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Study of *Lactoferrin* Gene Polymorphism in Iranian Holstein Cattle Using PCR-RFLP Technique

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Abstract: Lactoferrin (Lf) is a glycoprotein and a member of a transferrin family and plays an important role in defense mechanism of mammary gland of milk producing animals. The amount of lactoferrin increases during inflammatory process and viral infections. The purpose of present study was to analyze *lactoferrin* gene polymorphism in semen samples of Iranian Holstein cattle using molecular techniques, for the first time. DNA was isolated from 160 Iranian Holstein bulls' semen specimens and *lactoferrin* gene polymorphism was obtained with PCR-RFLP method using *Eco*RI enzyme. Two alleles of this gene, *A* and *B* and three genotypes *AA*, *AB* and *BB* were found in the studied population. Genotype *AA* is related with lower somatic cell count (SCC) and genotype *AB* with higher somatic cell count. The *A* and *B* allele's frequencies were 67.74% and 32.26%, respectively. The alleles controlled the occurrence of three genotypes: *AA*, *BB* and *AB*, of frequencies equal to 32.50%, 10% and 57.50%, respectively. The results showed high frequency of *A* allele and significantly *AB* and *AA* genotypes in relation to the highest and lowest somatic cell count, respectively ($P<0.01$). Furthermore there were significantly fewer *BB* homozygote in comparison with those expected ($P=0.10$). These findings showed that *lf* gene can be used as a genetic marker for somatic cell concentration in Iranian Holstein cattle and *A* allele of this gene is suitable marker for susceptibility or resistance to mastitis and microbial infections in dairy cows.

Key words: *Lactoferrin* gene · PCR-RFLP · Polymorphism · Holstein cattle

INTRODUCTION

Lactoferrin is one of the proteins present in bovine milk, which plays a role in the innate host defense. It is a single-chain protein of approximately 690 amino acids and a molecular weight of 77kDa [1]. The bovine lactoferrin (*blf*) gene was mapped to bovine chromosome 22 (BTA 22), contains 17 exons and spreads on about 34.5 kbp of a genomic DNA. Exons from 2 to 4 and from 9 to 12 code for the first globular domain of each lobe and exons from 12 to 15 code for another one [2]. Lf is an iron-binding glycoprotein that is structurally closely related to the iron-transport protein transferrin (Tf) in the plasma and discovered in 1939 as "the red protein from milk" [3]. Lf was first purified from human and bovine milk in 1960 [4]. This protein is present in a variety of tissues and cell types and its expression is under different regulatory controls. Lf is expressed and secreted by glandular epithelial cells and found in the secondary granules of PMN. It is present in mucosal secretions including tears, saliva, vaginal fluids, semen, nasal and bronchial secretions, bile, gastrointestinal fluids, urine, blood plasma, amniotic fluid and milk [5].

Lf has been ascribed several biological functions, including regulation of iron homeostasis, cellular growth and differentiation, host defense against microbial infections, anti-inflammatory activity and protection against cancer development and metastasis [6]. The antibacterial power of Lf against bacteria, some yeast, fungi, viruses and parasites has been investigated. In addition, the modulatory effect of Lf on inflammatory response and activation of the immune system have been reported previously. This protein works as an

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antimicrobial compound through chelating the iron ion, making this essential ion unavailable to the invading pathogens [7, 8].

The *lf* gene size varies between 23 and 35 kbp among different species [9]. An *lf* gene has developed during evolutional mutations in a transferring gene. Two features distinguish Lf from Tf; Lf contains a strongly basic region, which is very flexible and has relatively high isoelectric point as well as being strongly cationic, which is a major factor in the ability of Lf to bind to many different cell types and anionic molecules [10]. The concentration of bovine Lf (bLf) in milk is high in the colostrum and in udder secretions during dry periods [11]. During innovative medicines initiative (IMI), the concentration of bLf increases and is dependent of the severity of the infection [12]. In IMIs, Lf is both bacteriostatic and bactericidal [13].

Lactoferrin has antibacterial properties and bactericidal activity results from a direct interaction between Lf and the bacterium. The first of these properties arises from its ability to chelate Fe^{3+} and thus prevent some bacteria from obtaining sufficient quantities of iron necessary to thrive [14]. Iron starvation is generally bacteriostatic; while the bacteria cannot reproduce, if the body or other microbes have not been able to clear the bacteria. That lactoferrin has a bacteriostatic effect on some bacteria is not itself a problem. A number of clinical antibiotics are bacteriostatic and highly effective in people and animals with normal immune functions [15].

