multiparous Holstein dairy cows with a history clinical mastitis (n=38), and cows without mastitis as control group (n= 51). In this study, we detected type of SNP in the 5' untranslated region (5'-UTR, position +32) using a designed Allele specific PCR that validated following sequencing method. We did not found evidence for a significant association between SNP in the 5'-UTR (+32: G/G) Lf gene and the mastitis in dairy cattle in Iran. The results of this study did not provide any support to considered 5'-UTR polymorphism (position +32) as a marker for resistance/susceptibility to mastitis in dairy cattle.

Key Words: SNP - lactoferrin gene, mastitis, dairy cattle

ÖZET

LAKTOFERRİN GENİNDE ANLATIM YAPMAYAN 5' BÖLGESİNDEKİ (5'UTR) TEK NÜKLEOTİD POLİMORFİZMİNİN BELİRLENMESİ VE SIĞIRDA KLİNİK MASTİTİS İLE İLİŞKİSİ

Laktoferrin (Lf) süt üreten hayvanların doğal bağışıklık sisteminde ve meme bezinin savunma mekanizmasında önemli bir rol oynamaktadır. Viral enfeksiyonlar ve yangı sürecinde laktoferrin miktarında artış gözlenmektedir. Sığır Lf geninde birçok polimorfizm saptanmıştır. Bu çalışmanın amacı Siyah Alaca süt sığırında Lf geninin anlatım yapmayan 5' bölgesinde (5'-UTR, pozisyon +32) tek nükleotid polimorfizminin belirlenmesi ve Allel-spesifik polimeraz zincir reaksiyon (PCR) metodu kullanılarak klinik mastitis ile ilişkisinin ortaya koyulmasıdır. Birden çok doğum yapmış olan, klinik olarak mastitis hikayesi bulunan (n=38) ve mastitis hikayesi bulunmayan kontrol grubu (n=51) olmak üzere toplam 89 adet Siyah Alaca sütçü sığırdan kan örnekleri toplanmıştır. Bu çalışmada, sekanslama metoduyla doğrulanan tasarlanmış allel spesifik PCR yöntemi ile anlatım yapmayan 5' bölgesinde (5'-UTR, +32) SNP saptanmıştır. Lf geninin 5'-UTR (+32: G/G) bölgesinde saptanan SNP ile İran'da süt sığırlarında mastitis arasındaki ilişkinin önemli olduğuna dair bir kanıt bulunamamıştır. Bu çalışmanın sonucu 5'-UTR (pozisyon +32) polimorfizminin, süt sığırında mastitise direnç/yatkınlık işaretçisi olduğuna dair herhangi bir destek sağlamamıştır.

Anahtar Kelimeler: SNP - laktoferrin geni, mastitis, süt sığırı

Introduction

Inflammatory conditions of the udder (mastitis) represent a major problem in dairy cattle management. It is the most costly disease for dairy cattle. Producers suffer a huge loss due to veterinary treatment costs or, in some cases, necessary culling of the infected animals. The milk of cows afflicted with mastitis is not suitable for consumption, which also leads to a reduction in the profitability of the production process (Wojdak-Maksymiec and Kmiec, 2006).

