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The Effect of the TNFα Gene Alleles in Holstein Cows on Reproductive Function

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Abstract

SNP polymorphism in the promoter part of the TNFα gene at the 824 A → G position in Holstein cows of the Bayserke-Agro LLP was represented by the following genetic variants: AA - 22.4%, AG - 63.8%, GG - 13.8%; the frequency of the A and G alleles was 0.54 and 0.46. In the studied population, the excess occurrence of the AG +21.49 heterozygous genotype was detected, and in other genotypes, a deficit of homozygous variants of GG and AA was observed by -11.16 and -10.32 of individuals, respectively. The reproductive function indicators were high in cows with the GG genotype: the interval between calving and fertilizing insemination was 259 days, the insemination index was 2.63, the proportion of animals inseminated after more than 91 days was minimum 52% in individuals of the GG homozygous genotype.

Keywords: promoter part of the TNFα gene, PCR-RFLP, reproductive function of cows, DNA markers.

INTRODUCTION

Currently, genetic factors are regarded as fundamental in reducing the reproductive function of cows observed in recent years. Studies have shown the effect of the T and C alleles of the Toll-like receptor 4 (TLR4) on the immune function of cells (apoptosis and migration), the insemination index of cows, and the duration of infertility (sterility). The area 4 of the intron portion of the TLR4 gene was amplified using the following primers: the forward F - 5′-TCTTTGCTCGTCCCAGTAGC-3′, and the reverse R - 5′AAGTGAATGAAAAGGAGACCTCA-3′, 3 of the exon part of the TLR4 gene: F-5′ AGACAGCATTTCACTCCCTC-3′, and R - 5′-ACCACCGACACACTGATGAT-3′. The SNPs polymorphism in the 4 intron and 3 exon parts of the TLR4 gene was detected by PCR-RFLP analysis, the insemination index was low in individuals with the TC heterozygous genotype (polymorphism in exon 3 of the TLR4 gene) compared to carriers of the homozygous CC genotype, 1.6±0.2 and 2.2±0.2, respectively. A similar correlation was observed for the duration of the period from calving to fertilizing insemination. Thus, in individuals with the TC genotype, this indicator was 100.7±6.9 days, in animals with the CC genotype - 136.6±9.0 days; SNPs polymorphism in the intron portion of the TLR4 gene did not influence the reproductive performance of cows. The percentage of apoptosis of polymorphonuclear (PMN) leukocytes treated with lipopolysaccharide was generally lower in individuals with the TC genotype compared to the CC genotype, the rate of migration of PMN leukocytes and the production of IL-1β in PBMC were higher in animals with the TC genotype as compared to those with the CC genotype. The authors recommend the use of DNA polymorphism by the TLR4 gene locus as a tool for assessing reproductive potential and immune activity of individual cows [1].

The study conducted by scientists in 1996 revealed the localization of the TNFα (tumor necrosis factor) gene on the 23rd chromosome and identified 4 alleles of the TNFα gene in cattle of six breeds from different geographic regions. The TNFα gene allele frequency varied from 0 to 0.61 in different breeds of representatives of *B. Taurus and B. Indicus*; the heterozygosity level was high (0.80) in individuals of local breeds, and low (0.22) - in Frisian breeds. To detect alleles of the TNFα gene, the authors used forward F- 5′AGCCTCAAGTAACAAGCCG-3′ primers and reverse R 5′ TCTACTCTCCACATCCTGG- 3′ primers [2].

The Japanese scientists established the effect of polymorphism in the promoter part of the TNFα gene and the SNP replacement of one nucleotide in the exon part of the indicated gene on the immune status and reproductive function in cows. In the study of the population of dairy cows, the authors identified the following genetic variants by the TNFα locus: A/A, A/G, G/G and T/T, T/C, C/C in the promoter and exon parts of the gene, respectively.