The second property is also bacteriostatic. Bacteriostatic activity of Lf is based on its ability to sequester iron and thereby prevent growth of bacteria. Coliforms have a high requirement for iron and are more susceptible to Lf than other mastitis-causing bacteria [16, 17]. Lf disrupts the type IV secretion system (T4SS), interfering with the export of certain proteins necessary for virulence and for uptake of the bacteria into animal cells [18].

The third of these properties is bactericidal. Inherent within the protein, or possibly released from the protein, is a smaller fragment that acts as a protein antibiotic. Some bacteria can use the iron chelating property of lactoferrin to their own advantage [1]. "Lactoferrin does not inhibit the growth of all iron requiring microorganisms; some pathogens of mucosal surfaces, such as *Helicobacter pylori*, *Neisseria* sp., *Treponema* sp. and *Shigella* sp. have receptors for lactoferrin (sometimes species specific) and acquire iron directly there from allowing their growth and pathogenicity in adverse host environments" [1].

The other antimicrobial mechanisms of Lf may be related to the inhibition of bacterial biofilm formation or inhibition of intracellular invasion by blocking bacterial adhesion to host cells [19-21]. Lf modulates the immune response by decreasing free radical formation and by down-regulating LPS-induced cytokines [22]. Lactoferrin's combined anti-bacterial and anti-viral activities suggest it as a promising anti-diarrhoeal agent, because this condition can be caused by either bacteria or viruses. Lf is also being tested as a therapeutic for conditions not caused by infection. Those suffering from diseases that increase gut permeability, such as Crohn's disease, or from infections that result in transient increases in gut permeability, are prone to absorbing microorganisms, toxins and allergens in higher amounts [23].

Somatic cell count (SCC) in semen and milk constitutes a good diagnostic tool that allows early detection of either subclinical or acute form of mastitis and is therefore a valuable component of monitoring programs [24, 25].

In locus of *lf* gene two different allelic variants were mapped. These variants code three different genotypes including *AA*, *AB* and *BB*. Genotype *AA* is associated with lower SCC, genotype *AB* with higher SCC. These genotypes associate to susceptibility or resistance to mastitis in dairy cows [26, 27].

Results have been published on in vivo studies on the use of bovine Lf alone or with antimicrobials in the treatment and prevention of bovine mastitis. Intramammary bovine Lf-treatment of *Staphylococcus aureus* mastitis combined with penicillin G was shown to increase cure rate as compared with the antibiotic alone and had also some effect on mastitis caused by a penicillin-resistant isolate [28-31].

Studies of *lf* gene expression have revealed factors related to Lf production and secretion. The bovine *lf* gene sequence and identified polymorphisms account for Lf variants and differences in Lf expression [9]. Therefore, observation on polymorphism of *lf* gene is important for evolution studies and determines the association between this gene and bacterial infection and other disease such as mastitis in cattle. The objectives of present study were detection of *lactoferrin* gene polymorphism in Iranian Holstein cattle for the first time, using PCR-RFLP technique.

MATERIALS AND METHODS

Semen Samples Collection: The semen samples from 160 Iranian Holstein bulls were collected randomly by means of an artificial vagina during June and December 2010, being careful of avoiding contamination with bacteria present in the prepuce. Prior to taking the samples, the prepuce was washed with a 1% benzalkonium chloride solution drying with sterile cotton. Semen samples were diluted according to standard procedures and sent to the laboratory in refrigerated boxes. Each of semen specimens was stored at -70°C for further use.

DNA Extraction: Total DNA was extracted from semen specimens using DNP™ Kit (CinnaGen, Iran), according to the manufacturer's recommendations. The total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell [32]. The extracted DNA of each sample was kept frozen at -20°C until used.

Gene Amplification: The primers were used from the study of Wojdak-Maksymiec *et al.* [3] for detection of bovine *lactoferrin* gene in milk. For detection of *lf* gene PCR reaction was performed in a final volume of 25 µL containing 40 ng of template DNA, 20 pmole of each primer (5'-GCCTCATGACAACTCCCACAC-3' and 5'-CAGGTTGACACATCGGTTGAC-3'), 10X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1 mM $MgCl_2$, 0.25 mM of dNTPs and 1 U of *Taq* DNA polymerase (Fermentas, Germany). This solution was initially denatured at 95°C for 5 min, followed by 30 cycles of denaturation (94°C for 1 min), annealing (62°C for 1 min), elongation (72°C for 1 min) and a final extension at 72°C for 5 min. The amplified products were detected in 1% agarose gel electrophoresis. Aliquots of 10 µL of PCR products were applied to the gel. Constant voltage of 80 V for 30 min was used for products separation. After electrophoresis, the gel was stained with Ethidium Bromide and images were obtained in UVIdoc gel documentation systems (UK).