Although advances have been made in controlling mastitis, but most of the treatments are still ineffective. Selection of cows for low somatic cell count has been used to reduce the incidence of mastitis, but this can also result in selection of non desirable traits. With worldwide concern about the use of antibiotics in farm animal, the development of new selection techniques are needed to reduce reliance on antibiotic treatment of mastitis. One possible solution could be selection of cows with higher innate levels of antimicrobial proteins in their milk. This approach has the potential to reduce the use of antibiotics and their possible exposure to consumers, while improving the health of dairy cattle and profitability of dairy operations (Person et al., 2007*)*. Lactoferrin (Lf) is a protein which exerts several functions related to innate immunity. Lf was first known for its iron chelating properties, the basis of two of its activities, bacteriostasis and protection against oxygen radicals catalyzed by free iron (Legrand et al., 2004). Lf is a component of the natural protection systems within the body. Non-specific, multifunctional Lf is present in milk and such external secretions of the body as saliva, bile, tears and sperm. It is released by secondary granules of neutrophils and epithelial cells in high concentrations in response to inflammatory stimuli (Baggiolini et al., 1970; Harmon and Newbould, 1980; Plaffl et al., 2003). Lactoferrin is composed of 690 amino acids (Zheng et al., 2005). The traditional functions of lactoferrin are: iron binding exhibiting growth of bacteria and an iron source for infants (Tsuji et al., 1989). However, the bacteriostatic and bactericidal mechanisms of lactoferrin are very complicated. Lf can bind by its N-terminal end to the bacterial cell membrane and increases sensitivity to lyzosyme, antibodies and antibiotics (Diarra et al., 2003; Li et al., 2004). The concentration of Lf in milk from healthy cows is low (0.1–1.0 mg/ml) and rapidly increases in cows with sub-clinical and clinical mastitis and is positively correlated with somatic cell count (SCC)-higher SCC and higher lactoferrin concentrations (Hagiwara et al., 2003; Kawai et al., 1999). The bovine lactoferrin gene is located on chromosome 22 spanning approximately 34.5 kb of genomic DNA and comprises 17 exons ranging from 82 (exon 1) to 225 bp (exon17) (Zheng et al., 2005). Single nucleotide polymorphisms (SNPs) are the most abundant genetic variations in mammalian genomes, occurring in regulatory, exon and intron regions of genes (Teng et al., 2006). Seyfert and Kuhn (1994) found 16 polymorphisms in *Lf* gene. Lately, Li et al. (2004) found 3 new SNPs in the promoter region, and also 4 SNPs in exons 4, 8, 9, 15 and one in intron 4. None of these SNPs has been verified as a potential genetic marker for production traits in dairy cattle. Kaminiski et al. (2006) found SNP (G/C) in position +32 and assessed its associations with milk performance traits, including SCC. The aim of this study was to identify the single nucleotide polymorphisms in the region 5'-untranslated region (5'-UTR) of the lactoferrin gene using Allele-Specific polymerase chain reaction (PCR) technique and its relationship with clinical mastitis in Iranian Holstein dairy cattle.

Materials and Methods

Animal and sample collection

This study was carried out on registered multiparous Iranian Holstein cattle at the farm of Farzis milk and meat producing complex in Shiraz, Fars province, south of Iran. Shiraz is located at a latitude of 29° 38′ N and longitude 52° 36′ E. Its altitude is 1296 m above sea level. The climatic condition is relatively rainy mild winters and hot dry summers with the average temperature of 17 °C ranging between 5 and

30 °C (Ansari-Lari and Abbasi 2008). The cows were kept under the same weather and management conditions in a similar manner. Cows were fed standard rations (total mixed ration) including mainly alfalfa, corn silage, beet pulp, cotton seed, soybean, corn and barley. The cows were milked three times daily with the use of a pipeline milking machine.

A total of 89 multiparous Holstein dairy cows were selected with determined history of clinical mastitis in the previous lactation. A total number of 38 cows with clinical mastitis and 51 cows without clinical mastitis were selected as case and control groups, respectively. Blood samples (2 ml) were collected from each cow via caudal venipuncture into tubes containing EDTA anticoagulant and then were sent to the laboratory and stored at -20º C for subsequent DNA extraction.

Genomic DNA extraction

Samples were removed from the -20° C freezer and then were melt and Vortex for 10 seconds. DNA was extracted using a DNA isolation kit (MBST, Iran) according to the manufacturer's instructions. The DNA was quantified spectrophotometrically, and the integrity assessed via agarose gel electrophoresis (0.8%). Extracted genomic DNA samples were stored at -20ºC for subsequent analysis.

