



Effect of Prolactin Gene Polymorphisms on Egg Weight of White Leghorn and Hy-line Brown Laying Hen Strains

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Abstract: This study was conducted at Poultry farm and Poultry Physiology laboratory /Animal Resources Department / College of Agriculture / The University of Baghdad during a period from 26/September/2013 to 1/June/2014 to find out the association of prolactin (PRL) gene polymorphisms with egg weight of laying hens. Two strains of laying hens, White Leghorn (n=100) and Hy-line Brown (n=60) were used in this study and reared in individual cages. Blood samples were collected from a brachial vein at 41 weeks of age. The PRL gene polymorphisms were determined using PCR-RFLP technique and *AluI* restriction enzyme. Two alleles C and T and three genotypes wild type CC (homozygous), heterozygous CT and mutant TT (homozygous) have been got. The size of the target gene fragment was 439 bp. A significant ($p < 0.05$) difference was showed in the Brown strain between genotypes CC and TT, the genotype CC was higher than TT in egg weight (EW) at 34 week of age, in White strain there was a significant ($p < 0.05$) differences between CC and CT, also, CC with TT genotypes in EW at 30 week of age. The T allele frequency was 0.66, whereas, C allele frequency was 0.34 for Brown strain. In White strain, the C allele frequency was 0.98, whereas T allele frequency was 0.02. The distribution percentage of polymorphism of PRL gene in Brown strain were 56.60, 37.74 and 5.66 % for CT, TT and CC genotype respectively, whereas, the distribution percentage of polymorphism of PRL gene in White strain were 97.75, 1.12 and 1.12 % for CC, CT and TT genotype respectively, and the differences among percentages was significant ($p < 0.01$). The allele frequency and distribution percentage of polymorphism for PRL gene was measured according to Hardy-Weinberg equilibrium.

Key words: Egg weight, Laying Hens, Polymorphism, Prolactin gene

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Introduction

Chickens are good models for studying the genetic basis of phenotypic traits, because of the wide diversity among domestic chickens selected for different purposes (1). An important purpose of modern breeding in the poultry industry is to create high performance poultry lines and breeds in two main directions meat and eggs (2), then they indicate that using molecular genetic markers potentially will greatly

increase the intensity of selection and will most effectively uncover the productive potential of birds.

Historically, chickens play an important role in molecular genetics as compared to other animals. Prolactin (PRL) is one of the pituitary hormones which is important role in regulation of different physiological functions, such as nesting behavior and implicated in the regulation of broodiness in birds (3). Several studies analyzed the frequencies of PRL genotypes were mostly in

Leghorn and native breeds, and found significant differences between the distributions of genotype groups (4). PRL in birds has an important role in the function of the reproductive system (5, 6). Chicken prolactin gene contains 229 amino acids compared with human PRL (227 amino acid). PRL is a single chain polypeptide. Which has a molecular weight of 23kDa (6). The gene located on chromosome 2 in birds (7, 8) and chromosome 6 in humans (9). The avian PRL gene consists of five exons and four introns (10, 11). The purpose of this study aimed to determine the effect of genetic polymorphism of prolactin gene on egg weight.

Materials and Methods

The field and laboratory works of this study were carried out at Poultry farm and poultry physiology laboratory /Animal Resources Department / College of Agriculture / The University of Baghdad, during the period from 26/September/2013 till 01/June/2014. The study was lasted 14 weeks. Two strains of commercial laying hens, Brown and White strain, 27 weeks of age were used. Hy-line Brown strain (60 hens) and White Leghorn strain (100 hens) were reared inside individual cages with dimension (40 × 40 × 50 cm). Birds were fed with diet (17.2% crude protein and 2823 Kcal/Kg metabolizable energy). Five ml blood of each bird were collected via brachial vein at 41 weeks of age into tubes containing EDTA and stored under -20°C till the time of DNA extraction. Total genomic DNA was extracted from the whole chicken frozen blood for molecular studies and it was applied using genomic DNA purification kits

(Delta / Spain) and the Procedure of DNA extraction from whole blood was as follows:-

1- 100µl of blood had been poured into a 1.5ml eppendorf tube and added 800 µl of cell lysis buffer.

