

ORIGINAL ARTICLE

Polymorphism of prolactin, growth differentiation factor 9, and calpastatin genes and their effects on weight traits in Awassi lambs

Khaleel Ibrahim Jawasreh¹, Zuhair Bani Ismail²

¹Department of Animal Production, Faculty of Agriculture, Jordan University of Science and Technology, Irbid, Jordan

²Department of veterinary Clinical Science, Faculty of Veterinary Medicine, Jordan University of Science and Technology, Irbid, Jordan

ABSTRACT

Objectives: This study was conducted to determine the correlation among prolactin gene (PRG), growth differentiation factor 9 (GDF-9), and calpastatin (CAG) genes polymorphism with growth traits in Awassi lambs.

Materials and Methods: Records of 779 Awassi lambs from 264 dams and 15 sires were used. The sex, type of birth (single *versus* twins), weight at birth, average daily gain (ADG), and the adjusted weight at 60 days of age were determined for each animal. Blood samples were collected from all lambs to determine PRG, GDF-9, and CAG polymorphism using polymerase chain reaction-Restriction fragment length polymorphism.

Results: Three PRG genotypes (AA, AB, and BB) were determined with a frequency of 0.88, 0.05, and 0.07, respectively. The frequency of each allele was 0.84 and 0.16 for A and B alleles, respectively. For GDF-9, there were only two genotypes detected (AB and BB) with a frequency of 0.96 and 0.04, respectively. The frequency of each allele was 0.92 and 0.08 for A and B alleles, respectively. For CAG, two genotypes were also detected (AB and BB) with a frequency of 0.92 and 0.08, respectively. The frequency of each allele was 0.96 and 0.04 for A and B alleles, respectively. A significant ($p \leq 0.04$) effect of PRG genotype on birth weight was detected but this effect was not significant on ADG and weight at weaning. There were no associations between any of the pre-weaning growth traits and GDF-9 and CAG variants.

Conclusion: The results of this study show that PRG could be used to select dams with a high frequency of dystocia to reduce birth weight of newborn lambs and therefore conserve the dam's reproductive functions and improve lamb survivability.

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Introduction

Sheep are considered an important source of livelihoods in many countries in the Middle East. Awassi sheep or fat-tailed sheep are hardy and multipurpose breed that is kept for meat, milk, and wool production [1,2]. Growth performance of lambs is one of the most detrimental factors of meat production. In fact, strong relationships have been determined between the marketing weights and different growth traits in lambs, including birth and weaning weights and average daily gain (ADG) [2,3]. Therefore, selection for these traits in the early stages of the animal's life may result in the overall improvement of the animal's productivity [1].

Growth traits are substantially affected by various environmental factors such as sex, type of birth (singles *versus* twins), dam weight, lambing year, and season [2,4]. Growth performance is also under the control of many genes that can be used for selection of individual animals based on their phenotypic expression.

Awassi sheep selection programs have long been concentrated on milk production traits [7]. In recent years, there has been a great increase in the demand for red meat in the Middle East. Therefore, improving meat production of Awassi lambs by genetic manipulation and selection based on phenotypic performance could significantly improve their profitability. It has been determined that the

Correspondence Khaleel Ibrahim Jawasreh ✉ kijawasreh@just.edu.jo 📧 Department of Animal Production, Faculty of Agriculture, Jordan University of Science and Technology, Irbid, Jordan.

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Table 1. Primers used to study the polymorphism of PRG, GDF-9, and CAG genes in Awassi sheep.

Primers	Sequence	Annealing temperature (°C)	Digestion enzyme	Product size
PRG	F: ACCTCTCTCGGAAATGTTC A	56	<i>HaeIII</i>	1,200-bp
	R: GGGACACTGAAGGACCAGAA			
GDF-9	F: GAAGACTGGTATGGGGAAATG	58	<i>HhaI</i>	462-bp
	R: CCAATCTGCTCCTACACACCT			
CAG	F: TGGGGCCCAATGACGCCATCGATG	63	<i>MspI</i>	622-bp
	R: GGTGGAGCAGCACTTCTGATCAC			

heritability of growth performance is much better than that of milk production in sheep [2,6]. Previous work in lambs has shown that selection for growth performance led to superior performance when compared to results of selection for milk production [5,6]. In this study, the effects of the dam, sex of birth, year of birth, and type of birth (single or twins) on pre-weaning growth performance parameters, including birth and weaning weights, ADG, and marketing weight of Awassi lambs were determined. In addition, the effect of prolactin, growth differentiation factor 9, and calpastatin (CAG) genes polymorphism on these growth traits were also determined.

