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Novel single nucleotide polymorphism in the prolactin gene of Awassi ewes and its role in the reproductive traits

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Abstract

This study was conducted to determine the genetic variation of the prolactin (*PRL*) gene in 5' flanking region and its role with several reproductive traits in Awassi sheep. One-hundred six Iraqi sexually mature and healthy Awassi ewes that aged between 2 and 2.5 years were included in this study. Ewes were classified into two the main divisions: ewes producing twins and ewes producing single offspring. Two genotypes (AA and AT) were observed in Awassi sheep. While genotype AA was detected in Awassi ewes that produced twins, genotype AT was detected in ewes that produced a single offspring. The sequencing reactions identified nine SNPs in the prolactin gene at the 5′ flanking region in Awassi sheep, differing from the prolactin reference sequence (GenBank accession number X16641.1). Genotype AT possessed one single nucleotide polymorphism SNP substitute comparison with the AA genotype in Awassi ewes. The association analysis revealed that the AA genotype is characterized by significantly higher levels of the progesterone concentration, twinning ratio, fecundity, and prolificacy than the AT genotype. In conclusion, a new SNP (g.1209 A>T) was discovered within the ovine flanking region which potentially influences prolactin gene expression. These results showed that the genotype AA associated with high prolificacy of Awassi sheep may be used as a selection criterion for improving the reproductive performance of Iraqi Awassi sheep.

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Introduction

Sheep (*Ovis aries*) are a very diverse type of farm animals in their physiological traits involving litter size and prolificacy. These characteristics were found to be attributed to the effect of a single or a closely related group of the genes (1), which stimulated research interest previously (2) and also going after this study (3,4). Prolactin gene situated on chromosome 20 in sheep that included five exons separated by four introns (5) which are positioned on chromosome 23 in cattle (6), and situated in birds on chromosome 2 (7). The prolactin gene (*PRL*) encodes an essential hormone involved in many activities for lactation, osmoregulation (8), and the regulation of reproductive functions (9). The genetic variation of the *PRL* gene and its relationship with phenotypic traits in the living organism have been the focused the most of the research. In avian species, the C-5961T polymorphism of the prolactin gene was statistically associated with the egg production traits, where the CC genotype was associated with greater egg production traits and larger egg weight compared with the CT genotype in six breeds of Chinese native ducks (10). In farm animals, Ozmen and Kul (11) reported for the first time that 48 SNPs of the *PRL* gene were investigated by PCR- Restriction Fragment Length Polymorphism (RFLP) and sequencing methods for three breeds of sheep (Sakiz, Akkaraman, and Awassi). In all populations examined, AA genotype was significantly associated with an increase in milk production, whereas the animals carrying the genetic pattern BB had a high percentage of fat in milk. The polymorphisms of the *PRL*

gene was also studied in Barki, Damascus, and Zaraibi goat breeds using RFLP and DNA sequencing methods (4).

The *PRL* gene polymorphism of PRL/*Rsa*I marker and its relationship with higher milk traits was also reported in Gir and Kankrej cattle breeds (12). Cattle with the AA genotype were associated with the higher milk yield and less fat percentages as compared to the cattle of the other genotypes (12). The previous study in Montebeliard cows showed that the AA genotype yielded the most milk comparison to other genotypes (13). Most SNPs of the *PRL* gene were found and investigated in 5'-flanking regions (14). The SNP 24-bp of the prolactin promoter region at the position -358 in Fars native chickens showed a significant relationship with higher egg production traits (15). In cattle, a novel SNP A/G change at locus -1043 of the bovine prolactin gene at the 5' flanking region was revealed by using PCR- Single Strand Conformation Polymorphism (SSCP) and the Sanger nucleotide sequence methods (16). In addition, two SNPs were identified in the bovine *PRL* gene of the enhancer region at positions a1167g and c1286t were evaluated as potential markers of profitability traits in beef cows and calves (17). Genotype analysis in Chinese Holsteins of two SNPs of the *PRL* genes at the 5′-regulatory region demonstrated that the AG genotype was statistically related with higher milk yields, whereas the AA genotype was related with higher fat contents (18). The polymorphism of the ovine *PRL* gene at the 5′ flanking region has only received limited attention. Keeping this in view, this study was undertaken to determine SNPs of the *PRL* gene at the 5' flanking region and its association with reproductive performance in Iraqi Awassi sheep.

