The influence of TLR4 gene polymorphisms on milk quality and composition of Lithuanian Holstein cows

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Abstract

This study investigated bovine TRL4 gene c.9421C>T, c.2021C>T and c.-10C>T polymorphisms and their relationship with somatic cell count and indicators of milk composition. Blood samples were collected from 152 Lithuanian Holstein dairy cows. The method of polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) was used to genotype TLR4 gene polymorphisms. The data concerning somatic cell count and milk composition indicators (fat, protein, lactose) were analysed. The influence of genes and statistical significance of differences between different genotypes was evaluated by the one-factor dispersion analysis (ANOVA). Results showed that c.9421C>T was significantly associated with somatic cell count in milk. Also a significant association between the genotypes of c.9421C>T and somatic cell count was found (P<0.05). Cattle with the TT genotype showed the lowest somatic cell count. TLR4 gene polymorphisms c.2021C>T and c.-10C>T have no significant effect on mastitis resistance and milk composition. Analysis of the combined genotypes TC/CC of c.2021C>T and c.9421C>T allowed us to determine the possible association of SNPs with the somatic cell count and lactose content. The study showed a significant association between the somatic cell count (SCC) and TLR4 polymorphism c.9421C>T in Lithuanian Holstein cows. SCS of cows with a TT genotype was significantly lower and indicated the association of TT genotype with resistance to mastitis in c.9421C>T and allele T might be the beneficial allele for mastitis resistance.

Key words: Lithuanian Holstein cow, milk, single nucleotide polymorphisms, toll-like receptor 4

Introduction

Mastitis is one of the most prevalent diseases of dairy cattle, which causes huge economic losses to the dairy industry worldwide (Ruegg, 2003). The somatic cell count (SCC) in milk is regarded to reflect the degree of mastitis. The mammary gland tissue is protected by two defence systems of the immune system: innate or nonspecific immunity and the acquired or specific immunity. Both, innate and acquired immunity, interact in the attempt to provide protection against mastitis causing microorganisms (Burvenich et al., 2015). Quantitative trait loci (QTL) for mastitis and SCC have been identified in nearly all chromosomes of the bovine genome (Rupp et al., 2015). Ten different TLRs (TLR1 to TLR10) have been identified in cattle. Among them, TLR2, TLR4, TLR6 and TLR9 genes have been found associated with mastitis resistance in cattle and play a role in innate immunity (Yang et al., 2008).
The genes that are strong potential markers for resistance/susceptibility to udder inflammation include genes encoding PAMP-recognition receptors (PRRs) (Sharma et al., 2006). TLR4 is a candidate gene for resistance to a large number of diseases. Toll-like receptor 4 (TLR4) recognizes pathogen ligands and mediates signalling to initiate innate and adaptive immune responses (Wang et al., 2007). TLR4 was the first mammalian TLR identified, and it is consequently the best described of the family (Takeda et al., 2003). The structure of bovine TLR4 gene is very complex. This gene encodes 841 amino acids (Wang et al., 2007; El-Domany et al., 2019). The TLR4 gene in cattle about 3739-bp, contains an open reading frame of 2526-bp encoded 841 amino acids (El-Domany et al., 2019). The bovine TLR4 gene coding region is 2526 bp long, consists of 3 exons and is located on chromosome BTA 8 (White et al., 2003). Bovine TLR4 is highly polymorphic with more than 40 SNPs (single nucleotide polymorphisms) of the bovine TLR4 gene have been found to date, which means an average of 1 SNP per 90 bp (Wang et al., 2007). TLR4 gene discovered across 14 breeds of cattle (Sharma et al., 2006; Wang et al., 2007; Opsal et al., 2008).