Materials and Methods

Ethical approval

The project was approved by the Jordan University of Science and Technology animal care and use committee (JUST-ACUC) (Approval No. 16/4/21/70).

Animals

This study was performed at Al-Khanasry Research Station located at the Eastern North of Jordan, located at 32°30'N and altitude of 860 m above sea level, with an annual rainfall of less than 150 mm [7]. The flock is allowed to graze freely for 2 months of the year and fed forages and concentrates the rest of the year.

Following birth, the lambs are kept continuously with their dams until 21 days. After that, the lambs are only allowed to suckle freely for 12 h of the day for 40 days while they were separated from their dams for 12 h. Creep feeding is provided to lambs until weaning at 60 days of age. Creep feed in the amount of 0.75 kg/lamb is provided freely. Creep feed is composed of commercially available concentrate (Local supplier, Jordan) containing 68% barley, 15% bran, 15% soybean, and 2% salts, vitamins, and limestone. Freshwater is provided to the lambs freely.

In total, records of 779 Awassi lambs from 264 dams and 15 sires were used in the study. The following data were extracted from each record: sex, birth year (2010,

2011, and 2012), type of birth (single *versus* twins), and weight at birth (BW). The ADG and the adjusted weight at 60 days of age (AWW) were determined for each animal. The following formula was used to determine the AWW: $AWW = [(weaning\ weight - birth\ weight) / days\ at\ weaning \times 60] + birth\ weight$.

PCR-RFLP

Approximately 5 ml of whole blood was collected via jugular vein puncture from 208 Awassi ewes and 27 Awassi rams, as individuals in the pedigree and parents of the lambs, using vacutainer needle and Ethylenediaminetetraacetic acid-containing tubes. Blood tubes were stored at 4°C until analysis.

DNA was isolated from whole blood using EZNA blood DNA kit (OMEGA Bio-tek, USA) according to manufacturer's recommendations. The quantity and quality of isolated DNA were verified using 1.5% agarose gel electrophoresis.

The polymorphism of the prolactin gene (PRG), growth differentiation factor 9 (GDF-9) gene, and calpastatin (CAG) gene were determined using PCR-RFLP. The primer composition, PCR conditions for each gene are presented in Table 1.

The PCR reaction was prepared in a final volume of 20 µl containing 10 µl nuclease free water, 2 µl of genomic DNA (100 ng/µl), 2 µl primer forward, 2 µl primer reverse, and 4 µl (5 U/µl) of 10× buffer (HOT FIREPol DNA Polymerase; Solis BioDyne, Estonia), then specific PCR protocol for each primer was applied depending on the annealing temperature using specific restriction digestion enzyme for each of the genes, as described in Table 1.

Imputation and data analyses: The relationship among all individuals from the pedigree with known genotypes was used to predict the genotype of the non-genotyped relatives using GENPROB software (Gene Probe, USA) using segregation analysis as described previously [8]. The accuracy of the predicted genotypes was estimated by obtaining the genotype probability index (GPI) [9,10]. The probability of each genotype with a GPI of 0% was excluded from the analysis. Only two alleles (A and B)

Table 2. Allele and genotype frequencies for the studied genes in Awassi sheep.

Locus	No.	Alleles	Allele frequencies	Genotypes	Observed frequencies	Expected frequencies	χ^2
PRG	683	A	0.84	AA	0.88	0.71	219*
	43			AB	0.05	0.27	
	53	B	0.16	BB	0.07	0.03	
GDF-9	667	A	0.98	AB	0.96	0.96	0.021
	28	B	0.02	BB	0.04	0.04	
CAG	625	A	0.96	AB	0.92	0.92	0.016
	53	B	0.04	BB	0.08	0.08	

* $p < 0.05$ according to the Hardy Weinberg Equilibrium (HWE) test.