Materials and methods

Animals and samples collection

The study was conducted according to the regulations of the international recommendations for the care and use of livestock animals (19). The animal experimentation was approved by Al-Qasim Green University (Approval No. 12.10.15). One-hundred six Iraqi sexually mature healthy Awassi sheep were included in this study. Sheep were classified into the two main divisions according to litter size after parturition (71 ewes that produced twins and 35 ewes that produced a single offspring) with weight ranging from 40-60 kg and age between 2 to 2.5 years. Animals were raised in the Barakat Abu al Fadhl Al-Abbas (AS) The station for raising sheep (Al-Khafeel Co., Karbala, Iraq) from October 2014 to May 2015. For all animals, the maintenance and the nutrition were similar and remained consistent with proper animal welfare recommendations for the care and use of livestock animals (19). Pedigree information for the subject ewes was updated annually with regard to the identification number of each lamb, its sire and dam, the breed code, and the date of birth. The number of ewes joined per ram varied between 20-25.

Hormonal assays

The blood samples 5 ml were collected to determine the progesterone and the oestradiol levels in the plasma of pregnant ewes were measured in 90, 120, and 150-day intervals. The collected samples were centrifuged and the serum kept at -20°C until the performing of enzyme-linked immunosorbent assay (ELISA) test for hormonal assays (20). The sexual hormone concentrations were determined in the third month, and the fourth month of pregnancy, as well as in the post-parturition period according to manufacturer's instructions mentioned in Calbiotech (Cat No. ES180S, Spring Valley, USA) and Monobind incorporations (Cat. No. 4825-300A, Lake Forest, USA) respectively.

DNA isolation and PCR amplification

The genomics DNA from blood was isolated by the high salt method with some modifications (21). Briefly, RBC lysis carried out by using 5 mL of distal water and the pellet was resuspended in 0.9 mL of TNES lysis buffer (10 mM Tris-ClpH 7.7, 0.4 M NaCl, 2 mM EDTA, 0.5% SDS). The exact genomic position of the ovine *PRL* gene at the 5' flanking region was described according to GenBank acc. no. X16641.1 (Figure 1, A). One pair of the primers was used to amplify the ovine prolactin gene at the 5' flanking region dependent on the reference gene by (22). The sequences of primer used in this study were as follows: F:5'-AGGTCAGAGAATTAAAGCT-3', R:5' GGAAGTGACAGTGGTTTT-3'. The PCR reaction was performed using the *Accu*Power PCR PreMix (Bioneer, South Korea). Each 20 µl of PCR premix contained 250 µM of dNTPs, 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, 1 U of Taq DNA polymerase, and 1.5 mM of $MgCl_2$. The PCR reaction mixture was completed with 10 pmol of each primer and 10 - 30 ng of genomic DNA (23). The program of PCR amplification were: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation (94°C for 30 seconds), annealing (51.0°C for 30 seconds), extension (72°C for 30 seconds), and a final extension step (72°C for 5 minutes) (24). The amplicons were visualized electrophoretically in 1.5% agarose gel (25). Photos were taken by using the gel document unit (Chemidoc, Biorad, USA). After it was confirmed that all electrophoretic PCR bands were specific with length (161 bp) then be submitted to the next genotyping step.

SSCP analyses

The initial denaturation of the PCR amplicons, as well as SSCP protocol, were performed according to Al-Shuhaib *et al.* protocol (26). The SSCP analysis was carried out in 10% polyacrylamide gels (37.5:1) at 200 V for 4 h in Tris Borate EDTA (TBE) (0.5X) buffer at a constant temperature of 20°C using (216 × 110) mm mini-wide gels with (1.0 mm) gel thickness (JY-CZ-B, Junyi-Dongfang Electrophoresis Equipment). Then, the amplified fragments were visualized by using the PAGE gel-red staining method.

DNA sequence analysis

The sequences of prolactin gene fragments purified and sequenced by using the Macrogen Incorporation (Macrogen - Korea) then analyzed by the multiple sequence alignment program according to DNA Star, EditSeq/ Clustal W, with the sequences published in the GenBank database taken as a reference to identify the polymorphisms. In order to identify the novel SNP of *PRL* gene, the ovine sequence obtained in the present study was compared with the bovine for prolactin 5' flanking regulatory region (GenBank acc. no. X16641.1) due to lack of the DNA sequence of the ovine *PRL* gene.