The interval between calving and first ovulation was short in cows with the heterozygous A/G genotype and the homozygous G/G genotype compared to animals with the homozygous A/A genotype. Polymorphism of the promoter part of the TNFα gene in cows had no effect on the apoptosis rate of PMN leukocytes, but the transmigration rate was significantly higher in animals with the A/A and A/G genotype than in animals with the homozygous G/G genotype. A correlation was found between the mRNA expression level of promoter part of TNFα gene and the formation of interleukin 8 (IL-8), which performed the protective function in the body. Thus, the expression of mRNA of PMN leukocytes and peripheral blood mononuclear cells was higher in cows with the A/A genotype than in those with the G/G genotype. The findings indicate that the polymorphism of the TNFα gene alleles has a significant effect on immune function and reproductive performance in cows [3].

The PCR and real-time PCR diagnostic methods were used for genotyping of individuals by the locus of the TNFα gene and studying the effect of SNP polymorphism at the 824 A → G position on the expression level of this gene in cows with viral leukemia (BLV) and in animals resistant to this disease. It has been established that the polymorphism of the TNFα gene at the 824 A → G position had a complex effect on the TNFα gene expression in cows infected with BLV. In healthy cows, significant differences in the expression of the TNFα gene were not detected at any of the levels analyzed.

Conversely, leukemia virus infection (BLV) and the progression of enzootic bovine leukemia (EBL) resulted in the differentiation of the TNFα gene expression at the mRNA and protein levels, but differences in the amount of transcript and percentages of the mTNFα and cells showed a reverse trend. The

lowest mRNA levels and the highest percentage of PBMC expressing the mTNFα protein were identified in BLV-positive cows. The alleles of the TNFα gene were identified by PCR-RFLP analysis using Sac I restriction enzyme, and three groups of genotypes were identified: AA (168 bp, 81 bp), AG (249 bp, 168 bp, 81 bp) and GG (249 bp) [4].

Associative data and some studies have shown that inhibition of the TNFα gene expression contributes to the obesity of the liver with the energy deficiency in dairy cattle. Experimentally, under in vitro cultivation conditions, the authors have demonstrated the ability of TRLP to inhibit TNFα signaling on primary cattle hepatocytes with recombinant TNFα. TRLP was injected under the skin to four lactating Holstein cows for 24 hours at an interval of 4 hours at a rate of 0.15 and 3.0 mg per kg of live weight, and recombinant TNFα was injected intravenously at a dose of 5 μg per 1 kg of live weight of a cow. According to the results of the study, injection of the recombinant TNFα and TRLP for 2 hours reduced the amount of non-esterified fatty acid (NEFA), which indicated a change in the metabolic process in cows. Despite the fact that TRLP inhibited cattle TNFα signals, the use of recombinant TNFα for 7 days did not alter the metabolism in cows with negative energy balance [5].

Initial protection of the endometrium of the cow's uterus against the pathogenic effects of microbes is due to three innate immune systems: Toll-like receptors (TLR), antimicrobial peptides (AMP) and acute phase proteins (APP). Endometrium of the uterus in cows is infected with bacteria, which are often the main etiological factor of the pathology of the uterus. The endometrial epithelium of the uterus is the first defensive line against pathogenic agents, and the TLR is an important component of the innate immune system to detect pathogen-associated molecular patterns-PAMP.

It is known that the family of TLR of the mammal includes 10 receptors of this family, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10, where Toll-like TLR1, TLR2 and TLR6 receptors recognize bacterial lipids, and TLR3, TLR7, TLR8 and TLR9 recognize viral nucleic acids. The studies have also shown that the TLR9 receptor recognizes bacterial DNA [6].

The APP include peptides, such as haptoglobin and serum amyloid A that provide nonspecific protection against microbes. These peptides are synthesized in the liver, and the protective function is performed on the endometrial mucosa. The expression of genes controlling the APP peptides synthesis is regulated by steroid sex hormones [7, 8].

Mucin-1 (MUC-1) is a glycosylated transmembrane protein of the epithelial cell of the endometrial mucosa, which also plays a major role in the microbial protection of the endometrium [9].