There are several studies reporting associations between TLR4 polymorphism and milk traits in dairy cows. Beecher et al. (2010) revealed an association between variation in TLR4 and milk fat and protein percentage in late lactation in Irish cattle. In Iranian Holstein cattle Noori et al. (2013) described an association between TLR4 exon 3 variation, and somatic cell score and milk-related traits, while Zhou et al. (2017) have determined how polymorphisms of the TLR4 gene influences milk fat content in Chinese Holstein cows. Based on the findings of Ögorevc et al. (2009) who examined the role of TLR4 in pathogen recognition and subsequent initiation of the inflammatory and immune response, and on differential expression of the gene during mastitis, TLR4 has been proposed as a candidate for increasing mastitis resistance in breeding programs.

The purpose of our research was to investigate TLR4 c.9421C>T, c.2021C>T and c.-10C>T polymorphisms and to evaluate their correlation with somatic cell count and milk productivity traits in Lithuanian Holstein cows.

### Materials and methods

#### Sample collection and DNA extraction

Blood samples were collected from 152 Lithuanian Holstein dairy cows. 5 mL of blood was collected from each animal jugular vein in tubes containing 2.7 % EDTA and stored at -20 °C. Analyses were done in Lithuanian University of Health Sciences, Dr. K. Janušauskas Laboratory of Genetic (Kaunas, Lithuania). Genomic DNA was isolated from cows’ blood using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the recommended protocols. Primers applied for genotyping are shown in Table 1.

**TABLE 1. TLR4 gene polymorphisms and oligonucleotide primers**

| SNP    | Primers sequence (5’-3’)                                                                 | Restriction enzyme | Position | Amplicon size, bp | Fragment size, bp |
|--------|--------------------------------------------------------------------------------------------|--------------------|----------|-------------------|------------------|
| c.9421 C>T | AGACACCATTTTCACTCCCTC ACCACCGACACACTGATGAT                                              | AluI               | Partial exon3 | 382               | 118, 142*        |
| c.2021 C>T | GGGTCTAGTCTACAAGTTCC AGATGAAAGATGACGCAG                                               | BsiHKAI            | Partial exon3 | 367               | 75, 292          |
| c.10 C>T  | CGTACCACCGACTGCTTTGG GCCGTGAATAGGCCTTGACACC                                           | BstUI              | Partial exon3 | 405               | 159, 246         |

*77, 32 and 13 bp in agarose gel is not visible*
PCR and polymorphism detection

Primers for 3 SNPs in TLR4 (c.9421C>T, c.2021C>T and c.-10C>T) T4CRBR2, rs8193069, rs8193041) gene were designed (NCBI Reference sequence: NC_037335.1). The method of polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) was used to genotype TLR4 gene polymorphisms. The PCR reactions for c.2021C>T and c.-10C>T were performed in 25 µL volume of reaction mixture containing 15.0 µL MasterMix (Thermo Fisher Scientific, Waltham, MA, USA), 0.5 µM (10 pmoL) of each primer pair (Table 1) using Applied Biosystems 2700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA), 2 µL (50 ng) genomic DNA and 7.0 Nuclease-free water. The PCR mixture for c.9421C>T contained 1.5 µL (50 ng) DNA, 0.4 µL (10 pmol) of each primer, 2 µL 10x PCR buffer, (0.4 µL) 200 µmol dNTPs and 5 U of Taq DNA polymerase in a total volume of 20 µL. The primers and optimal conditions of PCR of TLR4 SNP’s are presented in Table 1 and Table 2. After amplification, 10 µL of PCR product were digested with a selected restriction enzyme (Table 1) according to producer recommendations (Thermo Fisher Scientific, Waltham, MA, USA). The visualization of the different genetic types was carried out by the 2 % agarose gel electrophoresis. The ethidium bromide was added to agarose to a final concentration of 0.5 µg/mL (Thermo Fisher Scientific, Waltham, MA, USA). Fragment identification was performed in ultraviolet light, using MiniBIS Pro Video Documentation System (DNR Bio-Imaging System, Neve Yamin, Israel).

| SNP         | Predegeneration | Generation | Annealing | Extension | Cycle | Extension |
|-------------|-----------------|------------|-----------|-----------|-------|-----------|
| c.9421 C>T  | 95 °C 5 min     | 94 °C 30 s | 62 °C 30 s | 72 °C 40 s | 35    | 72 °C 40 s |
| c.2021 C>T  | 94 °C 4 min     | 94 °C 30 s | 53.9 °C 30 s | 72 °C 30 s | 35    | 72 °C 10 min |

Traits and statistical analysis

Genetic parameters for milk quality (somatic cell count, thousand/ml) and milk composition (fat, protein and lactose percentage) in Lithuanian Holstein population were estimated.