Association study and statistical analysis

The variance studies of *PRL* genotypes with the reproduction traits were performed by using (SPSS, v 23). The two main statistical analyses were performed in the current study, including the litter size and the hormonal concentration analysis. With regard to a litter size of Awassi ewes, the following linear mixed model was employed; $Y_{ijklmn} = \mu + G_i + P_j + LS_k + A_l + S_m + e_{ijklmn}$. where Y_{ijklmn} is the observed trait, μ is the overall mean effects of the traits value with the fixed effect (G_i , P_j , LS_k , A_l), G_i effect of the i^{th} genotype (i=AA, AT), P_j effect of the j^{th} parity (j=Primiparous, 2nd parity, 3rd parity), LS_k effect of the k^{th} lambing season (k=autumn, winter, spring, summer), A_l effect of the l^{th} *age of ewes* (l=2 year, 2.5 years), while S_m is the random effect of the m^{th} sire (m=1, 2, 3, 4, 5), and e_{ijklmn} is the random error. Whereas the mean hormone concentrations between genotypes were analyzed by using the ANOVA-repeated measures, with the following model; $Y_{ijk} = \mu + G_i + P_j + (GP)_{ij} + p_{i(j)} + e_{ijk}$. where μ is the overall mean traits, G_i is the main effect of genotype (fixed w/$\sum$i G_i=0), P_j is the main effect of physiological stage (gestation and post-parturition) (fixed w/$\sum$j P_j =0), $(GP)_{ij}$ is the effect of interaction, $P_{i(j)}$ is the main effect of subjects N (0, σ^2_p), and e_{ijk} is random error assumed $e_{ijk} \sim N(0; \sigma^2)$. The multiple pairwise comparisons between the main factors were performed by using the Tukey-Kramer test, which is statistically significant at $P < 0.01$. Reproductive traits (fecundity and prolificacy) and twinning ratio were analyzed by using Chi-square test, while the genotypes, alleles frequencies, and deviation from Hardy-Weinberg test were estimated by Popgen32 (27). The prolificacy percentage was calculated as the following equation. Prolificacy (%) = (number of lambs born/total number of ewe delivered) ×100. While the fecundity was calculated as the following equation. Fecundity= (number of lambs born/ number of ewe lambed).

SNP genotype effects estimation

The SNP showed the significant association with the phenotypic traits, differences between the means of each the genotype and the allelic frequencies were used to estimate additive effects (28). The following formula was utilized to find the additive genetic variance (Var_A) imputed to a SNP: $Var_A = 2p_iq_i\alpha^2_i$. Where q and p were the allelic frequencies for the jth SNP predicted across the entire population, αi - SNP allele substitution effect were obtained from a linear regression model in a statistical program, in which the genotypes recorded as a variate of 0, 1 and 2 copies of a particular allele.

Results

The polymorphism investigations revealed the two types of banding pattern (genotypes); the genotype (AA) was only detected in Awassi ewes producing twins lambs, while the genotype (AT) was only detected in ewes producing single lamb (Figure 1B). The sequencing results confirmed the genotypes observed in this study. The several single nucleotide polymorphisms (SNPs) were obtained between the two resolved genotypes, as well as between the genotypes and the prolactin (*PRL*) gene reference sequences are shown in Figure 1C. In this study, we are the first to identify nine SNPs of the *PRL* gene in the 5′ flanking region in Awassi sheep that are different from the prolactin reference sequence (g.1209-1369). DNA sequencing analysis revealed that the AT genotype had one SNP (g.1209 A > T) substitute than the AA genotype in Awassi sheep.