As a material for the study of gene expression of the family of TLR and MUC-1, APP the authors used non-pregnant cow's uterine tissue. The level of expression at the loci studied was determined by the RT-PCR method of studying the mRNA samples isolated from the endometrial mucosa of the uterine horns and body. The expression level of the TLR of the uterine body endometrium, the ipsilateral and contralateral uterine horns relative to the functioning yellow body did not differ. It was also established that endometrial epithelial cells provided for the expression of the TLR 1-7 and TLR9 genes, and stromal endometrial cells provided for that of the TLR 1-4, TLR6, TLR7, TLR9 and TLR10 genes. Thus, the main components of innate immunity are TLR associated with molecular pattern pathogens (PAMP), which lead to an increase in the expression of AMP and APP. Thus, epithelial cells play a crucial role in the innate immune protection of endometrium of the cows' uterus against bacteria that often cause infertility in cows [6].

Under in vitro conditions for the cultivation of granulosa oocyte-cumulus cells, Brazilian scientists evaluated the effect of the GnRH preparation on the mRNA expression level of the TNFα, TNFR1 and TNFR2 systems in bovine cattle. Thus, the addition of the GnRH hormone to the growth medium activated the level of TNFα, TNFR1 and TNFR2 mRNA expression after 3, 6, 12 hours of cultivation, compared to 0 and 24 hours after cultivation. The study of the dynamics of the change in the amount of mRNA was carried out using the Real-Time PCR method. It should be noted that the expression level of the TNFα, TNFR1 and TNFR2 genes in antral follicles under in vivo and in vitro conditions of cultivation in *Bos taurus* individuals depends on the concentration of the GnRH gonadotropin, which has a great practical importance in regulating the reproductive function of cows [10].

Development of DNA markers of the reproductive function of animals, dairy and beef production and their introduction in the selection practice, the creation of a population of animals resistant to diseases, and prediction of useful traits are among actual problems of molecular and population genetics [11, 12]

The objective of the study was the genotyping of Holstein cows by the TNFα gene locus using the PCR-RFLP analysis, and studying the effect of alleles of the gene under study on the manifestation of reproductive function.

MATERIALS AND METHODS

The experiments were carried out in 2015-2017 on 152 Holstein cows of the Canadian selection from the breeding company Bayserke-Agro LLP, Almaty region. The blood for the study was taken from the jugular or tail vein into a vacuum tube with EDTA. DNA was isolated by phenol method, an equal volume of buffer 100 mM Tris-20 mM EDTA-10 mM NaCl, pH=8.0 was added to 1 cm^3 of the blood sample and centrifuged for 5 minutes at 5,000 g. The precipitate was washed one more time in the same manner and suspended in 400 μl of buffer. Then 5 μl of proteinase K (20 mg/ml) and 25 μl of a 10% solution of sodium dodecyl sulfate (SDS) were added to the suspension, gently mixed, and incubated for 3 hours at 55 °C. Then phenol (pH=8.0) was added in equal volume, and the resulting mixture was shaken for 15 minutes, then centrifuged at 10,000 g for 15 minutes and the upper aqueous phase containing DNA was gently withdrawn. 1/10 of the 3M sodium acetate volume and two volumes of cold ethanol were added to the resulting aqueous solution of DNA. The DNA passed into a visible state and was washed with 70° ethanol to remove the residues of salts and phenol. The DNA was slightly dried at room temperature and dissolved in the TE buffer.

The TNFα gene region was amplified on the Effendorf (Germany) amplifier using primers:

F 5′- GAGAAATGGGACAACCTCCA -3′

R: 5′- CCAGGAACTCGCTGAAACTC - 3′ [13].

The composition of the reaction mixture was as follows: 2.5 μl of 10 X PCR buffer, 1.5 μl of 25 mM MgCl2, 1.25 μl of 25 μM of forward and reverse primers, 2 μl of 0.2 mM of each dNTP concentration, 0.2 μl of Taq Polymerase with the 5u/μl activity, 3 μl of DNA and 13.3 μl of distilled water. The final volume of the mixture was 25 μl, the number of cycles was 35, and each cycle was as follows: 30 s – 94°C, 30 s – 60°C, and 30 s – 72 °C. The length of the resulting TNFα gene amplification was 249 bp (Figure 1). To detect SNP polymorphism at the 824 A → G position, the Sac I restriction enzyme was used which has a GAGCT/C restriction site. After fragmentation of the PCR product, the following fragments appeared on the electrophoregram depending on the animal genotype: in individuals with heterozygous AG genotype - 249, 168 and 81 bp, in in individuals with homozygous AA and GG - 168, 81 bp and

249 bp, respectively (Figure 2). To visualize the results of electrophoresis, the Infinity VX2 3026, WL/LC/26M X-Press, Vilber Lourmat (USA) gel documenting system was used, and the pUC19/MspI plasmid (Thermo Fisher Scientific) was used as the DNA marker.

