THE UTERINE MILK PROTEIN (UTMP) GENE IS ASSOCIATED WITH MILK PRODUCTION TRAITS IN IRANIAN HOLSTEIN BULLS

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Uterine Milk Protein (UTMP) the member of Serine Protease Inhibitor superfamily secreted from uterine endometrium during pregnancy under influence of progesterone. Specific functions of UTMP include protease inhibition, growth control, and direct nutrition of the conceptus. This investigation was performed in order to study polymorphism of UTMP gene and its association with milk production traits in Iranian Holstein bulls. Genomic DNA was extracted from 100 semen samples of proven bulls. In order to amplify 568bp-fragment including same part of intron 3, whole exon 4 and downstream of UTMP gene, a pair of primer was designed. PCR products were digested with BsrI enzyme. The allele frequencies of a, b and c were 0.135, 0.69 and 0.175, respectively in studied population. Four genotypes AB, BB, BC and CC were observed with frequencies of 0.27, 0.49, 0.13 and 0.11, respectively. Genotypes AA and AC were not observed in this study. The chi-square (X^2) test revealed deviation from Hardy-Weinberg equilibrium. Data were obtained from animal breeding centre of Iran for the first lactation during 1993-2008 to estimate some genetic parameters. Heritability of milk yield, fat yield, protein yield, fat percent and protein percent were 0.28, 0.21, 0.22, 0.32 and 0.34 respectively. Results indicated significant association between UTMP Genotypes and milk, fat and protein yield traits. Meanwhile, Bulls with CC Genotype had higher milk, fat and protein yield compared to other genotypes (p<0.05). These results suggested UTMP as a candidate gene influencing milk

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production traits might be implemented in breeding programs to improve the production performance of Iranian Holstein cattle.

Key words: UTMP gene, Heritability, Polymorphism, Milk production, Holstein cattle

INTRODUCTION

The Uterine Milk Proteins (recently known as SERPINA14) are a member of large Serpin superfamily of serine protease inhibitors (IRVING *et al*., 2000; MAHMOUD and NAWITO, 2012) which are secreted from the uterine endometrium mostly under the influence of progesterone(SPENCER and BAZER, 2004),in many ungulate animal species such ascattle (ULBRICH *et al*., 2009; MATHIALAGAN and HANSEN, 1996),Sheep (ING and ROBERTS, 1989), Goat (TEKIN *et al*., 2005), Buffalo (KANDASAMY *et al*., 2010) and Pig (MALATHY *et al*., 1990). The UTMP plays different biological roles including direct nutrition to the conceptus, growth control, protease inhibition, suppression of the local maternal immune system for sustaining pregnancy, and has a weak anti proteinase activity against pepsin (HANSEN, 1998). One of the most important regulator of uterine immune function is Uterine milk protein due to inhibiting of lymphocyte proliferation (SKOPETS *et al*., 1992), and reduce cytotoxic activity of NK cells (LIU and HANSEN, 1993; TEKIN and HANSEN, 2002). In sheep, from Day 30 of pregnancy till term, UTMP becomes a main protein in the Uterine fluid (MOFFATT *et al.*, 1987). Synthesis of UTMP in the endometrium is limited to glandular and luminal epithelial cells (HANSEN, 1998). ThemRNA level of ovine UTMP is quiet high in the intercaruncular endometrium between Day 20 and Day 60 of gestation, increases three times between Day 60 and Day 80, and then declines slightly after Day 120 (STEWART *et al.*, 2000). It has been reported that UTMP in bovine can be expressed predominantly in the uterine endometrium during pregnancy period (KHATIB *et al*., 2007). UTMP along with OPN which is an extracellular matrix protein, serve as excellent markers for endometrial gland differentiation and overall uterine secretory capacity during pregnancy in ewes (SPENCER *et al*., 1999; STEWART *et al*., 2000).UTMP gene consists of 8639 base pair with 4 exon which locating on chromosome 21(BTA21)within 321.6 kb of PI.UTMP and OPN genes have vital effects on milk production and health traits in cattle (KHATIB *et al*., 2007). KHATIB *et al*. (2007) investigated the expression pattern of UTMP in fetal and adult cattle tissues and studied its association with production traits in 2 independent Holstein populations: University of Wisconsin (UW) and Cooperative Dairy DNA repository (CDDR) identified an A/G SNP at position 1179 and an A/G SNP at position 1296 in bovine mRNA UTMP and SNP at position 1296 (in exon 4) was associated with a significant increase in productive life in 2 independent Holstein cattle populations, where the G allele expression at that position was associated with the longer productive life, while GG genotype of UTMP had a significant association with lower embryo survival rate (KHATIB *et al*., 2009), but in other study there was no significant association between observed SNPs and traits under study in buffalo (JEROME *et al*., 2015). JAKARIA *et al*. (2016) observed haplotype and nucleotide diversity of UTMP gene in Bali cattle. Researchers showed that OPN, FGF2, ABCG2, STAT5A, UTMP and ABCG2 have considerable association with milk production and health traits in dairy cattle (MOUSAVIZADEH *et al*., 2013; SALEHI *et al*., 2015; SADEGHI *et al*., 2009).