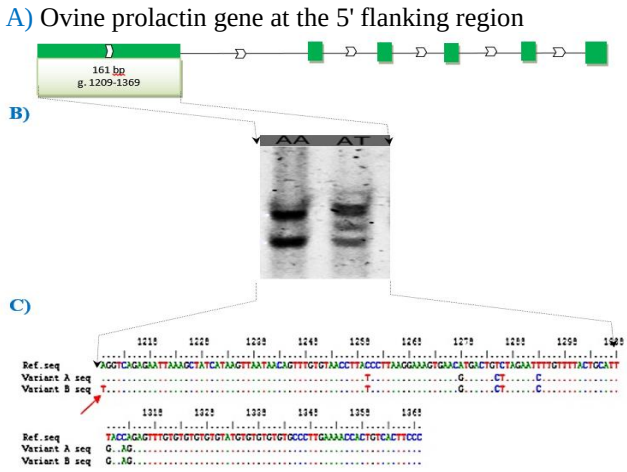

Figure 1: A schematic diagram of the current study to assess the *PRL* gene polymorphism in the Awassi sheep. A) The exact genomic position of the ovine *PRL* gene at the 5' flanking region was described according to GenBank acc. no. X16641.1. B) Two SSCP patterns are visible, corresponding to AA and AT genotypes. C) DNA sequencing alignment results for prolactin gene two genotypes with their reference sequence (GenBank acc. no. X16641.1) using DNA STAR, the Editseq software. Using ClustaW alignment, several point mutations (SNPs) are observed between the prolactin reference sequence and the two genotypes AA (Variant A) and AT (Variant B) themselves, with one mutation (A/T) was observed in single Awassi ewes at locus 1209 as shown by the arrow.

The genotype and allele frequencies and the hardy-weinberg equilibrium (HWE) results of the ovine *PRL* gene at the 5' flanking region are presented in Table 1. The value of chi-square was 6.768, meaning that the population under study is not in HWE, which was statistically significant at $P < 0.05$. It is unlikely that the deviation observed was the result of genetic drift or migration. The directional selection may be one possible reason for the deviation because the litter size at birth is a trait easily monitored by farmers.

Table 1: Genotype, allele frequencies and genetic diversity parameters for 5' flanking region of ovine *PRL* gene of Awassi breed

Genotype frequencies		Allele frequencies		*Ho*	*He*	*Ne*	*χ2*
AA	AT	A	T	0.3302	0.2770	1.3806	6.768
0.67	0.33	0.83	0.17				

Abbreviations: χ2 - chi-square at $P<0.05$, Ho - observed heterozygosity, He - Expected heterozygosity, *Ne*-effective allele number.

All identified SNPs in this study are not reported by the International Sheep Genomics Consortium in the public database European Variation Archive (https://www.ebi.ac.uk/eva/) and are not detected in the *PRL* gene at the 5′ flanking region of the Small-Tail Han sheep. The novelty of SNPs was confirmed by Ensembl genome browser 97 for ovine species (http://www.ensembl.org/index.html). The association analysis results of this study revealed that the significant difference ($P < 0.01$) in progesterone level between the observed genotypes during pregnancy and the post-parturition months (Table 2). The AA genotype was characterized by significantly higher levels ($P < 0.01$) of progesterone than the AT genotype and higher levels in the third month and fourth month of gestational periods than post-parturition. No significant difference ($P > 0.01$) in estradiol levels was observed for either genotype.

Furthermore, the genotype effect prediction confirmed that the AA genotype was associated with higher progesterone concentration ($P < 0.01$), litter size, fecundity, and prolificacy. The greater SNP additive genetic variance percentage with the reproductive traits (>1%) is represented in Table 3.

Table 2: Association of *PRL* genotypes at the 5′- flanking region with estradiol/ progesterone hormones concentrations in single and twin Awassi breeds

Genotype	LSM ± SE (pg/ml) of progesterone hormone			LSM ± SE (ng/ml) of estradiol hormone		
	3rd Month	4th Month	Post-parturition	3rd Month	4th Month	Post-parturition
AA (71)	14.144±0.542 aA	13.403±0.542 aA	8.353±0.542 aB	25.172±2.266	29.275±2.266	14.744±2.266
AT (35)	7.018±0.542 bA	8.605±0.542 bA	2.248±0.542 bB	27.831±2.266	30.788±2.266	13.228±2.266
Significant	**	**	**	NS	NS	NS

Least square Mean (LSM) and standard errors of those means (SE). ** ($P<0.01$), A, B different capital letters refer to statistically difference in the rows ($P <0.01$), a,b different lowercase letters refer to the statistical difference in columns ($P <0.01$).

