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ANALYSIS OF LACTOFERRIN GENE POLYMOPHISM AND ITS ASSOCIATION TO MILK QUALITY AND MAMMARY GLAND HEALTH IN HOLSTEIN-FRIESIAN COWS

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Lactoferrin (LTF) is a glycoprotein, a member of transferrin gene family which plays an important role in immune mechanisms in the mammary glands of cows. The amount of lactoferrin increases during inflammatory processes and viral infections. The aim of this investigation was to monitor the distribution of lactoferrin gene genotypes and its connection to milk quality and the occurrence of mammary gland diseases in 46 Holstein-Freisian cows of different age (2-7 years) on a farm near Belgrade. DNA was isolated from blood samples, and the polymorphism of lactoferrin gene was deterimined by PCR-RFLP method using the restriction enzyme Eco RI. We found two alelic forms of this gene in cows included in these experiments (A and B) and two genotypes (AA and AB) in a ratio 71.7% to 28.3%. The genotype BB was not found in this sample. In order to determine the degree of differences between genotypes we used discriminant analysis which has shown that there is a statistically significant difference between genotypes AA and AB with respect to productive parameters. When analysed separately, the only parameter which differed significantly (p=0.021) between two genotypes was total milk production. Individuals with observed genotypes are most similar for the amount of milk fat (p=0.271). There is no statistically significant difference in the number of somatic cells in milk samples between the examined genotypes.

Key words: cow somatic cells, lactoferrin gene, mammary gland, PCR-RFLP, polymorphism

INTRODUCTION

There is an ever-increasing trend in modern animal husbandry to obtain genetically selected individuals more resistant to mammary gland diseases (Rupp *et al.,* 2003). Ogorevc *et al*. (2009) examined the expression of genes related to mammary gland diseases in cows. They mention 934 candidate genes involved in mammary gland development, milk production, sensitivity and resistance to mastitis. Moreover, these authors point to 15 candidate genes (BolA-13, IL8RA, TLR4, C5AR1, CD14, IFNG, IL1B, IL6, IL8, LBP, SAA3, TLR2, TLR4, TNF, LTF, β-4 defensin) useful in monitoring the mechanisms of the development of an infectious disease as well as natural resistance of cows to mastitis (Ogorevc *et al.,* 2009). Nowdays, there is ample evidence about the significance of lactoferrin and other 14 candidate genes in monitoring the distribution of specific genotypes of various cow breeds in different geographical regions (Arnould *et al.,* 2009; O' Halloran *et al.,* 2009, 2010)

Lactoferrin (LTF) is a bioactive glycoprotein found in many exocrine secretions including tears, saliva, milk etc. There are numerous data about the antibacterial effects of lactoferrin both *in vitro* and *in vivo* (Levay *et al.,* 1995; Conecely, 2001; Chaneton *et al.,* 2008). Lactoferrin is synthesised in neutrophil granulocytes, macrophages and udder epithelial cells (Harmon, 1980). The amount of lactoferrin in the milk varies from 0.020-0.035 mg/mL, depending on time of lactation. The main function of lactoferrin is to protect the mammary gland from infection by coliform microorganisms, especially in the phase of involution. This function is achieved by activation of phagocytosis and of the complement system. In addition to bacteriostatic effects, lactoferrin has an ability to protect mammary gland parenchyma from deleterious effects of reactive oxygen species (Legrand *et al.,* 2004; Adlerova *et al.,* 2009). Its bacteriostatic activity is inhibited by citrate anions, which are present in milk and colostrum in a much higher concentration than lactoferrin itself. The activity of lactoferrin is highest in the dry period, when it can achieve concentrations in mammary gland secret of 20-100 mg/mL. At the same time, the concentration of citrate is decreased, while the concentration of bicarbonate is increased (Legrand *et al.,* 2004). Lactoferrin concentration starts to increase 2-4 days after the cesation of milking, and it further increases in the dry period as a consequence of an increased neto synthesis of lactoferrin in the period of udder involution (Legrand *et al.,* 2004). The investigations of Oliver *et al*. (2000) have shown that lactoferrin can act as a stimulator of the process of phagocytosis of bacteria, therefore allowing removal of bacteria from the udder. In addition to antibacterial, lactoferrin also has antiviral, antifungal and antiparasitic actions. Moreover, lactoferrin has a catalytical effect in some enzyme reactions. It is also assumed that lactoferrin has immunomodulatory effects and that it can even slow growth of tumors (Adlerova *et al.,* 2008; Pawlik *et al.,* 2009). Some authors classify lactoferin in acute phase proteins, because its concentration increases during an acute