Lactoferrin Gene Polymorphism: Restriction fragment length polymorphisms (RFLPs) were used for analysis of *lf* gene polymorphisms. PCR products were digested with *EcoR*I enzyme (Roche applied science) in a total volume of 20 µL (10 µL reaction solutions, 2 µL enzyme buffers, 0.2 µL enzyme and 7.8 µL distilled water) and placed in the incubator at 37°C for 4 h. Restriction fragments were analyzed electrophoretically in 2% agarose gel in TBE buffer.

Statistical Analysis: Analysis of polymorphic patterns in *lf* gene was performed using the SPSS version 17.0 computer software (SPSS, Chicago, IL). The probability of Hardy-Weinberg equilibrium associated with the observed genotypic frequencies of *lf* gene was determined using the χ2-test.

Allele's frequency and their standard error were calculated as follows:

$$N_A = 2\times NAA + NAB \Rightarrow F_A = \frac{2NAA+2NBB}{2N}$$

Also standard error of mean allelic frequencies of *lf* gene was calculated as below:

$$s.e=\sqrt{\frac{pq}{2n}}$$

To test deviation from Hardy-Weinberg equilibrium test of the two X^2 and G^2 were used as follows:

$$x^2 = \frac{\sum(0-e)^2}{e} \qquad G^2 = -2(LnLo - LnL1)$$

RESULTS

PCR-RFLP technique was used to reveal polymorphism of *lf* gene in semen samples of Iranian Holstein cattle. PCR products for *lf* gene on agarose gel showed a fragment of about of 301 bp (base pair). Two alleles of *lf* gene, *A* and *B*, were found in the studied population of Iranian Holstein cattle. The *EcoR*I digestion produced a mixture containing fragments of 301 bp (allele *A*, no sequence recognized by the restriction enzyme), 201 bp and 100 bp (allele *B*). Agarose gel electrophoresis of PCR products after digested with *EcoR*I enzyme were showed in Figure 1.

The frequency of alleles *A* and *B* were 67.74% and 32.26%, respectively. The alleles controlled the occurrence of three genotypes *AA*, *BB* and *AB*, with their frequencies of 32.50%, 10% and 57.50%, respectively. The observed and expected genotypes frequencies of *lf* gene in semen samples of Iranian Holstein cattle were shown in Table 1.

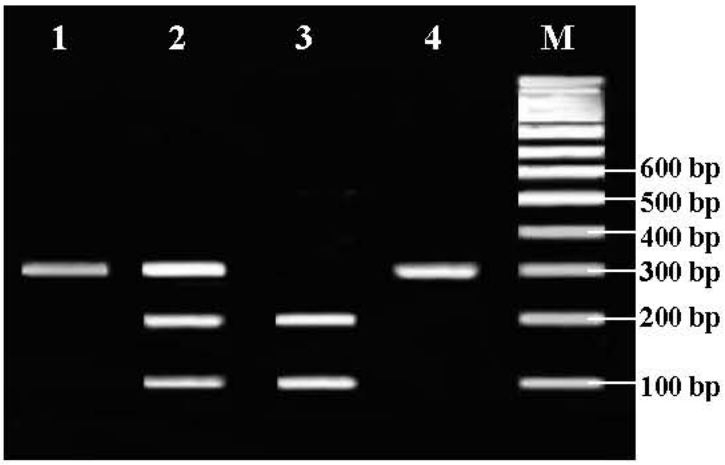

Fig. 1: Gel electrophoresis of PCR product after digested with *EcoR*I restriction enzyme for detection of *lactoferrin* gene polymorphism in semen samples of Iranian Holstein cattle. Line M: 100 bp DNA ladder (Fermentas, Germany), line 1: uncut sample (301 bp), line 2: *AB* genotype (301 bp, 201 bp and 100 bp), line 3: *BB* genotype (201 bp and 100 bp) and line 4: *AA* genotype (301 bp).

Table 1: Genotypes frequencies of *lf* gene in the analyzed population.

lf genotype	Observed frequency Number (%)	Expected frequency Number (%)	Chi-square
AA	52 (32.50)	40 (25)	1.89736
AB	92 (57.50)	80 (25)	1.34164
BB	16 (10)	40 (50)	3.79473
Total	160 (100.00)	160 (100.00)	7.03373

Statistically significant ($P<0.01$) deviations were found in the analyzed population between the observed distribution of *lf* genotypes and their expected distribution estimated according to the Hardy-Weinberg law.