χ^2 = Chi-square value; PRG = prolactin gene; GDF-9 = growth differentiation factor 9 gene; CAG = calpastatin gene; N = number of animals in each genotype.

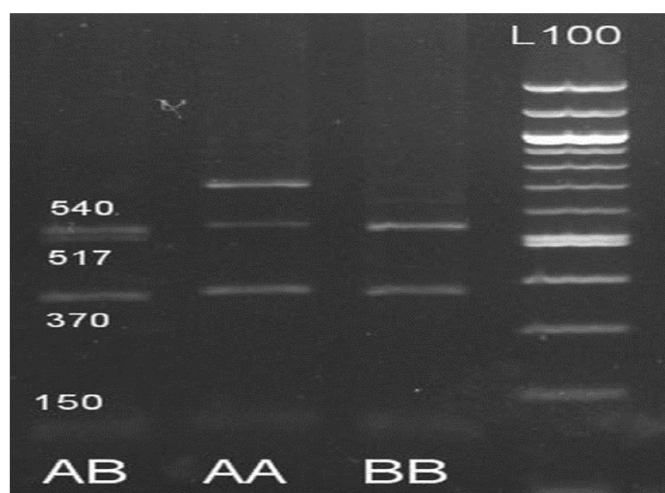


Figure 1. PCR-RFLP results for PRG gene using *HaeIII* restriction enzyme on 3% agarose gel.

were identified for the PRG, GDF-9, and CAG genes. To guarantee the quality of the analyses, data were edited and abnormal records or out of biological limit were deleted. Gene and genotype frequencies and the Hardy-Weinberg test (based on Chi-square test) were calculated as previously described [1].

Mixed model was used to evaluate the sex, year of birth, type of birth, PRG, GDF-9, and CAG as fixed effects, and dam nested in sire and residual as random effects using ANOVA. Statistical analysis was performed using SAS/STAT software version 14.2 (SAS, USA).

Results

Three genotypes (AA, AB, and BB) of the PRG with observed frequencies of 0.88, 0.05, and 0.07, respectively were detected (Table 2). The frequency of different alleles was 0.84 and 0.16 for A and B allele, respectively (Table 2). For both alleles A and B, analysis detected the presence of three fragments of different sizes (Fig. 1).

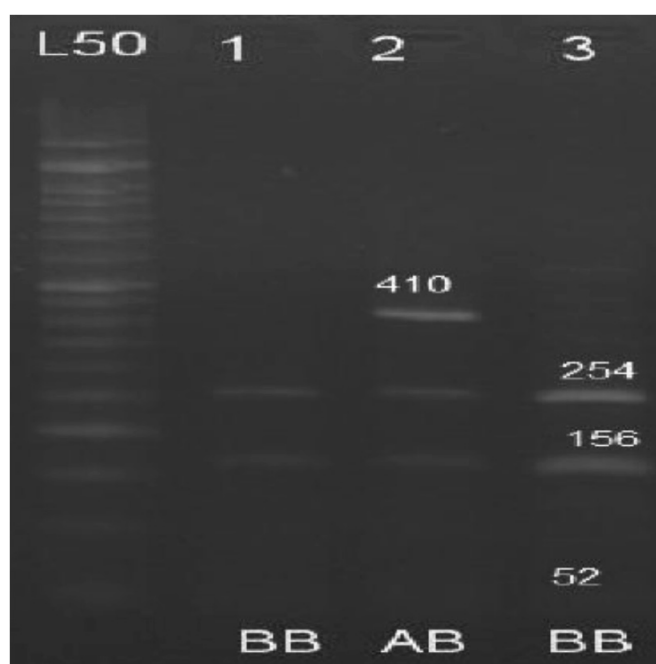


Figure 2. PCR-RFLP results for GDF-9 gene using *HhaI* restriction enzyme on 3% agarose gel.

For the GDF-9 gene, there were only two genotypes detected (AB and BB) with observed frequencies of 0.96 and 0.04, respectively (Table 2). The frequencies of each allele were 0.98 and 0.02 for allele A and B, respectively (Table 2). The *HhaI* restriction enzyme resulted in products of different sizes in homozygous animals (BB) and four different products in heterozygous (AB) individuals (Fig. 2).