The data concerning SCC and milk composition indicators were derived from the results of monthly test milkings. Fat, protein and lactose content was determined by the infrared instrument LactoScope FTIR (Perten Instruments, Stockholm, Sweden). Somatic cell count was performed by the heavy-duty counter-measurer Somascope (Perten Instruments, Stockholm, Sweden) which operates by the fluoro-opto electronic method. Milk analysis was performed at State Enterprise “Pieno Tyrimai” in an accredited central milk testing laboratory in Lithuania.

The analysis was performed using data accumulation and analysis program SPSS 22.0 (Statistical Package for Social Science 22 for Windows). The following descriptive statistics were used for the exploratory characteristics: arithmetic mean and its error; frequency distributions for qualitative variables were calculated genotype and allele frequencies for the studied population of cows. Using the one-factor dispersion analysis (ANOVA, post-hoc Fisher LSD criterion, α = 0.05) influence of genes and statistical significance of differences between different genotypes were evaluated. The results are considered reliable when P<0.05. Using the Kolmogorov-Smirnov test the assumption of the continuity of the variables was verified.

Results

Allele C was detected in 77 heterozygous and 43 homozygous samples out of 152 samples for the c.9421C>T. After carrying out genotyping allele T was detected in 145 heterozygous and 5 homozygous samples out of 152 samples at the c.2021C>T. Meanwhile, allele C was detected in 1 heterozygous and 151 homozygous samples at the c.-10C>T polymorphism.
The genotypic and allelic frequencies are shown in Table 3. In two polymorphisms - c.9421C>T and c.2021C>T all three possible genotypes (TT, TC and CC) were identified. The most equal distribution of genotypes established at c.9421C>T, which means that this polymorphism is evenly distributed among dairy cattle. Genotype TC was the most common genotype in two c.9421C>T and c.2021C>T polymorphisms though allele T was more frequent in c.9421C>T. TC frequency was 44.7 % bigger in c.2021C>T than in c.9421C>T. TT genotypic frequency was the least (3.3 %) in the c.2021C>T. Both alleles (T and C) and two genotypes (TC and CC) with very uneven distribution were observed in c.-10C>T. The TT genotype was not detected in cows may be due to the insufficient number of cows.

**Table 3. Allele and genotype frequency of the TLR4 SNP’s in Lithuanian Holstein cows**

| SNP     | Genotype | Number of cows | Genotype frequency | Allele | Allele frequency |
|---------|----------|----------------|--------------------|--------|------------------|
| c.9421 C>T | CC       | 43             | 0.283              | C      | 0.536            |
|         | TC       | 77             | 0.507              | T      | 0.464            |
|         | TT       | 32             | 0.210              | -      | -                |
| c.2021 C>T | CC       | 2              | 0.013              | C      | 0.490            |
|         | TC       | 145            | 0.954              | T      | 0.510            |
|         | TT       | 5              | 0.033              | -      | -                |
| c.-10 C>T | CC       | 151            | 0.993              | C      | 0.997            |
|         | TC       | 1              | 0.007              | T      | 0.003            |
|         | TT       | 0              | 0                  | -      | 0                |

The association of TLR4 gene SNP’s with somatic cell count was analysed (Table 4). A significant association between the genotypes of c.9421C>T and SCC was found (P<0.05). Cattle with the TT genotype showed the lowest SCC in comparison to the cattle with TC genotype. A non-significant association between the genotypes TC and CC of c.-10C>T was found, but SCC in cattle with TC genotype was on average 209.0 thousand/mL lower by following with CC genotype. Cows with CC genotype had the biggest influence on SCC in c.2021C>T.