inflammatory reaction and in response to viral infections (Kanyschkova., 2001). The concentration of lactoferrin is dramatically increased upon infection of a quarter of the udder with bacterial species *Streptococcus uberi*. However, other infectious agents do not cause a significant increase in lactoferrin concentration (Pawlik *et al.,* 2009). Interestingly, under *in vitro* conditions, lactoferrin inhibits growth of certain bacterial species capable to cause mammary gland diseases, and the strongest activity is observed against *Esherichia coli* and *Pseudomonas aeruginosa* (Kutila, 2004; Chaneton *et al.,* 2008). On the other hand, there are some inconsistent results concerning lactoferrin effects on mastitis caused by *Staphylococcus aureus* and *Klebsiella pneumoniae (*Kutila, 2004). Conecely (2001) discusses that lactoferrin belongs to a group of transferrin proteins, together with ovotransferrin (a type of transferrin in eggs), melanotransferrin (melanocytic transferrin protein) etc. An immunomodulatory role of lactoferrin inhibiting secretion of two very important mediators of inflammation (TNF-α and IL-1β) is described by Conecely (2001) and also by Puddu *et al*. (2009). Also, there are reports that lactoferrin changes an expression of specific receptors on lymphocytes and thereby actively changes the functions of lymphocytes allowing selectivity in recognition and neutralization of specific antigens (Legrand *et al.,* 2008). The bovine lactoferrin gene is mapped at the chromosome 22q24 and it comprises 17 exons. In a gene bank, there are specific sequences with a mutation recognizable by a restriction enzyme *Eco RI.* This polymorphism is described at intron 6 of the LTF gene (Seyfert and Kuhn, 1994). There are two alelic forms at the LTF locus (*A* and *B*), giving three possible genotypes: *AA, AB, BB* (O Halloran *et al.,* 2009). RFLP analysis of the genetic polymorphism of the lactoferrin gene and its connection to udder infections can have both theoretical and practical importance (Zhao *et al.,* 2008; 2009; O' Halloran *et al.,* 2009; Woydak-Maksymiec *et al.,* 2012).

The aim of the present work was to identify allelic forms of lactoferrin gene (lactoferrin A and lactoferrin B) and respective genotypes (*AA, AB* and *BB*) in heifers and cows of Holstein-Friesian breed, and to evaluate the possible relation with milk quality and number of somatic cells that point to subclinical and clinical forms of mastitis.

MATERIALS AND METHODS

Animals

The experiment initally comprised 60 Holstein-Freisien cows (HF) chosen randomly on a farm near Belgrade. However, 14 cows were eliminated from the experiments due to certain medical problems which were not directly related to the experiment. Within the group of monitored individuals there were 14 (30.4%) cows in first lactation, 11 (23.9%) cows in the second lactation, 9 (19.6%) cows in the third lactation and 12 (26.1%) cows in the fourth lactation. The experiment

was performed from January 2012. to December 2012. All cows were kept under the same zoohygienic and zootechnical conditions a tie stall rearing system. Also, all cows were given approximatelly the same meal, depending of production category. The cows were milked with a milkng machine twice a day.

Sampling was done once a month in the period from the 4th until the 6th day of the month, and milk samples for cytological analysis were taken at the same time. We took a single sample from each individual included in the experiment (from all four quarters). Before the samples were taken we applied sanitary cleaning of the udder and disinfection of the top of the mammary gland papilla using a wet tissue with 70% ethanol.

The content of lipids and fats in the milk can be determined in various ways. In order to investigate a large number of milk samples, usually instrumental methods compatible with standard methods are used. In these experiments, analysis of the qualitative milk composition was done on automatic analyser Milcoscan® (Foss, Denmark).