MATERIALS AND METHODS

DNA samples

100 Semen samples of Iranian Holstein proven bulls were obtained from Animal Breeding Center in Karaj, Alborz province of Iran. Genomic DNA was extracted using High Pure PCR Template Preparation Kit (Roche Company kit, CAD No=11796828001) along 7μl Di Thiothreitol (DTT) and also modified Salting-Out method per sample. The quality and quantity of the extracted DNA were assessed by spectrophotometer (PicoDrop, England) andalso Agarose gel electrophoreses.

DNA amplification with PCR-RFLP

The primers were designed with Oligo software (version 7) based on available bovine genomic sequences (GeneBank accession numbers: ID NC_007319.4). A 568 bp-fragment including same part of intron 3, whole exon 4 and downstream of UTMP gene was amplified using a pair of primer with the following nucleotide sequences: forward 5' TTGGTCTGGGGCTAACTC3' and reverse: 5' TTGCTTCTCTGCCTATGTCA 3'. The amplification reactions were performed in a total volume of 20 μl using Hot Start Taq plus PCR Master Mix kit (Qiagen Company kit, Tehran, Iran, CAD No= 28104) which included 50 ng of genomic DNA, 1μl of each primer (10 pM), 10 μl of PCR kit (10X), 2 μl CoraLoad (10X) in 35 Cycles (initial denaturation: 95℃, for 5 min; denaturation: 95°C, for 1 min; annealing: 51℃, for 1 min; extension: 72℃, for 1 min;) followed by a final extention: 72℃ for 5 min. PCR products were assessed by electrophoresis on 2% agarose gel.

A single digestion reaction consisted of 8 μl of PCR product, 0.8 μl of BsrI (BseNI) enzyme (Fermentase), 2 μl buffer 10XB and 19.2 μl nuclease free water. The final reaction volume of 30 μl was incubated at 65℃ for 16h. The digestion products were separated on 3% agarose gel by electrophoresis. The 50 bp DNA ladder (Fermentase) was used in each gel as molecular size standard.

Statistical analysis

The genotype and allele frequencies of UTMP variants were calculated using PopGene 32 software version 1.31 (YEH, 1999), the Hardy-Weinberg equilibrium was analyzed with Chi square test in this population.

The genetic parameters for milk production traits (milk yield, fat yield, fat percent, protein yield and protein percent traits) in Iranian Holstein cattle were estimated during years 1993 to 2008 with Restricted Maximum Likelihood algorithm (REML) under univariate Animal Model for the first lactation using ASREML software (GILMOUR *et al.*, 2009). Records of first lactation were obtained from Animal Breeding Centre of Iran. The data were edited by FoxPro and Excel software. In this study, the recodes of 305 days and twice milking per day were applied. Only records of cows with calving age between 18 to 38 months with more than 90 days of lactation were included and animals without records were removed. Statistical description of data for milk production traits is shown in Table1.Pedigree information is presented in Table 2.

In this study, we used GLM procedure of SAS software version 9.2 in order to identify factors affecting on trait changes and drive an appropriate model.

$$Y_{ij} = \mu + HYS_J + Age_i + A_i + e_{ij} \qquad (1)$$

Where Yij represent milk related traits, μ is population mean, A_i is animal effect as random effect, Age_i age at first calving as covariate factor, HYS_j fix effect of herd -year- season at calving and e_{ij} is random residual effect.