For CAG gene, two genotypes were also detected (AB and BB) with observed frequencies of 0.92 and 0.08, respectively (Table 2). The allelic frequencies of CAG gene were 0.96 and 0.04, respectively for A and B alleles (Table 2). The *MspI* restriction enzyme produced two fragments in case of homozygous genotype (BB) (336 and 286 bp),

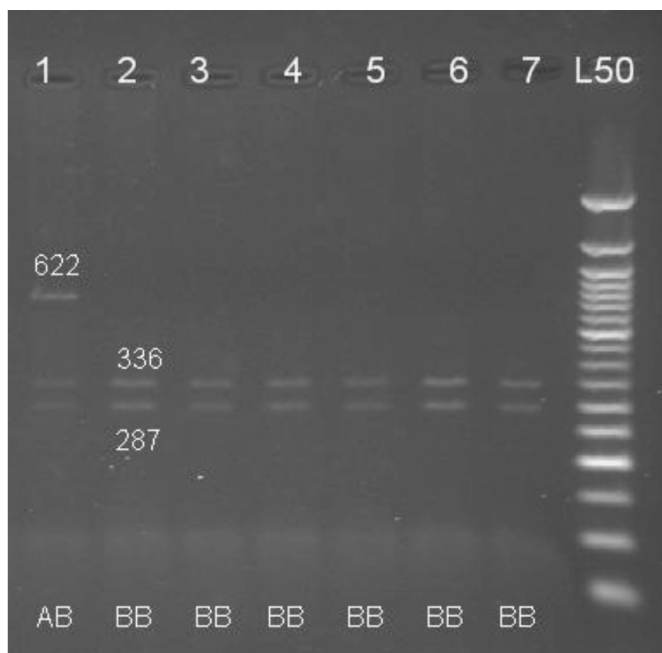


Figure 3. PCR-RFLP results for CAG gene using *MspI* restriction enzyme using 2% agarose gel.

and three fragments in case of AB genotype (622, 336, and 287 bp) (Fig. 3).

The probability of the sources of variation for BW, AWW, and ADG in Awassi lambs is presented in Table 3. The dam (sire) was found to significantly ($p \leq 0.0001$) affect all studied growth traits. Males were heavier ($p \leq 0.05$) than females at birth. Birth weight was also affected significantly ($p \leq 0.001$) by the type of birth where single born lambs were heavier at birth than twins.

The means (\pm SE) for BW, AWW, and ADG in Awassi lambs are presented in Table 4. There was a significant ($p \leq 0.04$) association between PRG and birth weight. Homozygous (AA) individuals weighed 1 kg more (at birth) than the heterozygous (AB) individuals. However, no obvious association between pre-weaning growth traits and GDF-9 and CAG genes variants was detected.

Discussion

In this study, three different genotypes of PRG were detected in Awassi lambs. Similar results were obtained previously in Chios sheep in Cyprus [11]. The majority of animals in this study carried the AA variant with an observed frequency of 0.876. These results are also in congruent with reports in Chios sheep, Serra da Estrela sheep, and Merino sheep [11,12]. However, lower allele A frequency than allele B frequency was found in East

Table 3. Probability of the sources of variation for BW, AWW, and ADG in Awassi lambs.

Source	BW	AWW	ADG
Dam (sire)	0.0001*	0.0001*	0.0001*
Sex	0.0407*	0.9051	0.9267
Year	0.1281	0.4013	0.2534
Type of birth	0.0001*	0.6733	0.1837
PRG	0.0401*	0.9957	0.8413
GDF-9	0.7871	0.9969	0.8413
CAG	0.5040	0.7663	0.7685

* $p \leq 0.05$; PRG = prolactin gene; GDF-9 = growth differentiation gene; CAG = calpastatin gene.

Friesian sheep [5]. The observed differences in allelic frequencies in sheep could be due to breed differences or the presence of founder effects in the respective populations.

Two genotypes of GDF-9 gene (AB, BB) were detected using *HhaI* restriction enzyme digestion in Awassi lambs. It is interesting that the homozygous (wild genotype) for the A allele (AA) (52 and 410-bp) that was reported in Chios sheep, Baluchi sheep, Belclare and Cambridge sheep, and Karagouniki sheep breeds were not detected in Awassi lambs [13–15]. Mutant genotype (BB) had been found to exert higher frequency than the heterozygous and wild genotypes (AB and AA, respectively) in Chios breed and Karagouniki breed in Cyprus [15].