Primers

The single nucleotide polymorphisms (SNPs) in the 5' untranslated region (5'- UTR, position +32 from transcription start site) of lactoferrin promoter gene (G/G at Lf +32) were genotyped using a designed double PCR amplification of the specific alleles. In the present study, three primer sets (I, II and III) were used for PCR. Allel-specific primers (HajR2, HajF2) for each allele were designed using the software Primer Premier 5 based on the +32 area of 5'-UTR lactoferrin gene. All oligonucleotide primers used in this study were synthesized by CinnaGen Company in Iran. Main characteristics of the primers and expected length of produced Fragment by each pairs of primers are presented in Table 1.

Allele specific PCRs and genotyping

Primer combination II was used to identify G allele in 5'- UTR and contained primers HajF2, LFR1-R, Lac-1, Lac-2; primer combination III also contained four primers (HajR2, LFR1-F, Lac-1, Lac-2) for detection of C allele. The PCR reaction (25 µl) was performed in 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 250 µM of each dNTP, 10-20 pmol of each primer (Cinnagen Inc., Tehran, Iran), and 0.5 U *Taq* DNA polymerase (Fermentas; Glen Burnie, Maryland) using 2 µl of DNA extracted as template.

Amplification was performed with a Bio-Rad thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) by using a single denaturation step (5 min at 94ºC), followed by a 35-cycle program, with each cycle consisting of denaturation at 94ºC, for 1 min, annealing at 65-68 ºC for 45 sec, and extension at 72 ºC for 45 sec, with a final extension; a final extension step (72 ºC for 7 min) was also used. The PCR products were analyzed by agarose gel electrophoresis (3%) after ethidium bromide staining and visualized under ultraviolet transillumination.

Sequencing

The validity of our designed Allele-specific PCR was verified by testing 21 individuals including three polymorphisms. Related PCR products generated by LFR1-F and LFR2-R primers (Table 1) were sequenced using capillary DNA analyzer (ABI 3730, Applied Biosystems, Foster City, California) after sequencing reactions with a Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems). The PCR reaction condition for primer combination I was the same as that for two previously described PCR with primer combination II and III with the exception of annealing temperature of 57.8 ºC.

Point mutations were screened by DNA sequence analysis for +32 site in 5'UTR after multiple alignment using ClustalW alignment option available in the MEGA 4 software.

Table 1. Characteristics of the specific primers used in PCR.
Tablo 1. PCR yönteminde kullanılan spesifik primerlerin özellikleri.

	Primer	(5`- 3`) Sequence	Purpose (references)	Annealing Temperature (ºC)	Length (bp)
Primer Combination I	LFR1-F	5' -GACAGCCTTTGGGCACTTAG-3'	Sequencing (O'Halloran, et al., 2009)	57.8	1068
	LFR1-R	5' -GGGTAGGACAGAAGCGACAG-3'			
Primer Combination II	HajF2	5'-TTCGTTCCGGAGTCGCCCCAGGA**A**<u>**A**</u>**G**-3'	G allel specific detection (designed in this study)	65-68	408
	LFR1-R	5'-GGGTAGGACAGAAGCGACAG-3'	(O'Halloran, et al., 2009)		
	Lac-1	5'-GCCTCATGACAACTCCCACAC-3'	Internal control (Wojdak-Maksymiec and Kmiec 2006)		301
	Lac-2	5'-CAGGTTGACACATCGGTTGAC-3'			
Primer Combination III	HajR2	5' -GGGGACGAAGAGCTTCATGGCTG<u>**T**</u>**G**-3'	C allel specific detection (designed in this study)	65-68	709
	LFR1-F	5' -GACAGCCTTTGGGCACTTAG-3'	(O'Halloran, et al., 2009)		
	Lac-1	5' -GCCTCATGACAACTCCCACAC-3'	Internal control (Wojdak-Maksymiec and Kmiec, 2006)		301
	Lac-2	5' -CAGGTTGACACATCGGTTGAC-3'			