Table 1. Statistical description of data for milk production traits.

Traits	N	means	Minimum	Maximum	Coefficient of variation	Standard Deviation
Milk yield	239019	6786.30	2074.68	11312.18	22.08	1498.5
Fat yield	216415	214.780	47.9	545.3	25.25	54.25
Fat percent (%)	216208	3.2	1.66	6.06	15.8	0.5
Protein yield	116989	222.933	50.83	435.82	18.71	41.71
Protein percent (%)	116968	3.1	1.52	5.3	8.68	0.26

Table 2. Summary of pedigree file for milk production traits.

No. animals in pedigree file	No. founders	No. Individuals with Both known parent	No. Non founder	No. Sire	No. Dam	No. Of inbreed	Average inbreeding Coefficients
362044	37963	301015	324081	7157	208972	204513	0.009

Variance and covariance components were estimated and breeding value was predicted using Restricted Maximum Likelihood method based on average information algorithm ASRMEL software (version 3.1). In the next step of our study, Predicted breeding values of 100 proven bulls, that their genomic DNA was previously extracted, were applied.

The least square method of GLM procedure of SAS was used to test the association of UTMP genotypes with milk production traits. The linear model was as follow in equation (2):

$$Y_{ij} = \mu + S_i + G_j + e_{ij} \quad (2)$$

Where Y_{ij} is the breeding value for milk related traits; μ is the least square means of the traits, S_i is the fixed effect of sire i, G_j is the effect of the jth genotype (j=1, 2, 3, 4) in ith animal and e is the random residual effect.

Regression analyses were performed in which (BV) for milk yield, fat, protein yield and fat, protein percent were considered as dependent variables, and the genotype was the independent variable. Genotypes were coded as AA (0), BB (0), BC (1) and CC (2) to identify allele substitution effects. The average effect of allele substitution effects is known as regression coefficient (α) estimated as follows in equation (3):

$$Y_i = \mu + \beta_1 x_{1i} + \beta_{2r} x_{2ir} + e_i \quad (3)$$

Y_i is the breeding value of $_i$th bull for related traits, μ is population mean, β_1 is regression coefficient of the breeding values on the corresponding value of their sire, x_{1i} is sire breeding value for corresponding trait of the $_i$th bull, β_{2r} is regression coefficient of the bull breeding values for trait on the number of copies of the rth UTMP alleles, X_{2ir} is the number of C alleles (0, 1, 2) for the rth allele for it h bull and e_i is the residual random effect.

RESULTS AND DISCUSSION

The PCR amplified a 568-bp fragment including same part of intron 3, whole exon 4 and downstream of UTMP gene (figure1). Three alleles A, B and C were detected using *BsrI* restriction enzyme. Allelic frequencies for A, B and C were 0.135, 0.69 and 0.175, respectively. Four genotypes namely AB, BB, BC and CC were observed. The genotype frequencies of AB, BB, BC and CC were 0.27, 0.49, 0.13 and 0.11, accordingly (figure2). The x^2 test showed deviation from Hardy-Weinberg equilibrium in studied population (P<0.05). The UTMP allelic and genotypic frequencies and chi-square (x^2) value are summarized in Table 3. Estimation of Variance components and genetic parameters of production traits for Iranian Holstein dairy cattle are given in Table 4.
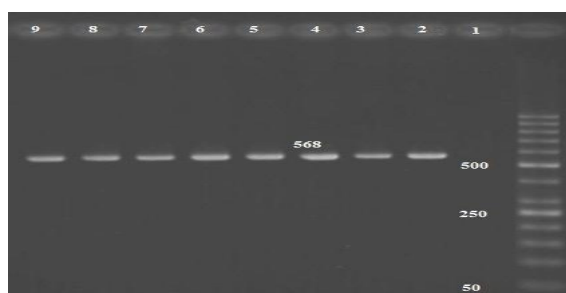