**Table 4. Effects of different polymorphisms genotypes on somatic cell count**

| SNP     | Genotype | SCC thousand/mL (mean±SE) |
|---------|----------|---------------------------|
| c.9421 C>T | CC       | 309.35±43.33              |
|         | TC       | 362.51±48.57              |
|         | TT       | 176.47±21.92              |
| c.2021 C>T | CC       | 121.5±82.5               |
|         | TC       | 311.54±29.54              |
|         | TT       | 289.2±84.55               |
| c.-10 C>T | CC       | 309.68±28.51              |
|         | TC       | 100.00                    |

a, b - means with different letters differed significantly at P<0.05 (post-hoc Fisher LSD criterion)
was found in cattle with genotype TT. The results of c.9421C>T showed cows homozygous for the T allele had higher milk fat percentage than those of TC genotype while cows with genotype TT had no significant association with the milk protein content. Cows homozygous for the C allele had the biggest impact on lactose percentage. Meanwhile, no significant association between two genotypes of c-10C>T and fat, protein and lactose content was found.

**TABLE 5. Effects of different SNP's of TLR4 gene on milk composition indicators**

| SNP          | Number of cows | Genotype | Fat, %   | Protein, % | Lactose, % |
|--------------|----------------|----------|----------|------------|------------|
| c.9421 C>T   | 43             | CC       | 4.14±0.08 | 3.43±0.04  | 4.56±0.02  |
|              | 77             | TC       | 4.24±0.08 | 3.49±0.03  | 4.54±0.03  |
|              | 32             | TT       | 4.03±0.11 | 3.45±0.06  | 4.62±0.02  |
| c.2021 C>T   | 2              | CC       | 4.69±0.33 | 3.62±0.23  | 4.67±0.06  |
|              | 145            | TC       | 4.14±0.05 | 3.46±0.02  | 4.57±0.01  |
|              | 5              | TT       | 4.65±0.23 | 3.52±0.08  | 4.48±0.04  |
| c.-10 C>T    | 151            | CC       | 4.17±0.05 | 3.46±0.02  | 4.57±0.01  |
|              | 1              | TC       | 3.97      | 3.5        | 4.66       |

One-factor dispersion analysis (ANOVA) evaluated the influence of the TLR4 gene polymorphisms (c.2021C>T, Gen2 and c.9421C>T, Gen3) and the differences between different genotypes (Table 6). Six combination genotypes of the two TLR4 gene polymorphisms were detected in analysed Holstein cows. Most combined genotypes were associated with the fat content and SCC. Cows with the combined genotype of A6 produced respectively 0.65 % and 0.58 % more fat reach milk than those of the A5 and A3 genotypes (P<0.05). Milk with the biggest lactose content was produced by cows of A5 group (P < 0.05). SCC mean of A5 combination genotype group had the lowest SCC by following A3 and A4 groups (P < 0.05). A1 genotype combination had the lowest SCC, but since only one cow had this genotype the results are questionable. Cows with A4 genotype combination showed the highest SCC.

**TABLE 6. Combination effects of c.2021C>T and c.9421C>T polymorphisms (mean±SE)**

| Combination genotype (Gen2_Gen3) | Number of cows | Fat, %   | Protein, % | Lactose, % | SCC thousand/ml |
|----------------------------------|----------------|----------|------------|------------|-----------------|
| A1 (CC/TC)                       | 1              | 4.36     | 3.39       | 4.73       | 39              |
| A2 (CC/TT)                       | 1              | 5.01     | 3.84       | 4.61       | 204             |
| A3 (TC/CC)                       | 41             | 4.07±0.08 | 3.41±0.05  | 4.57±0.02  | 312.0±48.05     |
| A4 (TC/TC)                       | 73             | 4.24±0.08 | 3.49±0.03  | 4.56±0.02  | 366.8±49.02     |
| A5 (TC/TT)                       | 31             | 4.00±0.10 | 3.44±0.06  | 4.62±0.02  | 175.6±22.62     |
| A6 (TT/CC)                       | 5              | 4.65±0.23 | 3.52±0.08  | 4.48±0.04  | 289.2±84.55     |

a, b - means with different letters in each column differed significantly at P<0.05 (post-hoc Fisher LSD criterion)
Discussion