2- Mix tube gently, then incubate at 25°C temperature for 30 minutes then spin for 5 minutes at 6000 rpm.

3- Supernatant discard , then pipette the pellet with the remaining solution and repeat steps 1 and 2 two more times – the pellet will become white .

4- 500 µl were added of complex materials lysis buffer to eppendorf tube (proteinnase K or RNase is optional in this stage), mixed well then 100 µl were added of high salt solution , mixed well then 600 µl were added of chloroform to eppendorf tube.

5- Mixed the tube well for 2 minutes then spin it for 2 minutes at 10000 rpm.

6- Supernatant transfer to a new 1.5 ml eppendorf tube.

7- 2.5volume were added of cold absolute ethanol (-20°C) to the supernatant and shake it gently , then DNA will appear as white threads.

8- Spin the tube for 5 minutes at 5000-10000 rpm then discard supernatant carefully and dry pellet at room temperature.

9- 100 µl were added of rehydration buffer and dissolved the DNA with gentle pipetting then storage DNA tube in 20°C. The DNA concentration samples were estimated by using NanoDrop (BioSpec-nano / Shimadzu Corporation / Tokyo- Japan): 1µl of extracted DNA was placed on the highly sensitive microdetector to detect concentration in ng/µl and the purity detected by noticing the ratio of optical density (OD) 260/280 nm to detect the

contamination of samples with protein. The accepted 260/280 ratio for pure DNA was between 1.7-1.9 (12). PCR products were amplified using primers as previously described according to (4). Primers were supplied by Alpha DNA Company, as lyophilized product of different picomols concentrations. Lyophilized primer was dissolved in a free DNase/RNase water to give a final concentration of 100 pmol/ μ l and stored

as a stock in -20°C . The solution is prepared to pull 10 μ l from stock solution (100 picomols/ μ l) and diluted with 90 μ l of deionized water to get a final concentration of solution which is 10 Picomols / μ l. The Primers were designed according to the sequence of PRL gene (13). Sequences of primers for prolactin gene used in this study presented in Table 1.

Table (1): Sequences of primers for prolactin gene used in this study.

Gene	Primer	Sequence of primers 5' \leftarrow \rightarrow 3'	PCR Product size (bp)
PRL gene (promoter region)	Forward	AGAGGCAGCCCAGGCATTTTAC	439
	Reverse	CCTGGGTCTGGTTTGAAATTG	

PCR amplification

The prolactin gene polymorphisms were analyzed using PCR- RFLP method. PCR amplification was carried out in a final volume 15 μ l: 50- 90 ng/ μ l genomic DNA, 1 μ l of each primer, 200MM dNTP, 1.5mM Mgcl, 10 \times PCR buffer and 1.0 U Taq DNA polymerase. According to (14), 8 μ l for PCR product was digested at 37°C overnight, after digestion the products were subjected to 1.5% agarose gel-electrophoresis and the genotypes were determined with the ultraviolet trans-illuminator by ethidium bromide staining.

Agarose Gel check for PCR amplification

The PCR reactions were tested by agarose gel electrophoresis. A 3% gel was prepared by mixing 1.5 gm agarose

and 50 ml of 1 x TBE buffer and heating the mixture to boiling point in a microwave oven. When the solution was approximately 60°C , gel solution poured into a gel chamber. A slot former was fixed at one end. The gel solution became solid within 10 min and then placed in an electrophoresis chamber containing 1 x TBE buffer added to 15 μ l of PCR product, then the reaction mixture was loaded into a well and loading the marker (100bp DNA-ladder). The electrophoresis was run at 60V for 2 $\frac{1}{2}$ hours. Ethidium bromide was added to the sample by (1:5) loading dye, then placed on an UV-screen, the ethidium bromide stained DNA fragments emitted fluorescent light as a visible bands. The gel was photographed. The amount of DNA was estimated by comparing the brightness of the fluorescent sample band to the brightness of primers bands.

