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Effect of a novel polymorphism of the *LF* and *TLR4* genes on milk yield and milk compositions in dairy goats

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Abstract

In total 593 goats were genotyped on 5 single nucleotide polymorphisms (SNP): one SNP at *Lactoferrin* in exon 4 and four SNPs at *Toll-like receptor 4* in exon 3 by using SNaPshot minisequencing. The most prevalent genotype was TT (546) in *LF* and genotype combination TTCCCCGC (216) and TTCCCCCC (204) in *TLR4*. There is possible influence of *LF* and *TLR4* on milk yield and milk components, therefore, further studies are needed to evaluate this possible association in more number of goat farms.

Keywords: goats, lactoferrin, milk components, single nucleotide polymorphism, SNaPshot, toll-like receptor 4

Introduction

The quality of dairy production is closely associated with production intensity, livestock management and profitability (Burriel, 2000). Milk components and quality of the goat milk is influenced by the type of feed, the stage and composition of the fodder, the season and the intensity of the breeding (Morand-Fehr et al., 2007). Besides breeding technologies; milk components are also affected by the genetic foundation of the individual. The use of candidate genes as genetic markers allows for more efficient selection and reduces the time required to achieve the breeding goals (Lende and Thompson, 1990). *Lactoferrin* (*LF*) is an iron-binding glycoprotein; a member of transferrin gene family (Ateya et al., 2016) located at goat's chromosome 22 (*NC_030829.1*). *LF* associates with protein content and milk acidity (Guo et al., 2010) and single nucleotide polymorphisms (SNP) could be used for marker assisted selection on mastitis susceptibility (Ateya et al., 2016). *Toll-like receptor 4* (*TLR4*) is located on the cell surface and initiates innate immune responses (Bilal et al., 2017). *TLR4* gene is located at chromosome 8 (*NC_030815.1*) in goats. This gene recognizes gram-negative bacteria and lipopolysaccharides (Akira and Takeda, 2004). In cattle, SNP on *TLR4* (*TLR4-2021*) associates with milk protein and fat content in late lactation (Beecher et al., 2010).

The aim of the study was to type polymorphism at the *LF* and *TLR4* loci and find out their association with milk yield and milk compositions in Czech dairy goats.

Materials and methods

In current study, total of 12,280 records from 593 animals from 3 different goats populations were used: White shorthair goat (467) Brow shorthair goat (60) and crossbreeds (66). In this study, all phenotypic data for milk production traits (milk yield, content of lactose, protein and fat in percentage and in kg) were obtained from database of the routine performance testing kept in the Czech-Moravian Breeders Corporation. Genomic DNA was extracted from blood using GeneAll® Exgene™ Blood SV mini. In this study were detected SNP g.7605C→T in exon 4 of *LF* gene (NCBI GenBank FJ609300) and 4 SNPs in exon 3 of *TLR4*(NCBI GenBank EF409985-EF409989), 5 alleles are distinguished and their combination makes the final genotype. To type SNPs at LF and TLR4 locus were used SNaPshot minisequencing according to Kyselová et al. (2016) (CZ29769U1). In first step exon 4 for *LF* (594 bp including adjacent areas) and exon 3 for *TLR4* (494 bp including adjacent areas) were amplified by multiplex PCR. Following primers were used:

LF_E4 F 5´ ACACGTCCAGGGAATGATGT 3´

LF_E4 R 5´ GGAGCTCAGAAGATGTACATTGG 3´

TLR4_E3 F 5´ GTATTCAAGGTCTGGCTGGTT 3´

TLR4_E3 R 5´ ATCATTGAAGCTCAGATCTAAAT 3´

PCR assay was performed in 14 µl reaction mixture containing 7 µl Aptamer HotStart Master Mix (Top Bio Ltd., Prague, Czech Republic), 2 µl template genomic DNA (20 – 60 ng), 0.07 µM LF_E4 F primer, 0.07 µM LF_E4 R primer, 0.1 µM TLR4_E3 F, 0.1 µM TLR4_E3 R and 4.5 µl deionized H_2O. Thermal cycling conditions included: an initial denaturation step at 95 ˚C for 3 min; followed by 35 cycles of 95 ˚C for 30 s, 59 ˚C for 30 s, 72 ˚C for 30 s; after 35 cycles extension step at 72 ˚C for 5 min and final cooling to 4 ˚C (Biometra Thermoblock: 050-801 TGradient 96, Biometra, Goettingen, Germany). PCR product was verified by electrophoresis on a 2% agarose gel in TBE stained with ethidium bromide. After purification using 0.5 U FastAP Thermostatie Alkaline Phosphatase and 10 U Exonuclease I (Thermofisher Scientific, USA) at 37 °C for 30 min and 15 min inactivation in 80 °C, second PCR (SNaPshot PCR) with specific oligonucleotide single based extension (SBE) primers were used. SBE primers detect 5 SNPs and had following sequences:

SBE LF_E4 F 5´ $(AT)_2$GCCAGAAGTCCTGCCACA 3´

SBE TLR4_E3 97R 5´ $(AT)_6$AGCTCCAGTGCAGGAAACTT 3´

SBE TLR4_E3 14R 5´ $(AT)_9$CAACTTCCTTTCATTTTTAAATTCTCC 3´

SBE TLR4_E3 125R 5´ $(AT)_{13}$GAGATCTAGATACTGAAGGCTTGGTAG 3´

SBE TLR4_E3 143R 5´ $(AT)_{19}$CAAAATCAGTGTGAGAACAGCAG 3´

SNaPshot PCR 6 µl reaction mixture contained 2.5 µl SNaPshot Multiplex Ready Reaction Mix (ThermoFisher Scientific, USA), 1.5 µl purified PCR product, 0.25 µM SBE LF_E4 F, 0.15 µM SBE TLR4_E3 97R, 0.3 µM SBE TLR4_E3 14R, 0.8 µM SBE

TLR4_E3 125R and1.2 µM SBE TLR4_E3 143R. Thermal cycling was conducted in 25 cycles of 96 ˚C for 10 s, 50 ˚C for 5 s, 60 ˚C for 30 s; and final cooling to 4 ˚C (Biometra Thermoblock: 050-801 TGradient 96, Biometra, Goettingen, Germany). Final product was purified by 0.5 µl FastAP Thermostative Alkaline Phosphatase (ThermoFisher Scientific, USA) for 1 hour in 37 °C and then inactivated for 15 min in 80 °C.

The following mixture was used for fragmentation analysis: 0.5 µl SNaPshot product, 9 µl Hi-Di formamide (ThermoFisher Scientific, USA) and 0.5 µl GeneScan120 LIZ Size Standard (ThermoFisher Scientific, USA). After 5 min denaturation in 95 °C and final cooling, the mixture was analyzed in capillary sequencer DNA 3130 Genetic Analyzer (Applied Biosystems; Foster City, CA, USA). For data evaluation and determination *LF* and *TLR4* software GeneMapper v. 4.0 (Applied Biosystems; Forster City, CA, USA) was used. Influence of polymorphism at *LF* and *TLR4* loci on milk components and milk yield was calculated by using the least squares method (LSM) in SAS v.9.4 software. The fixed effects were: genotypes (*LF* and *TLR4)*, herd-year-season (3 months) of measurement (HYS), age, father and breed. The analyses were performed for following variables: milk yield (MY), percentage of fat (FP), kilograms of fat (FK), percentage of proteins (PP), kilograms of proteins (PK), percentage of lactose (LP) and kilograms of lactose (LK).

Results and discussion

In *LF* are possible variants CC, CT and TT. In *TLR4* are possible allele 01 (TCAC), allele 02 (GCCC), allele 03 (TCCG), allele 04 (TTCG) and allele 05 (TCCC) and their mutual combination. In Table 1 are recorded absolute and relative genotype frequencies.

Genotypes combination 0305 and 0505 were the most common genotypes. In White Shorthair, genotype 0305 (35.97 %) was more frequented than 0505 (33.62%). In Brown Shorthair the genotype 0505 (50%) predominated above the genotype 0305 (28.33%). Genotypes 0102, 0202, 0203, 0204 and 0405 were detected only in White Shorthair goat population. Genotype 0104 and 0404 did not have any individual.

Allele frequencies of genetic polymorphism at *LF* and *TLR4* genes are shown in Table 2. All SNPs except last *TLR4* SNP (this was in H-W equilibrium P<0.01) were in Hardy-Weinberg equilibrium (P<0.05)

Table 1. Absolute and relative genotype frequencies for each *LF* and *TLR4* genotype

Gene	Genotype	Absolute frequency	Relative frequency (%)
LF	TT	447	75.38
LF	CT	130	21.92
LF	CC	16	2.7
TLR4	0101	1	0.17
TLR4	0102	3	0.51
TLR4	0103	16	2.7
TLR4	0105	40	6.75
TLR4	0202	1	0.17
TLR4	0203	8	1.35
TLR4	0204	1	0.17
TLR4	0205	34	5.73
TLR4	0303	52	8.77
TLR4	0304	3	0.51
TLR4	0305	216	36.42
TLR4	0405	14	2.36
TLR4	0505	204	34.4

When calculating least squares means, all effects included in the model were statistically significant.