RESULTS

The results of SNP polymorphism identification in the promoter part of the TNFα gene at 824 A → G position in Holstein cows among 152 heads of the Bayserke-Agro LLP breeding company showed the shift in the frequency of the A allele compared to G, 0.54 and 0.46, respectively. The heterozygous genotype of AG prevailed in the studied group of animals, and its prevalence was 63.8%, the occurrence of homozygous genotypes was: GG -13.8% and AA - 22.4%.

In cows with the GG homozygous genotype (n=19), the interval between calving and fertilizing insemination was 259 days, in individuals with the AG heterozygous genotype (n=50) the value was 378 days, the intermediate position (290 days) was occupied by animals with the AA genotype (n=27). The correlation was observed between the interval from calving to fertilizing insemination and the insemination index in animals of all three groups, a low insemination index (2.63) was in cows with the GG homozygous genotype (duration of the interval was 259 days), and a high insemination index (3.76) was observed in heterozygous animals (duration of the interval was 378 days). In homozygous individuals with the AA genotype, the insemination index was 2.85, and the interval between calving and productive insemination was 290 days.

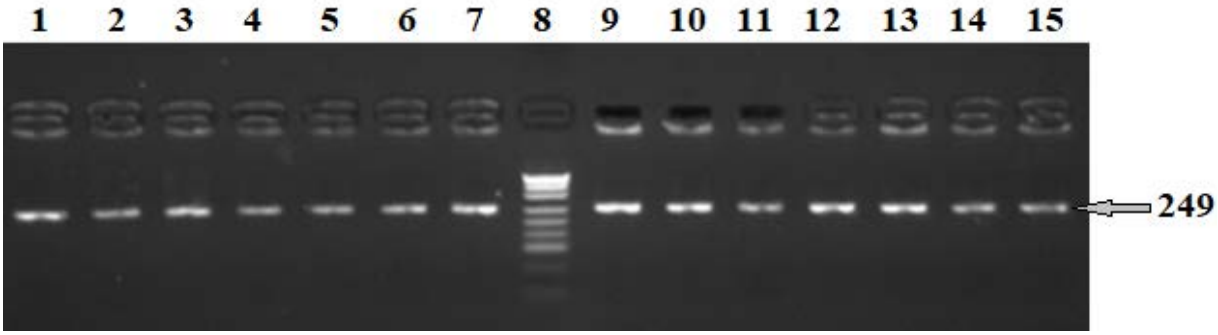

Figure 1. Electrophoregram of the TNFα gene, agarose 3%, traces 1–7, 9-15 - PCR product, trace 8 – pUC19/MspI DNA marker.

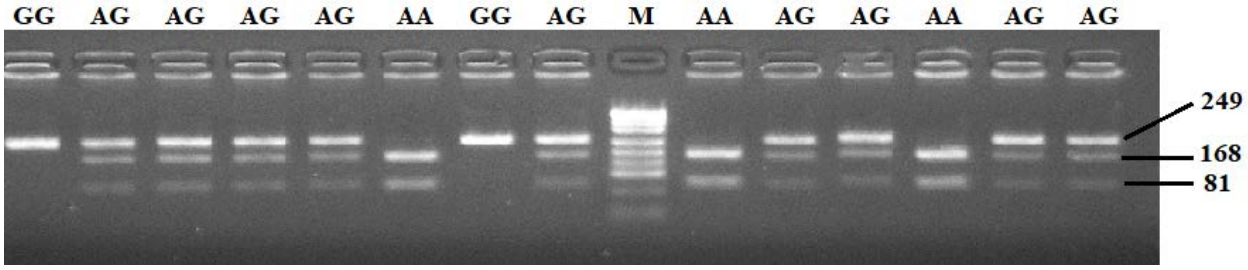

Figure 2. The electrophoregram of the Sac I restriction enzyme of the PCR product of the TNFα gene, agarose 3%, M-pUC19/MspI DNA marker, GG, AG, AA genetic variants.