Two genotypes (AB and BB) were also detected using *MspI* restriction enzyme of CAG gene in Awassi lambs. The AA genotype (the wild-type) was absent in this population. These results are similar to previously reported data in Awassi and Slovakian sheep [1,10]. However, three genotypes were detected in the Indonesian local sheep and Iranian sheep [6,16].

In the present study, the dam (sire) significantly ($p \leq 0.0001$) affected all studied growth traits. Previously, it was reported that sex of the newborn and type of birth (single *versus* twins) may significantly affect Awassi lambs' BW [2,14,18]. However, this effect was not lasting and actually did not affect the weaning weight and pre-weaning daily gain. It seems that this effect could be related to the intrauterine environment and hormonal effects during the prenatal life [19]. In Awassi lambs, it has been reported that the number of cotyledons plays a vital role in producing heavier weights at birth in males and singles than females and twins [17].

The association analysis showed that PRG has a significant ($p \leq 0.04$) effect on birth weight only. Homozygous (AA) individuals weighed 1 kg (at birth) more than the heterozygous (AB) individuals. Although a significant effect of PRG genotype on birth weight was detected, this effect was not significant on daily gain and weight at weaning. There were no associations between any of the growth traits

Table 4. Least squares means (\pm SE) for BW, AWW, and ADG in Awassi lambs.

Variables	N	BW (kg)	AWW (kg)	ADG (g)
Sex				
Male	133	3.80 \pm 0.33 ^a	18.2 \pm 3.0	0.23 \pm 0.04
Female	234	3.72 \pm 0.28 ^b	17.9 \pm 2.70	0.23 \pm 0.04
Type of birth				
Single	267	4.25 \pm 0.29 ^a	17.6 \pm 2.60	0.21 \pm 0.03
Twins	90	3.26 \pm 0.34 ^b	18.3 \pm 3.30	0.25 \pm 0.05
Year				
2010	165	3.52 \pm 0.25	19.9 \pm 2.90	0.27 \pm 0.04
2011	85	3.47 \pm 0.37	18.5 \pm 3.10	0.25 \pm 0.04
2012	105	3.28 \pm 0.47	15.5 \pm 3.60	0.18 \pm 0.05
PRG				
AA	346	4.26 \pm 0.38 ^a	18.0 \pm 3.40	0.22 \pm 0.05
AB	11	3.26 \pm 0.38 ^b	17.8 \pm 3.40	0.24 \pm 0.05
GDG				
AB	346	3.67 \pm 0.38	17.9 \pm 3.0	0.24 \pm 0.04
BB	11	3.85 \pm 0.50	18 \pm 4.50	0.22 \pm 0.06
CAG				
AB	343	3.90 \pm 0.25	17.3 \pm 1.80	0.22 \pm 0.02
BB	14	3.60 \pm 0.49	18.6 \pm 4.60	0.24 \pm 0.07

Different superscript letters between parameters in columns within each trait indicate statistically significant difference at $p \leq 0.05$.

PRG = prolactin gene; GDF-9 = growth differentiation gene; CAG = calpastatin gene; N = number of records.

and GDF-9 and CAG variants. There was a lack of obvious association between the growth traits and GDF-9 and CAG genes variants. Previously, CAG was found to significantly affect the birth weight and growth rate in Polypay and Targhee pure breeds and their crosses [3]. At the same time, no significant effects of CAG were detected on weaning and post-weaning weights [3].

Conclusion

In this study, the PRG appeared to strongly affect the weight of lambs at birth but not pre-weaning or weaning weight. PRG could be used to select dams with a high frequency of dystocia to reduce birth weight of newborn lambs and therefore conserve the dam's reproductive functions and improve her lambs' survivability.

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Competing Interests

The authors declare that they have no competing interests.

Authors' contribution

Khaleel Ibrahim Jawasreh designed the experiment and coordinated all research activities Lab work, statistical analysis, and drafting the manuscript. Zuhair Bani Ismail supervised fieldwork, data interpretation, and manuscript writing.

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