In Table 3 are shown least squares means (LSM) and standard errors (in brackets) for milk yield and milk components for each *LF* genotypes and for the most frequent *TLR4* genotypes.

Table 2. Allele frequencies of *LF* and *TLR4*

	LF	*TLR4 97R*	*TLR4 14R*	*TLR4 125R*	*TLR4 143R*
T	0.8634	0.9595	0.0152	-	-
A	-	-	-	0.0514	-
G	-	0.0405	-	-	0.3364
C	0.1366	-	0.9848	0.9486	0.6636
p^2	0.7396	0.9216	0.9604	0.9025	0.4356
2pq	0.2408	0.0768	0.0392	0.0950	0.4488
q^2	0.0196	0.0016	0.0004	0.0025	0.1156

Table 3. Resulting least squares means and standard errors for *LF* and *TLR4*

Gene	Genotype	MY	FP	FK	LP	LK	PP	PK
LF	TT	2.68^{a} (0.04)	3.32^{a} (0.04)	3.2^{a} (0.07)	4.52^{a} (0.01)	4.48^{a} (0.09)	3.08^{b} (0.02)	3.01^{a} (0.06)
LF	CT	2.62^{b} (0.05)	3.32^{a} (0.04)	3.16^{a} (0.07)	4.55^{b} (0.02)	4.42^{a} (0.09)	3.07^{a} (0.02)	2.97^{a} (0.06)
LF	CC	3.04^{c} (0.8)	3.45^{b} (0.07)	3.76^{b} (0.12)	4.56^{a} (0.03)	5.15^{b} (0.15)	3.03^{a} (0.03)	3.39^{b} (0.1)
TLR4	0105	2.82 (0.05)	3.26 (0.05)	3.41 (0.08)	4.48 (0.02)	4.81 (0.1)	3.02 (0.02)	3.18 (0.07)
TLR4	0205	2.64 (0.05)	3.4 (0.05)	3.33 (0.08)	4.58 (0.02)	4.56 (0.10)	3.07 (0.02)	3.05 (0.07)
TLR4	0303	2.66 (0.05)	3.33 (0.04)	3.29 (0.08)	4.49 (0.02)	4.49 (0.1)	3.1 (0.02)	3.07 (0.06)
TLR4	0305	2.75 (0.04)	3.31 (0.04)	3.34 (0.06)	4.52 (0.01)	4.7 (0.08)	3.06 (0.01)	3.14 (0.05)
TLR4	0505	2.61 (0.04)	3.37 (0.04)	3.26 (0.06)	4.52 (0.01)	4.49 (0.08)	3.07 (0.01)	3.03 (0.05)

[a, b, c] LSMs marked by different letters differ significantly (P<0.05) (only for *LF*), MY – milk yield, FP – fat percentage, FK – fat kg, LP – lactose percentage, LK – lactose kg, PP – proteins percentage, PK – proteins kg

For *TLR4*, there were different statistically significant differences between genotypes for individual properties. Except protein percentage and lactose percentage, there was statistical significant difference between genotype 0305 and 0505 (the most common genotypes). At *LF*, genotype CC had the best values for milk yield and milk components except protein percentage. At *TLR4* genotype 0305 had higher milk yield, fat content in kg, lactose content in kg and protein content in kg than genotype 0505. The most common genotypes for *LF* and for *TLR4* did not have the best values for milk yield and milk components. This could be caused due to the binding of both genes to other genes or association with other properties such as mastitis (Ateya et al., 2016; Firyal et al., 2018; Gondaira et al.,2018). Great importance to the composition of milk also has nutrition (Morand-Fehr et al., 2007; Kholif et al., 2018).

Conclusion

Current study describes genetic variability at *LF* gene in exon 4 and at *TLR4* gene in exon 3 and their association of genotypes on milk performance. New SNPs were described using new primers. Results regarding the *LF* and T*LR4* genes and their possible effect on milk production presented in this work or published in sheep and goats so far highlight the necessity of further investigation of both genes. Further studies are needed to evaluate the possible association between LF and TLR4 and milk production traits and possible influence on mastitis in goats and sheep.

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