Table 3: Percentages of g.1209A>T SNP additive genetic variance identified in the fragment of 5′- flanking region in *PRL* gene

Phenotypic traits	SNPs genetic variance (%)
Estradiol hormone at 3rd Month	-
Estradiol hormone at 4th Month	-
Estradiol hormone at Post-parturition	-
Progesterone hormone at 3rd Month	4.754
Progesterone hormone at 4th Month	6.986
Progesterone hormone at Post-parturition	11.521
Litter size	3.718
Fecundity	2.635
Prolificacy	1.211

The percentage of g.1209A>T SNP additive genetic variance that showed only a significant association with the phenotypic traits. With regard to twinning ratio, fecundity, and prolificacy, the results were 94.36%, 1.94, and 194.36%, respectively, for Awassi ewes producing twins, while the results were 5.71%, 1.05, and 105.71%, respectively, for ewes producing single lambs (Table 4).

The association analysis of the litter size in Awassi ewes is represented in Table 5. Litter size was statistically affected by *PRL* genotype ($p < 0.01$). The AA ewes had more lambs (1.95) than the AT ewes (1.20) (Table 5).

Table 4: The observed twinning ratio, fecundity, and prolificacy in both studied twin and single Awassi populations

Ewes	No.	Single No. (%)	Twin No. (%)	Fecundity	Prolificacy (%)	Total
Twin Awassi % of animals	71	4 (5.63^{b})	67 (94.36^{a})	194.36^{a}	1.94^{a}	138
Single Awassi % of animals	35	33 (94.28^{a})	2 (5.71^{b})	105.71^{b}	1.05^{b}	37
Level of sig		**	**	**	**	

** ($P<0.01$). Means with different superscripts in the same column differ significantly.

Table 5: The association of *PRL* genotypes with litter size in single (AA) and twin (AT) Awassi ewes

Genotypes	Litter size (LSM ± SE)
AA	1.95 ± 0.09^{a}
AT	1.20 ± 0.06^{b}
Level of sig.	**

Least square Mean (LSM) and standard errors of those means (SE) derived from General Linear Mixed-effects Models. ** $P<0.01$ means bearing different letters significantly different.

Discussion

In this study, the single-strand conformation polymorphism (SSCP) genotyping assay was used to assess the variance nature of the *PRL* gene at the 5′ flanking region and its relationship with phenotypic traits. The two genotypes, AA and AT, were detected in this study. The SSCP technique was used due to its ability to detect the potential presence of unknown variation (29). While other studies depending on the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay to assess polymorphism in the *PRL* gene. A study by Parihar and colleagues (30) used the PCR-RFLP assay in the prolactin receptor (*PRLR*) gene in Sahiwal and Hariana cattle and demonstrate that the GG genotype showed a higher milk yield value than TT and GT animals in the first lactation. Another association analysis study between PRL/*Rsa*I polymorphisms with the milk production and the meat traits were reported in water buffalo (9,31).

The sequencing reactions were indicated the presence of the one novel SNP (g.1209A>T) of the *PRL* gene at the 5′ flanking region in single Awassi ewes, which may be a causal factor because all ewes who carried this SNP produced single lambs. Our findings supported Chu and colleagues (22) study, which reported an SNP (G/T substitute at the locus 63 bp in the amplified fragment) of the ovine *PRL* gene at the 5′ flanking region, and this has been associated with the twinning rate in Small-Tail Han sheep. Association analysis study of the *PRL* gene was associated with the prolificacy of Awassi sheep (32). Identification of the polymorphism in the *PRL* gene of Chios sheep revealed five SNPs, with the association effect of the non-synonymous SNP (g.567G > A) with the litter size at lamb births (5).

The *PRL* gene expression depends on the 5′ flanking region sequence (33). The sequence polymorphism of the *PRL* gene at the 5′ flanking region may influence and regulate the activation of gene transcription (34) involved with development and reproduction (3,21,35). It is noteworthy to mention that causative mutation in the regulatory sequences of the *PRL* gene influences *PRL* gene expression (16). Variation in the flanking region, particularly those that result in changes of promoter binding sites, found to influence mRNA expression (34). In the 5' flanking region of the bovine prolactin gene, a distal regulatory element enhances the basal level of expression fivefold (16).