*The underlined bases are those modified from the original sequence to increase the specificity of the allele-specific PCR

Statistical analysis

The results were analyzed from the aspect of relationship between the polymorphism within 5' UTR, position +32 of the lactoferrin promoter region and clinical mastitis. The results obtained were subjected to statistical analysis by means of chi-square test for independence. Three categories of different mutation types of control (without clinical mastitis) and patient (with single or more occurrence clinical mastitis) groups were tested for significant differences by Pearson Chi-Square test for 2×2 contingency tables. All results were statistically analyzed at the $P \leq 0.05$ level of confidence using the SPSS statistical software (Version 15.0, SPSS Inc, Chicago, Illinois).

Results

To identify polymorphism in 5' UTR, position +32 of the Lf gene, best method of using Allele Specific PCR consisted two separate chain reactions that 301 bp product makes term as internal control. As expected, Allele Specific PCR analysis of internal PCR products from different 5' UTR, position +32 genotypes (GG, GC, CC) showed three distinct banding patterns (Figure 1). As the Allele Specific PCR method results were completely consistent with those of the direct sequencing method in examined samples (Figure 2), we used this methodology of genotyping for all cases in this study. GG genotype as wide type show no mutation in 5' UTR, position +32, whereas GC and CC genotypes show that mutation is present in one and both alleles, respectively. According

to the double tube allele- specific PCR results, three kinds of genotypes, named GG (709 bp, 301 bp), GC (709 bp, 408 bp and 301 bp), and CC (408 bp and 301 bp), were easily distinguished based on the SNP within the 5' UTR of lactoferrin gene.

The results of the present study showed the occurrence of polymorphism (G→C) within 5'-UTR in the both groups of with and without clinical mastitis (Table 2). Cows with and without clinical mastitis were compared regarding the mutation within 5'-UTR, position +32. There were no significant differences between the occurrences of different polymorphism within 5'-UTR, position +32 for the two groups of cattle (P > 0.05; Table 2).

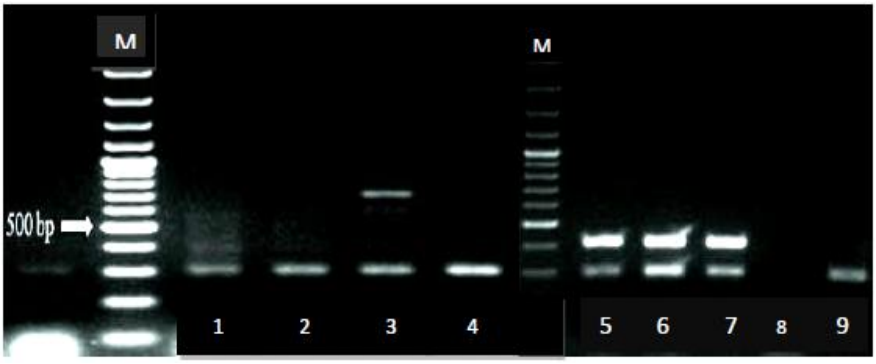

Figure 1. Electrophoresis figure showing reaction PCR products of some samples. Primer combination II (lane 1-4) and primer combination III (lane 5-9) were used to amplify 408 bp (G specific band) and 709 bp (C specific band), respectively. The fragment of 301 bp was also produced as internal control in the both primer combination. M: marker 100 bp. GG genotype samples: lane 1 and 2 does not produce C allele specific band (709 bp) using primer combination III (Table 1); whereas, lane 5 and 6 positive for G allele by producing 408 bp using primer combination II. Lane 4 and 7: PCR product for sequenced GG genotype (positive control). CC genotype: lane 3 positive for C allele by producing 709 bp using primer combination III and lane 9 does not produce G allele specific band (408 bp) using primer combination II. Lane 8: Distilled water (no template).