Fig1. PCR products of UTMP gene in2% agarose gel electrophoresis. Molecular marker is 50bp (Gene Fan Avaran co., Tehran, Iran).
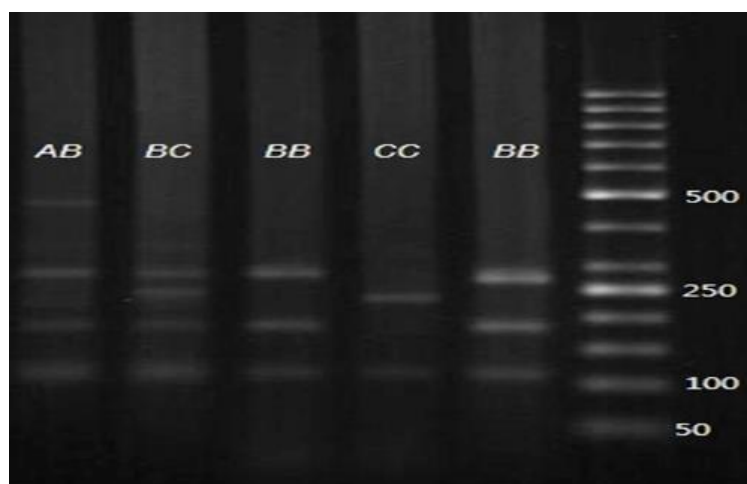

Fig 2. Restriction fragment length polymorphisms within the UTMP gene PCR product separated in a 3% agarose gel (PAGE). Molecular marker is M100 (Gen Fan Avaran co., Tehran, Iran).

Table 3. Genotypic and allelic frequencies with standard errors of single nucleotide polymorphism of bovine UTMP genes in Iranian Holstein bulls.

Genotypic frequency			Allele frequency				SE	x^2
CC	BC	BB	AB	A	B	C		p<0.001
0.11	0.13	0.49	0.27	0.135	0.69	0.175	0.034	**36.91**

Table 4. Heritability, genetic variance, residual variance and phenotypic variance and their standard errors in Iranian Holstein bulls.

Traits	Heritability± SE	Genetic Variance	Residual Variance	Phenotypic Variance
Milk yield (kg)	0.28± 0.0061	349724	905130	1254854
Fat yield(kg)	0.21± 0.0064	268.752	964.329	1233.081
Fat percent (%)	0.22± 0.0086	232.237	818.165	1050.402
Protein yield (kg)	0.32± 0.0065	0.0494093	0.103380	0.152789
Protein percent (%)	0.34± 0.0096	0.0133010	0.0253976	0.038699

The endometrium under the control of the secretion of progesterone in the uterine milk protein (UTMP) might be responsible for protease inhibition, nutrition of the conceptus, growth control, and suppression of the maternal immune system, as on the main protein. UTMP gene contains 8639 base pair with 4 exons which locating on chromosome 21 (BTA21) within 321.6 kb of PI. The uterine milk protein gene (UTMP) was selected for this study because of its possible roles in health traits. Results of the other researchers showed the association of the UTMP region with milk production in dairy cattle as well (KHATIB *et al.*, 2007; JAKARIA *et al.*, 2016).

Tables 5 shows the comparison of the least square means and their standard errors of breeding values for milk production traits between the genotypes of the UTMP polymorphisms. The results indicate that the least squares means of CC genotype for milk and fat yield were higher than the other genotypes and were significant. Significant differences between genotypes for fat and protein percent were not detected. Significant difference were observed between genotypes for protein yield and Bulls which CC genotype had a higher protein yield compared to the others and AB genotype had a lower protein yield (P<0.05). In addition, the allele substitution effects on milk production traits were estimated (Table 6). The average allele substitution effects of the B and C allele were significant for milk yield (-289.14±70.55; 404.85±74.46 kg), fat yield (-6.60±2.07; 10.05±2.17kg) and protein yield (-5.31±2.12; 9.93± 2.27kg), respectively. No significant association between alleles for protein percent were observed and for fat percent only allele C had a significant association (-0.045±0.018). Moreover, KHATIB *et al.* (2007) reported that UTMP mRNA SNP 1296 shows significant association with productive life in two independent Holstein cattle populations.

Result obtained in our study showed significant association between UTMP genotypes and milk, fat and protein yield traits. Meanwhile, Bulls with CC Genotype had a higher milk, fat and protein yield traits in comparison with other genotypes. These results suggested that UTMP

as a major gene which has a substantial impact on milk production traits might be implemented in breeding programs to improve the production performance of Iranian Holstein cattle.