The amount of produced milk from each experimental individual was determined on the basis of average values for monthly controls.

Cytological analysis of milk samples

Sampling of milk for somatic cell count was done once a month, from each cow during the period of standard lactation (305 days). Milk was taken from each quarter of the udder in an amount 5-10 mL and kept in sterile test tubes for single use. The test tubes were layed in almost horizontal position, and from each udder quarter several mL of milk were milked. Milk samples were transported to the lab in a hand refrigerator.

The number of somatic cells was evaluated by light microscopy. From each milk sample cytological slides were made the following way: 0.01 mL of milk was poured on a microscopic slide and spread on an area of 1 cm^2. In order to achieve this, we used cardboards with 1 cm^2 drawn squares under the microscope. After air drying of the slides (at least for 24 h), we removed fats with xylol, dried them again, fixed in ethanol for 5 min and stained with the previously prepared stain (37 mL ethyl-alcohol, 130 mL distilled water, 3 gr methilen blue, 20 mL fuchsin and 10 mL aniline).

Sampling of blood for molecular genetics analysis

A total of 10 mL of whole blood was taken from the *Vena coccigea media* in test tubes with potassium-ethylendiaminetetraacetic acid (K_2-EDTA) added as an anticoagulant. The blood was transported to the lab in a hand refrigerator with ice, and thereon used for DNA extraction.

DNA extraction

The DNA isolation was performed according to the protocol for Dneasy® Blood & Tissue kit Cat. No 69504 (Qiagen, Valencia CA).

PCR amplification of lactoferrin gene fragments

In order to amplify the monitored fragment of the lactoferrin gene (amplicon) of 301 bp in length we used a couple of primers: LAC-FW: 5'-GCC TCA TGA CAA CTC CCA CAC-3' and LAC RV: 5'-CAG GTT GAC ACA TCG GTT GAC -3'. (Woydac-Maksimiec *et al.,* 2006). The mixture for PCR was prepared in 25 µL minitubes and was composed of 12.5 µL KAPA 2G Robust HotStart ReadyMix (Kapa Biosystems), 1.25 µL of each primer and 10 µL of isolated DNA. The PCR reaction was achieved in apparatus Multi-Gene Gradient (Labnet International Inc). Temperature schedule was composed of an initial denaturation for 2 min at 95°C, followed by 40 cycles of the process of denaturation (15 sec at 95°C), hybridisation of primers (60 sec at 60°C) and primer elongation (15 sec at 72°C). The final elongation lasted for 8 min at 72°C.

Restriction framents length polymorphism (RFLP) analysis

Identification of lactoferrin genotype was done using RFLP method, after the digestion of PCR products by a restriction endonuclease *Eco RI*. RFLP reveals allels which differ for the presence or abscence of specific restriction sites (Botstein *et al.,* 1980).

Digestion of PCR products was performed according to recommendations of the *Eco RI* restriction enzyme manufacturer (Fermentas, USA), as a reaction in a total volume of 30 µL composed of 17 µL re-distilled water, 2 µL green buffer fast enzyme®, 1 µL *EcoRI* (5U/L) and 10 µL of PCR products. The reaction was performed for 3 h at 37°C. After the digestion, two allelic forms could be observed – an allelic form *A* as a fragment of 301 bp, and an allelic form *B* as two fragments of 201 bp and 100 bp (Fig 1).

The digested fragments were further electrophoretically separated in 2% agarose gel (Sigma-Aldrich, Germany) for 90 min in a tank containing TBE buffer (60 V, 50 mA). After staining with ethidium bromide the visualisation of obtained fragments was achieved on a UV lamp. The length of fragments was analysed by commercial O'RangeRuler™ 50bp DNA Ladder.

RESULTS

A total of 46 cows 2-7 years of age was included in this study. According to the results of χ^2-test (χ^2=9.29; p=0.098) individuals of different age were equally represented. Also, the examined individuals were uniformly distributed by lactation, from the first to the fourth (χ^2=1.13; p=0.770). The examined cows had only two lactoferrin genotypes: *AA* and *AB*. The genotype *AA* was statistically more (χ^2=8.696; p=0.003) presented (33 individuals, i.e. 71.7%), than the genotype AB (13 individuals, i.e. 28.7%).