Şekil 1. Bazı örneklerin PCR ürünlerine ait elektroforez görüntüsü. Primer kombinasyonu II (sıra 1-4) ve primer kombinasyonu III (sıra 5-9) sırasıyla 408bp (G spesifik bant) ve 709bp (C spesifik bant) ürün oluşumunda kullanılmışlardır. 301bp'lik parça aynı zamanda her iki primer kombinasyonunun iç kontrolü olarak üretilmiştir. M: marker 100 bp. GG genotip örnekleri: sıra 1 ve 2 primer kombinasyonu III kullanılarak (Tablo 1) C allel spesifik bandını üretmemiştir. Sıra 4 ve 7: GG genotipi için sekanslanmış PCR ürünü (pozitif kontrol).CC genotipi: sıra 3; primer kombinasyonu III kullanılarak C alleli için pozitif 709bp üretilmiş ve sıra 9; primer II kombinasyonu kulllanılarak G alleline spesifik bant (408bp) üretilmemiştir. Sıra 8: Distile su (örnek yok).

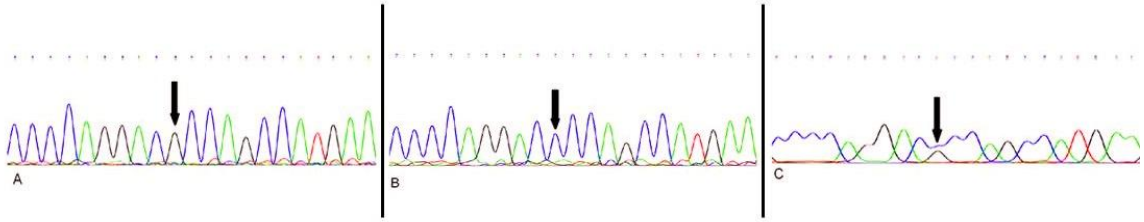

Figure 2. Partial chromatogram of the cow lactoferrin gene from different genotypes. A: Genotype GG, B: Genotype CC, C: Genotype GC.

Şekil 2. Sığır laktoferrin geninin farklı genotiplerine ait kromotogramın bir kısmı. A: Genotip GG, B: Genotip CC, C: Genotip GC.

Table 2. Classification of genotypes for cows with (n = 38) and without (n = 51) clinical mastitis based on the 5'-UTR Genotype (Position +32).
Tablo 2. Klinik mastitli olan (n = 38) ve olmayan (n = 51) ineklerde 5'-UTR Genotipine (Pozisyon +32) dayanılarak genotip sınıflandırılması.

Clinical mastitis	5'-UTR Genotype (Position +32) %(N)			Total
	Without Polymorphism (+32:GG)	Polymorphism Heterozygotes (+32:GC)	Polymorphism Homozygotes (+32:CC)	
With clinical mastitis	57.89 (22)	26.31 (10)	15.78 (6)	100 (38)
Without clinical mastitis	60.78 (31)	29.41 (15)	9.80 (5)	100 (51)

Discussion

Mastitis is a major problem in dairy cattle which affects the farmer economically through direct and indirect costs. These include discarded milk, drug and veterinary costs, decreased milk yield during remainder of lactation, extra labour, higher culling and deaths (Blowey and Edmondson, 2010). Over the past decades, the incidence of mastitis and milk cell counts have decreased due to the employed mastitis control measures by dairy farmers. Vaccination is considered for many years prior to control of mastitis, however, is not efficient enough. Another approach to control of mastitis is the selection of cows that are generally more resistant to mastitis. Selection for increased milk production will result in an unfavorable correlated increase in mastitis incidence, if mastitis is ignored in the breeding program (Heringstad et al., 2003). Increased production of milk for calves, cows suffer stress and increasing dilution of soluble factors and SCC. Negative correlation has been found between milk production and mastitis resistance that this indicates the allele specific in cows to mastitis resistance (Fleischer et al., 2001). To correct this adverse selection, recent genetic improvement has been considered in selection of mastitis resistance (Rupp and Boichard, 2003).