The bovine TLR4 gene is highly polymorphic (Wang et al., 2007; Mariotti et al., 2009) and some SNPs in the TLR4 gene have been associated with the somatic cell score (SCS) in cattle and milk traits (Sharma et al., 2006; Wang et al., 2007). Studies have reported that mutation in the TLR4 gene increases the susceptibility of cattle to mastitis (Sentitula et al., 2012). White et al. (2003) have identified several polymorphisms in the bovine TLR4 gene, many of which reportedly affect somatic cell count during mastitis. The study Razak et al. (2015) revealed that polymorphism is prevalent in the TLR4 gene and is significantly associated with mastitis in Holstein cross-bred cattle. El-Domany et al. (2019) study revealed TLR4 genes could partly be associated with an improvement in milk and reproductive performance in dairy cows which may lead to a brief and rigorous selection within dairy cows. The presence of these polymorphisms significantly affected the studied milk and reproductive parameters.

Our study was carried out to analyse three polymorphisms of the TLR4 gene and to determine their possible associations with SCC and milk composition traits in Holstein cows. Noori et al. (2013) showed the T allele in c.9421C>T was associated with lower fat percentage and lower SCS when compared to allele C. Our results obtained frequency of allele T dominated in c.9421C>T. Cows homozygous with T allele produced milk with the lower fat content (4.03 %) but lactose content was the biggest (4.62 %) by following homozygous C allele although no reliable associations have been identified. Zhou et al. (2017) reported also that the phenotypic variation in milk traits of dairy cows in pasture-fed New Zealand (NZ) Holstein-Friesian Jersey (HF×J) cross dairy cows was attributed to TLR4 gene polymorphism. Two variants were observed, allele C was associated with higher milk yields and but lower milk fat percentage, whereas T was associated with lower milk yields and higher fat and protein percentages. The study showed that SCS in cows with a TT genotype was significantly lower than that of the TC genotype (P<0.05). The result indicated that the cattle with the TT genotype were more difficult to be infected with mastitis than the cattle with CC genotype and allele T might be the beneficial allele for mastitis resistance. Sharma et al. (2015) demonstrated an association between the C allele (the most frequent) with lower SCS in Holstein cows. Wang et al. (2007) found no significant association between the genotypes in c.9421C>T and SCS for Chinese Holstein cattle (P>0.05) but Gupta et al. (2015) determined the frequency of CC genotype was significantly higher (P≤0.05) in healthy animals and indicated the association of CC genotype with resistance to mastitis in crossbred cows.

TLR4 c.2021C>T is a nonsynonymous SNP in exon 3 that corresponds to an amino acid change in the predicted transmembrane-cytoplasmic domain (Mariotti et al., 2009). This polymorphism is in locus rs8193069 of the TLR4 gene. Carvajal et al. (2013) found that for this SNP the most frequent variant in Holstein was the C allele (89 %) and allele T was found with low frequency (7 %) (Sharma et al., 2006) but our studies showed the more frequent T allele (51 %). Carvajal et al. (2013) also evaluated c.2021C>T of Chilean dairy cattle breeds associated with mastitis but no associations with SCS were evident. The results obtained in our studies also showed no significant association with SCS and the main milk composition indicators at this locus (P>0.01) while Beecher et al. (2010) found c.2021C>T association (P<0.05) with both milk protein and fat percentage (P<0.01) in late lactation in Holstein-Friesian cows. The study of Peng et al. (2010) determined the homozygous genotype of c.2021C>T to be significantly associated with incidences of mastitis. Sharma et al. (2015) suggested that c.2021C>T may affect TLR4 signalling and lead to inadequate induction of immune response genes, and therefore that it may have been selected against by natural selection.