The age structure of cows did not statistically significantly differ within both observed genotypes (χ^2=3.47; p=0.627). In addition, the distribution of individuals throughout lactations did not significantly differ between genotype *AA* and *AB*

(χ^2=2.43; *p*=0.487). Also, we did not find significant differences between the observed distribution of LTF genotypes in the analysed cow samples, and their expected distribution was within the limits of Hardy-Weinberg law (χ^2=1.25; *p*=0.536).

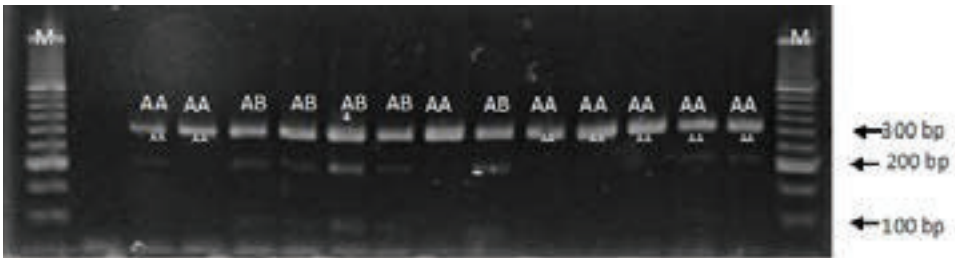

Figure 1. 2% agarose gel stained with ethidium bromide; primers LAC FW and LAC RV for the restriction enzyme *Eco RI* (Fermentas, DE)

The analysis of productive paremeters was done on the basis of average values for each individual. During this analysis, the arithmetic mean or mediana were used as average values. The arithmetic mean was used for homogenous data ($c_v \leq 30\%$), and the median for heterogenous data ($c_v > 30\%$). On the basis of the formed matrix and the obtained data, we determined basic statistical parameters and by using the Shapiro-Wilk test we checked the accordance of their distribution with the theoretical model of normal distribution (Table 1). In cow group *AA* genotype the average concentration of proteins in the milk samples was 3.29±0.02%, while in the population with *AB* genotype it was 3.22±0.03%. In a group of individuals with *AA* genotype the value of milk fat was 3.62±0.03%, and in the genotype *AB* it was 3.57±0.04%. The total milk production in individuals with genotype *AA* was 37.05±0.88 L, while in the genotype *AB* it was 32.96±1.84 L. The average number of somatic cells was higher in *AA* genotype (521005.64±89994.52), compared to *AB* genotype (3430266.54±83359.44)

Bearing in mind that data for total SCC were not homogenous ($c_v > 30\%$) and were distributed by the model of normal distribution ($p<0.01$ for *AA* genotype), we undertook their transformation. By doing transformation $\sqrt{x}$ coefficients of variations were decreased to 26.9% (for genotype *AA*) and 26.8% (for genotype *AB*). Also, after the transformation, the departures from the model of normal distribution were also decreased (p=0.763 for the genotype *AA*; p=0.619 for the genotype *AB*).

In order to determine the level of difference between genotypes, we applied discriminant analysis based on Squared Mahalanobis Distances. The results of this analyis (Table 2) point to the fact that genotypes *AA* and *AB* significantly differ in production parameters taken together (Wilks' Lambda: 0.77; approx. $F_{(4,41)} = 3.07$; p<0 .027). When analysed separately, only the milk production was significantly different between the two groups of cows with analysed genotypes (p=0.021). On the other hand, individuals of compared genotypes were most similar for milk fat quantity (p=0.271).