DISCUSSION

Lactoferrin is a member of the transferrin family, which is approximately 300-500 million years old [9]. Lf is found in all external secretions and biological fluids, including semen, milk, tears, pancreatic juice, saliva, mucous, bile, genital and nasal secretions and also in the secondary granules of PMN [9, 33]. Lf is involved particularly in the mechanism of alimentary immunity [34, 35]. This immunity results from the fact that possible infection factors have a limited availability of iron (as well as other growth agents, such as phosphorus and zinc), since its concentration in an organisms fluids is reduced [36]. Another function of Lf is to inhibit enteric absorption of iron in neonates thus belonging to those proteins capable of binding and transferring Fe^{3+} ions. Lf may also take part in intracellular destruction of bacteria performed by inducing hydroxyl radical formation, which is catalyzed by iron [37]. The fact that Lf appears in infected areas also due to its local synthesis [36]. For example, an infection results in 30-fold increase in the synthesis of the protein in secretory cells of the mammary gland [12].

Mastitis is the most important disease of dairy cows, causing the most significant economic losses [38]. Mastitis is an inflammation of the mammary gland that is mostly a response to an intramammary bacterial infection [39]. *S. aureus*, *streptococci* and *E. coli* are respectively among the most prevalent species of Gram-positive and Gram-negative bacteria that cause bovine clinical mastitis. Disease severity depends on the interaction between the host, the environment and the infectious agent. Frequency of new IMI is greatest during early involution, decreases during mid-stages and then increases prepartum. In mammary involution, the concentrations of antibacterial components, i.e. leucocytes, immunoglobulins and Lf increase while fluid volume decreases and thereupon the bovine mammary gland resists the IMI [38].

In this study *lactoferrin* gene polymorphism in semen samples of Iranian Holstein cattle were examined using PCR-RFLP method and PCR products were digested by *EcoR*I enzyme. The frequency of *A* and *B* alleles were

67.74% and 32.26%, respectively and three genotypes for *lf* gene were observed. The frequency of *AA*, *BB* and *AB* genotypes were 32.50%, 10% and 57.50%, respectively. Genotype *AA* is associated with lower somatic cell count and genotype *AB* is related to higher somatic cell count. The results showed significantly more heterozygous *AB* genotypes in relation to the expected rate of heterozygotes ($P<0.01$), whereas there were significantly fewer *BB* homozygotes in comparison with those expected ($P=0.10$).

Some studies were performed about *lf* gene polymorphism and its correlation with susceptibility or resistance to diseases in bovine. Ellison *et al.* [40] showed that Lf binds directly to the outer membrane of Gram-negative bacteria, causing a rapid release of LPS, with increased membrane permeability and damage to the cell wall. Tomita *et al.* [41], discuss twenty-five years of research and development of bovine Lf and bovine lactoferricin applications. SCC generally increases with advancing age and stage of lactation. An effect of lactation number, lactation stage and breed was reported by Schutz *et al.* [34] and Cameron and Anderson [42]. Seyfert and Kuhn [43] found two alleles, *A* and *B*, in the *lf* locus, which encoded three possible genotypes: *AA*, *AB* and *BB*. The frequencies of the alleles were 0.755 and 0.245 for *A* and *B* respectively, thus the results were similar, with a slightly higher frequency of the allele *A*. The study of Wojdak *et al.* [3] on associations between bovine *lf* gene polymorphism and somatic cell count in milk showed statistically significant associations exist between the SCC and *lf* genotype. The frequency of *A* and *B* alleles were 67.74% and 32.56%, respectively and the frequency of *AA*, *BB* and *AB* genotypes were equal to 37.90%, 2.42% and 59.68%, respectively [3]. Investigations of the same polymorphism, reported by Sender *et al.* [44] provided contrary results as the genotype *BB* animals showed the lowest somatic cell count and heterozygotes *AB* the highest. Because of the low frequency of the *BB* genotype, investigations on this polymorphism need to be continued. The findings obtained in Sender *et al.* [44] study confirmed the results of present research in semen samples of Iranian Holstein cattle.

These findings showed that lactoferrin is a multi-functional protein with many beneficial properties, which makes it a good candidate for a number of product applications and commercial and clinical applications. In present study high frequency of *A* allele were detected and therefore high frequency of *AA* and *AB* genotypes were observed. These results showed that *lf* gene can be used as a genetic marker for somatic cell concentration in semen of bulls and *A* allele of this gene as a marker for susceptibility/resistance to mastitis and microbial infections diseases in dairy cows.

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