The result indicated that allele C was the predominant allele with a frequency of 99.3 % in the c.-10C>T. The genotypic effect on SCS and milk composition parameters were not significant. The cattle with the CC genotype showed bigger SCS in accordance with TC genotype. We can be predicted that T allele may be could have a higher effect on SCS.

According to Sender et al. (2013) the best cumulative genotype does not identify individual genes, and animals are selected based on the estimated cumulative impact of multiple genes.
on resistance to mastitis. Mesquita et al. (2012) studied Brazilian Holsteins for TLR4 polymorphism indicated that animals with combined genotypes AACCCC, GGTCGG, and GACCGC presented the lowest SCS and have the potential to be applied as molecular markers for assisted animal selection to improve milk quality. Six combined genotypes of two TLR4 gene SNPs (c.9421C>T and c.2021C>T) were observed, though only one cow had a CC/TC and CC/TT combined genotype. Analysis of the combined genotypes revealed a significant correlation between the SCC and the genotype TC/TT (A5) (P < 0.05). There was also a significant association between this genotype and lactose content in milk (P<0.05). Accordingly, this genotype (TC/TT) could have the potential to be used as a genetic marker in the selection of animals with lower SCS and higher lactose percentage in milk.

**Conclusion**

The relationship between the bovine mastitis traits and the polymorphisms of TLR4 gene indicated that genotype TT of c.9421C>T was related to mastitis resistance and allele T might be the beneficial allele for mastitis resistance. The obtained results indicated that the selection of the TC/CC genotypes of the other two polymorphisms might contribute to a reduction of SCC in Lithuanian Holstein cows.

**Utjecaj polimorfizma gena TLR4 na kvalitetu i sastav mlijeka krava holstein pasmine u Litvi**

**Sažetak**

U okviru ove studije ispitivani su polimorfizmi c.9421C>T, c.2021C>T i c.-10C>T kravlje gena TLR4 te njihova povezanost s brojem somatskih stanica i sastavom mlijeka. Uzorci krvi izuzeti su od ukupno 152 krave holstein pasmine u Litvi. Za tipizaciju polimorfizma gena TLR4 korištene su metode polimeraza lančane reakcije (PCR) i polimorfizam dužine restrikcijskih fragmenata (RFLP). Također su određivani broj somatskih stanica i odabrani parametri kemijskog sastava mlijeka (mast, protein, laktoza). Za određivanje utjecaja gena i statističkog značaja razlika među ispitivanim genotipovima korištena je jednosmjerna analiza varijance (ANOVA). Rezultati su pokazali da postoji statistička značajna povezanost između genotipa c.9421C>T i broja somatskih stanica (P<0.05). Jedinke genotipa TT imale su najniži broj somatskih stanica. Polimorfizmi c.2021C>T i c.-10C>T gena TLR4 nisu pokazali značajan utjecaj na otpornost prema mastitisu niti na sastav mlijeka. Analiza kombiniranih genotipova TC/CC polimorfizma c.2021C>T i c.9421C>T omogućili su utvrđivanje povezanosti SNP-ova s brojem somatskih stanica i s udjelom laktoze. Ovim istraživanjem je utvrđena značajna povezanost između broja somatskih stanica i polimorfizma c.9421C>T gena TLR4 u krava pasmine holstein u Litvi. Broj somatskih stanica u krava genotipa TT bio je značajno niži i ukazivao je na povezanost ovog genotipa s otpornošću na mastitis u c.9421C>T. Stoga se alel T može smatrati poželjnim za otpornost prema mastitisu.

**Ključne riječi:** krave pasmine holstein, Litva, mlijeko, polimorfizmi jednog nukleotida, toll-like receptor 4
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