Table 1. Basic statistical parameters and levels of statistical significance (Shapiro-Wilk's test)

Genotype	Production parameters	Mean	Minimum	Maximum	Std.Dev.	St. Err.	Coef. of Var.	Shapiro-Wilk's *p*
AA	Proteins (%)	3.29	3.06	3.55	0.12	0.02	3.50	0.506
	Milk fat (%)	3.62	3.25	4.07	0.17	0.03	4.70	0.462
	Total milk production (L)	37.05	23,67	49,17	5,04	0.88	13.60	0.468
	Total SCC	521005.64	31200.00	2307000.00	516691.91	89944.52	99.17	<0.01
AB	Proteins (%)	3.22	3.05	3.46	0.11	0.03	3.32	0.286
	Milk fat (%)	3.57	3.39	3.89	0.14	0.04	3,99	0.421
	Total milk production (L)	32.96	16.50	40.83	6.64	1.84	20.14	0.073
	Total SCC	343026.54	28800.00	1030600.00	300556.75	83359.44	87.62	0.104

Table 2. Results of discriminant analysis

Production parameters	Wilks' Lambda	F-remove	p-level
Proteins (%)	0.82	2.47	0.124
Milk fat (%)	0.79	1.25	0.271
Total milk production (L)	0.88	5.79	0.021*
Total SCC	0.82	2.90	0.096

Individuals of genotypes *AA* and *AB* do not differ significantly in the number of SCC, although the average number of somatic cells is higher in group with genotype *AA*, when compared to cows with genotype *AB* (Table 1).

DISCUSSION

Milk of healthy cows contains, on average, 3.2% of total proteins, 3.9% of milk fats, 2.6% of casein and 4.6% of lactose. The composition of milk varies and depends on animal age, phase of lactation, nutrition, race etc. (Jensen *et al.,* 1991). In our investigation, the concentration of proteins was higher in the group of cows with *AA* genotype (3.29%), then in *AB* genotype (3.22%), but not statistically significant. The average concentration of milk fat between groups of cows with two analysed genotypes was the most similar parameter, probably due to homogeinety of observed individuals with respect to age, as well as due to identical rearing and nutrition conditions. In addition to composition, a very important parameter in the evaluation of milk quality is the number of milk somatic cells. The number of somatic cells is used as an indicator of mammary gland health and it directly influences on the price of milk on the market (Lindmark-Mansson *et al.,* 2006; Katić, 2007). The increased number of somatic cells in the milk is not always directly connected to inflammatory processes in the mammary gland, but also to the stage of lactation, milking hygiene, quality and accuracy of milking equipment, use of various pharmacological preparations, oestrus stage etc. (Heeschen, 1995; Katić, 1995). In the milk of healthy cows an average number of somatic cells is about 200 000/mL, wherease this number significantly increases in cows having subclinic mastitis (Paape *et al.,* 2002). Lactoferrin, as a glycoprotein and a member of the transferrin protein family, plays an important role in the defence system of the mammary gland aganist exogenous and endogenous infections. Therefore, lactoferrin acts in the second defence line against infection. A wide range of lactoferrin concentrations was detected in the milk of healthy cows. The values of lactoferrin in cow's milk vary from 1.5 μg/mL to 485.63 μg/mL. It is proven that lactoferrin is significantly related to the stage of lactation ($r=0.557$; $p<0.001$) and daily milk production ($r=-0.472$; $p<0.001$) (Cheng *et al.,* 2008). The concentration of lactoferrin can rise several times (up to 100 μg/mL) during the involution of the mammary gland (Welty *et al.,* 1976). This finding is in agreement with our results of a statistically