Table 5. Least Square means (LSM) and standard errors of breeding values for milk, fat and protein yield, and fat and protein percent in Iranian Holstein bulls with four UTMP genotypes.

	AB	BB	BC	CC
Milk yield (kg)	-117.05±108.63[a]	-33.86±66.39[a]	310.198±136.19[a]	**786.1±143.71[b]**
Fat yield (kg)	3.01±2.8[a]	-0.053 ±1.9[a]	7.34±3.8[a]	**20.3±4.1[b]**
Protein yield (kg)	20.36±3.2[a]	29.54±1.9[a]	33.79±4.1[a]	**50.52±4.1 [b]**
Fat percent (%)	-0.015±0.02 [a]	0.029±0.01 [a]	-0.085±0.03[a]	**-0.011±0.03[a]**
Protein percent (%)	-0.029±0.01 [a]	-0.034±0.009[a]	-0.053±0.01 [a]	**-0.044±0.02 [a]**

[ab] Within rows, means marked by the same superscripts do not differ each other significantly at (P≤0.05).

Table 6. Average allele substitution effects and standard errors of UTMP gene on milk production traits in Iranian Holstein bulls.

	A	B	C
Milk yield (kg)	-135±34.35	-289.14±70.55*	404.85±74.46*
Fat yield (kg)	-5.11 ±3.72	-6.60±2.07*	10.05±2.17*
Protein yield(kg)	-9.71 ±4.29*	-5.31±2.12*	9.93 ±2.27*
Fat percent (%)	0.029 ±0.03	0.031±0.01	-0.045±0.018*
Protein percent (%)	0.012 ±0.016	0.01±0.009	-0.017±0.01

* Statistically significant at the probability level at (P≤ 0.05)

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POVEZANOST UTMP GENA SA OSOBINAMA PROIZVODNJE MLEKA IRANSKIH HOLŠTAJN BIKOVA

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Izvod

Protein izlučevina materice (the uterine milk protein - UTMP) je član superfamilije serinskog inhibitora proteaze, koji se pod uticajem progesterona izlučuje iz endometrijuma materice tokom trudnoće. Specifične funkcije UTMP-a uključuju inhibiciju proteaze, kontrolu rasta i direktnu ishranu zametka. Ova istraživanja su sprovedena kako bi se proučio polimorfizam UTMP gena i njegova povezanost sa osobinama proizvodnje mleka iranskih bikova holštajn rase. Genomska DNK je ekstrahovana iz 100 uzoraka semena testiranih bikova. Da bi se pojačao 568bp-fragment koji uključuje isti deo introna 3, celi ekson 4 i donji deo UTMP gena, napravljen je par prajmera. PCR proizvodi su digestovani sa BsrI enzimom. Frekvencije alela a, b i c bile su 0,135; 0,69 i 0,175, respektivno u ispitanoj populaciji. U slučaju četiri genotipa AB, BB, BC i CC zabeležene su frekvencije od 0,27; 0,49; 0,13 i 0,11, respektivno. U ovom istraživanju nisu primećeni genotipovi AA i AC. Test Či-kvadrata (X^2) otkrio je odstupanje od Hardy-Weinbergovog ekvilibrijuma. Podaci korišćeni u ovom istraživanju su dobijeni od Centra za stočarstvo u Iranu, za prvu laktaciju tokom 1993-2008. godine, i korišćeni su u svrhu procene određenih genetičkih parametara. Vrednosti heritabiliteta za prinos mleka, prinos masti, prinos proteina, procenat mlečne masti i proteina su bile 0,28; 0,21; 0,22; 0,32 i 0,34, respektivno. Rezultati pokazuju značajnu povezanost između UTMP genotipova i osobina prinosa mleka, masti i proteina. Istovremeno, bikovi sa CC genotipom su imali veće vrednosti za prinos mleka, masti i proteina u poređenju sa drugim genotipovima (p <0,05). Ovi rezultati ukazuju na to da UTMP, kao kandidat gen koji utiče na osobine proizvodnje mleka, može biti primenjen u programima oplemenjivanja kako bi se poboljšale performanse u proizvodnji mleka iranske populacije holštajn goveda.