Innate immune is the projects selected for genetic improvement of cow's resistance to mastitis. Wide variety of mammary gland innate defenses is presented a large potential for selection of cows resistance to mastitis. Although resistance to mastitis is Multi-gene traits, in search of effective alleles resistance to mastitis is important (Rainard and Riollet, 2006). Looking for candidate genetic markers of potential value in Marker Assisted Selection in dairy cattle is lately very popular. Among many different candidates, Lf seems to be directly associated with udder health, SCC and mastitis (Kaminiski et al., 2006). Lf is one of the innate immunity agents which is iron chelating and have various function such as bacteriostasis and protection against oxygen radicals (Legrand et al., 2004). Several studies show increased concentrations of lactoferrin as an inhibitor of infection in cows with clinical and subclinical mastitis than the health state (Kawai et al., 1999). The milk Lf concentrations decreased with increasing age of the dairy cattle. The mean milk Lf concentration in the latter lactation period tended to be higher than those in the peak and middle periods (Hagiwara et al., 2003). It has been determined that low levels of lactoferrin caused rapid progress an inflammation in the early mastitis. In addition, the milk Lf concentrations increases rapidly during the mastitis; this reflects the role of lactoferrin against mastitis (Hagiwara et al., 2003; Kawai et al., 1999).

Bovine Lf gene is highly polymorphic. Seyfert and Kuhn (1994) reported the first bovine Lf gene variant, based on an EcoRI polymorphism. Seyfert and Kuhn (1994) also found 16 polymorphisms in Lf gene. A single SNP in position +32 (C/G) located in the promoter and was assessed its associations with milk performance traits, including SCC (Kaminiski et al., 2006). Daly et al. (2006) reported that fifteen different SNPs were

identified throughout the sequence of the bovine Lf gene promoter in five different cattle breeds (Holstein Friesian, New Zealand Holstein, Montebeliard, Normande, and Norwegian Red). O'Halloran et al. (2009) investigated the SNPs of Lf gene in the Irish bovine population (Six breeds, i.e., Holstein Friesians, Jersey, Jersey–Friesian crossbreeds, Norwegian Reds, Montbe´liard and Norwegian Red–Friesian crossbreed). Their results were as follows: Twenty-nine polymorphisms were identified within a 2.2 kb regulatory region. Nineteen novel polymorphisms were identified and some of these were found within transcription factor binding sites, including GATA-1 and SPI transcription factor sites. Forty-seven polymorphisms were identified within exon sequences with unique polymorphisms that were associated with amino acid substitutions (Jinming et al., 2010). In our study, we investigated whether the polymorphism is confirmed in the 5' untranslated region (5'-UTR, position +32) using Allele Specific PCR method and is associated with mastitis in Iranian Holstein cattle.

In the study of Kaminiski et al. (2006), 358 Polish Holstein cows were screened by the SSCP method giving the percentage of genotypes 62%, 31% and 5.9% for GG, GC, and CC, respectively in position +32. Lactoferrine allele C has double positive effect: increases protein yield and protein percentage and probably decreases SCC. They reported that the CC genotype had highest and lowest percentage of milk protein and SCC, respectively. However, there were no statistically significant correlations were found between CC genotype and SCC. In this study, we detected type of SNP in the 5' untranslated region (5'-UTR, position +32). Genotype GC had a tendency of lower mastitis, however, no significant differences were found between GC genotype and mastitis. We did not found evidence for a significant association between SNP in the 5'-UTR (+32:G/G) Lf gene and the clinical mastitis in Iranian Holstein dairy cattle. In conclusion, the results of this study do not provide any support to considered 5'-UTR polymorphism (position +32) as a marker for resistance/susceptibility to mastitis in dairy cattle.

However, further studies are needed to measure lactoferrin protein and evaluate all milk performance traits based on the 5' untranslated region (5'-UTR, position +32) genotypes.

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