significant difference in milk production between groups of cows with *AA* and *AB* genotype (Table 2). Wojdak-Maksymiec *et al*. (2006) monitored the distribution of lactoferrin genotypes in the population of Holstein-Fresien cows in Poland. They have confirmed the presence of two allelic forms of lactoferrin gene (*A* and *B*) which control the occurence of three genotypes: *AA*, *AB* and BB in ratios of 37.9%, 2.4% and 59.7%, respectively. It is noticable that there is a lower percentage of heterozygoes in comparison to our findings. At the same time, high level of SCC was detected in cows of *AB* genotype, and the lowest level of SCC in cows with *AA* genotype. In contrast, Sender *et al*. (2006) investigated the same polymorphism and found that animals with genotype *BB* exhibit the lowest somatic cell count, while animals with genotype *AB* had the highest SCC. In our investigation, there is no statistically significant difference in SCC between the two monitored genotypes, possibly due to a relatively low number of individuals included in this study. Similar observations were made by Sender *et al.,* 2010. However, it should be mentioned that the level of statistical significance for this parameter was 0.096 which is near the 0.05 level. Therefore, had we analysed more cows we might have obtained different statistical results. Nanei *et al*. (2012) examined the polymorphism of lactoferrin gene in 404 Holstein cows from Ishvan province (Iran). They used the same set of primers for identification of LTF allelic forms, as in our experiment. The frequency of LTF allel *A* was in the range 0.775-0.831, and the frequency of allel *B* was in the range 0.169-0.225. Two genotypes (*AA* and *AB*) were represented with 60.6% i 39.4%, respectively, while the genotype *BB* was not found. This result is in agreement with our observations (χ^2=2.391; p=0.122). The other group of authors also monitored the polymorphism of LTF gene using different primers and restriction enzyme, but, nevertheless, the results are very similar. Namely, Jemmali *et al*. (2011) monitored LTF polymorphism in 52 cows of Holstein race imported to Tunisia. By using *Hin fI* restriction enzyme to analyse DNA fragment of 1143±100 bp, they have found the presence of coding LTF sequences in all observed cows. All cows were homozygous for lactoferrin (the genotype *AA*). Zhao *et al*. (2009) monitored two groups of cows on a farm in China. The experimental group included 60 animals proven to suffer subclinical mastitis, based on California mastitis test (CMT). The control group numbered 60 animals proven to be healthy by CMT test. After the PCR and digestion of products with *Hin fI* enzyme, identification of the obtained fragments showed the presence of two allelic forms of the promotor of lactoferrin gene (*A* and *B*) with three possible genotypes (*AA*, *AB* and *BB*). In the same investigation, it was found that cows with genotype *AA* have more somatic cells in the milk than cows with *BB* genotype, which points to a more frequent occurence of subclinic mastitis in the *AA* genotype group. Interestingly, Sender *et al*. (2010) have found statistically significant differences among three genotypes in the average amount of milk produced. Namely, cows with *AA* genotype produce more milk and, probably, the artificial selection of cows for production of more milk resulted in the gradual decrease of *B* allele in HF cows.

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ANALIZA POLIMORFIZMA LAKTOFERIN GENA I NJEGOVA POVEZANOST SA KVALITETOM MLEKA I ZDRAVLJEM MLEČNE ŽLEZDE KOD HOLŠTAJN-FRIZIJSKIH KRAVA

MALETIĆ M, VAKANJAC SLOBODANKA, DJELIĆ N, LAKIĆ NADA, PAVLOVIĆ M, NEDIĆ SVETLANA, STANIMIROVIĆ Z

SADRŽAJ

Laktoferin (LTF) je glikoprotein, član familije transferina i igra važnu ulogu u odbrambenom mehanizmu mlečne žlezde krava. Količina cartoferina raste tokom inflamatornog procesa i virusne infekcije. Cilj ovog rada je bilo praćenje distribucije genotipova laktoferin gena i njihova povezanost sa kvalitetom mleka i pojavom oboljenja mlečne žlezde kod 46 krava Holštajn-Frizijske rase, različite starosti (2-7 godina) na farmi u blizini Beograda. Obavljena je izolacija DNK iz krvi, a polimorfizam laktoferin gena utvrđen je PCR-RFLP metodom pomoću restrikcionog enzima *Eco RI.* Kod životinja uključenih u ogled utvrđene su dve alelne forme, *A* i *B* i dva genotipa, *AA* i *AB* u odnosu 71,74% prema 28,26%. Genotip *BB* nije pronađen u datom uzorku životinja. U cilju utvrđivanja stepena

razdvajanja genotipova primenjena je diskriminaciona analiza koja je ukazala da se genotipovi *AA* i *AB* statistički značajno razlikuju prema proizvodnim parametrima posmatranim istovremeno. Pojedinačno, samo se po parametru ukupna proizvodnja mleka, krava analiziranih genotipova statistički značajno razlikuju (p=0.021). Grla posmatranih genotipova su najsličnija po količini mlečne masti (p=0.271). Nije ustanovljena statistički značajna razlika u broju somatskih ćelija u mleku između isptivanih genotipova.

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