Table 1. The degree of correspondence between the actual distribution of the SNP polymorphism of the promoter part of the TNFα gene in cows with the theoretical distribution, the allele frequency (n=152)

Indicators	TNFα genetic variants			Frequency of alleles	
	GG	AG	AA	A	G
The actual number of individuals (P emp)	21/13.8%	97/63.8%	34/22.4%	0.54	0.46
The theoretical expected number of individuals (P theor)	32.16	75.51	44.32		
Difference of the genotype (P emp-P theor)	-11.16	+21.49	-10.32		

Table 2. Indicators of the reproductive function of cows with different genotype by the locus of TNFα gene in cows (n=96)

Animals with TNFα genotype (n=96)	The interval between calving and fertilizing insemination (days)	Cows' insemination index	The number of successfully inseminated cows within more than 91 days after calving
GG (n=19)	259	2.63	9/47.36%
AG (n=50)	378	3.76	43/86.0%
AA (n=27)	290	2.85	17/62.96%

DISCUSSION

Analysis of the literature shows that most authors study polymorphism in the promoter of the gene since the level of gene expression depends on the functional activity of the promoter part of the relevant gene [7, 8].

If had been found that for all three genetic variants, in the cows there was a discrepancy between the actual distribution of genotypes and the theoretically expected number of genotypes, the excess occurrence of the AG +21.49 heterozygous genotype was detected, and in other genotypes a deficit of homozygous variants of GG and AA was observed, respectively, by -11.16 and -10.32 of individuals (Table 1). Similar results were obtained by foreign authors. Thus, the distribution of genetic variants of the promoter part of the TNFα gene in Japan's dairy cows was as follows: AA - 36 (16%), AG - 108 (48%) and GG 80 (36%) animal units [3]. The study of the SNP polymorphism of the promoter part of the TNFα gene at the 824 A → G position (n=127) in Holstein cows (Poland) showed more even distribution of genetic variants, namely: AA – 26.0%, AG -37.8% and GG - 36.2% [4].

The effect of alleles of the TLR1-TLR10 family, TNFα, AMP, and APP, on the immune function of cells (apoptosis and migration), the insemination index and the reproductive function of cows is now known. The mRNA functional activity in animals with various TNF-α genotypes is stable, but as demonstrated by the results of immunophenotypic analysis of the TNFα gene expression, significant differences have been noted in the percentage of the yield of peripheral blood mononuclear cells (PBMC) from peripheral blood expressing the mTNFα membrane protein.

It is known that the degree of pathogenic action of microflora on the endometrium of the cows' uterus depends on the level of gene expression: TNFα, TLR, AMP and APP. The experiments on genotyping 152 Holstein cows of the Bayserke-Agro LLP were conducted by the authors during the period from 2015 to 2017. Within this period 56 cows were dropped from the experimental group for various reasons (mortality and slaughter of animals, culling, and lack of information on the reproductive function). The analysis of the reproductive function of cows with different genotypes by the locus of the promoter part of the TNFα gene was carried out in terms of the following parameters: the interval between calving and fertilizing insemination, the insemination index, and the number of cows that were successfully inseminated after more than 91 days after calving.

As can be seen from Table 2, there is the association of the GG homozygous genotype GG with the reproductive function indicators in the group of animals under study (minimum interval between calving and fertilizing insemination, low insemination index, minimum number of cows (47.36%), the number of cows successfully inseminated after more than 91 days after calving). Thus, according to the results of Japanese scientists, the proportion of cows that showed ovulation within 3 weeks after calving in individuals with homozygous GG and heterozygous AG genotypes by the SNP locus of the polymorphism of the promoter part of the TNFα gene was the same, 59.5% and 57.1%, respectively, and the alleles of this gene did not influence the number of inseminations [3].

CONCLUSION

Thus, the studied locus of the promoter part of the TNFα gene in Holstein cows was polymorphic; the prevalence of genetic variants was as follows: AA - 22.4%, AG - 63.8% and GG - 13.8%. The positive effect of the GG genotype of Holstein cows on reproductive function had been established, and the reproductive abilities in the heterozygous animals were low.

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