Association analysis revealed that the AA genotype had a higher progesterone hormone concentration than the AT genotype. These differences may be due to the effect of prolactin on progesterone hormone release. PRL stimulates both the corpus luteum formation and the production of progesterone (14), as well as the influences gonadotropin release in sheep (17). The existence of PRL receptors in the bovine corpus luteum and granulosa cells suggests at least a supportive role in cattle reproduction (17). Furthermore, it has an influence on progesterone and relaxin production in the presence of prolactin receptors in endometrial cells (36). This association was confirmed by the genotype effect prediction. A significant association was found between AA genotype and the progesterone concentration, litter size, fecundity, and prolificacy.

Several studies were reported the avian species on the variation of the *PRL* gene and its correlated with production traits. A study showed that the genetic polymorphism of the *PRLR* gene is statistically correlated with egg quality traits in the Erlang Mountainous chicken (37). Six SNPs in the prolactin gene at the promoter region were detected in 177 individuals from White Leghorn and Yangshan chicken (34). Four SNPs in the *PRL* gene at the 5′ flanking region were identified in populations of native Yuehuang, Taihe Silkies and imported White Leghorn layer Chinese chickens were significantly correlated with the egg production (38). Another study was conducted to study egg production traits and reported 24 bp indel locus at the promoter region (*PRL*24). The "D" allele is associated with a higher number of eggs and higher egg weights of Kadaknath (39). In addition, a 5′-flanking region of prolactin gene was polymorphic in naked neck chicken with having a statistically significant association with egg quality traits (40). Whereas, limited research was reported in farm animals. So, this study was the first report of an association

of the *PRL* gene at the 5′ flanking region with the reproductive performance of Iraqi Awassi sheep.

Conclusion

In this study, a novel SNP (g.1209A>T) was discovered within the ovine flanking region, which potentially influences prolactin gene expression. These results showed that the genotype AA associated with high prolificacy of Awassi sheep may be used as a selection criterion for improving the reproductive performance of Iraqi Awassi sheep.

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Conflict of interest

The author declare that they have no conflict of interest.

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التباين الوراثي للنيوكليوتيدة المفردة الجديدة في جين البرولاكتين للنعاج العواسي ودورها في الصفات التكاثرية

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الخلاصة

أجريت هذه الدراسة لتحديد الاختلاف الجيني لجين البرولاكتين *PRL* في منطقة المحيطة من طرف ٥ وعلاقتها مع العديد من الصفات التكاثرية في الأغنام العواسية. تضمنت هذه الدراسة ١٠٦ نعاج عواسية عراقية ناضجة جنسيا وصحية تراوحت أعمارهم بين ٢ إلى ٢,٥ سنة. تم تصنيف النعاج إلى قسمين رئيسيين؛ النعاج التي تنتج التوائم والنعاج التي تنتج ولادة مفردة. لوحظ طرازان وراثيان AA و AT في الأغنام العواسية. بينما تم الكشف عن الطراز الوراثي AA في النعاج العواسية التي أنتجت توائم، كشف عن الطراز الوراثي AT في النعاج التي أنتجت نسلاً مفردا. حددت تفاعلات التسلسل الجيني تسعة طفرات نوع التباين الوراثي للنيوكليوتيدات المفردة في جين البرولاكتين عند المنطقة المحيطة من طرف ٥ ′ في الأغنام العواسية، تختلف عن تسلسل البرولاكتين المرجعي GenBank X16641.1. يمتلك الطراز الوراثي AT طفرة واحدة بديلة التباين الوراثي للنيوكليوتيدة المفردة مقارنة مع الطراز الوراثي AA في النعاج العواسية. كشف تحليل الارتباط أن الطراز الوراثي AA يتميز بمستويات أعلى بكثير من تركيز البروجسترون، نسبة التوائم، الخصب، والخصوبة من الطراز الوراثي AT. في الاستنتاج، تم اكتشاف طفرة جديد (g.1209 A>T) داخل منطقة المحيطة بالجين للأغنام مما قد تؤثر على التعبير الجيني للبرولاكتين، كما أظهرت هذه النتائج أن الطراز الوراثي AA مرتبط بالخصوبة العالية للأغنام العواسية والذي يمكن استخدامه كمعيار اختيار لتحسين الأداء التناسلي للأغنام العواسية العراقية.

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