Reagents of Gel Electrophoresis

Agarose, 1 X TBE Buffer, Bromophenol Blue in 1 % glycerol (loading buffer), Ethidium Bromide, DNA Ladder Marker (100 bp). The master mix of PCR 12.5 μ l was mixed

with (750 ng/ μ l) DNA and 0.6 μ l of each primer forward and reverse then the volume was completed up to 25 μ l with deionized distilled water according to the instructions of the company. PCR program is illustrated in Table 2.

Table (2): The PCR program used in this study

Steps	Temperature (°C)	Time	No. of Cycles
Initial denaturation	94	5min.	1
Denaturation	94	30 sec.	35
Annealing	62	30 sec.	
Extension	72	30 sec.	
Final extension	72	5 min.	1

Detection of PCR Products by agarose electrophoresis

Ten μ l of amplified products were analyzed by electrophoresis in 1.5% agarose gel which was stained with 0.5 μ g /ml ethidium bromide, at 5 volt/cm² for 90 min, in 1 X TBE buffer. After that, it was visualized under UV light using ultraviolet transilluminator. 9 μ l of amplified products was mixed with 5 Units of *AluI* enzyme, 0.5 μ l of enzyme buffer and 4 distilled water, then it was incubated for 3 hours in 37°C (15). All enzyme-digested mixtures were loaded in the well with 2% agarose gel stained with 0.5 μ g /ml ethidium bromide, at 5 Volt/cm² for 1 hour, in 1 X TBE buffer. After that it was visualized under UV

light using ultraviolet transilluminator (15). A 100-2000 bp DNA ladder was used, and the gel was photographed by a digital camera. The absence of SNP, that digested with *AluI* enzyme yielded 4 bands (54, 81, 144 and 160 bp) in CC genotype, while the presence of SNP, yielded in CT 5 bands (54, 81, 144, 160 and 304 bp), and in TT genotype yielded 3 bands (54, 81 and 304 bp).

Results and discussions

It was subsequently determine the genotypes of the test animals for PRL gene by using PCR-RFLP techniques and enzyme *AluI* restriction to identify genotypes within interested animal as showed in Figures 1, 2.

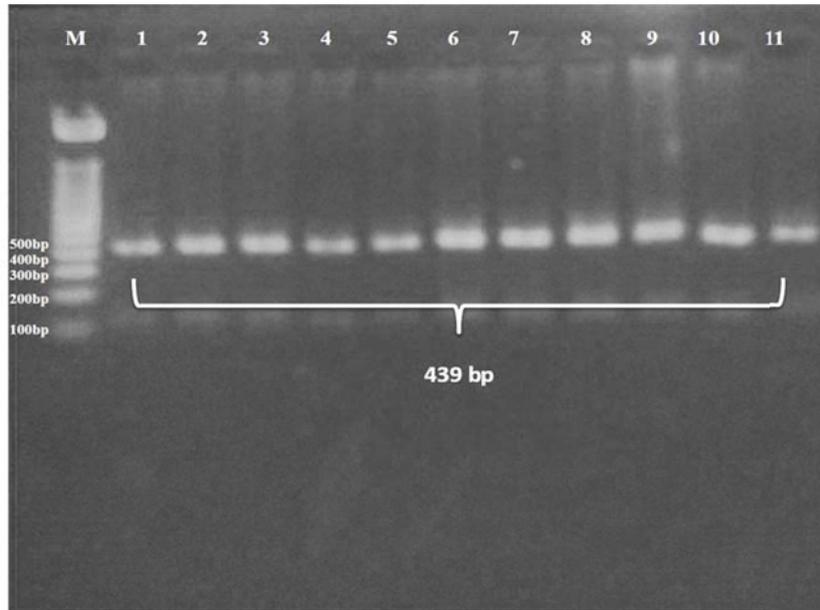


Figure (1): PCR product for target fragment in avian Prolactin gene.

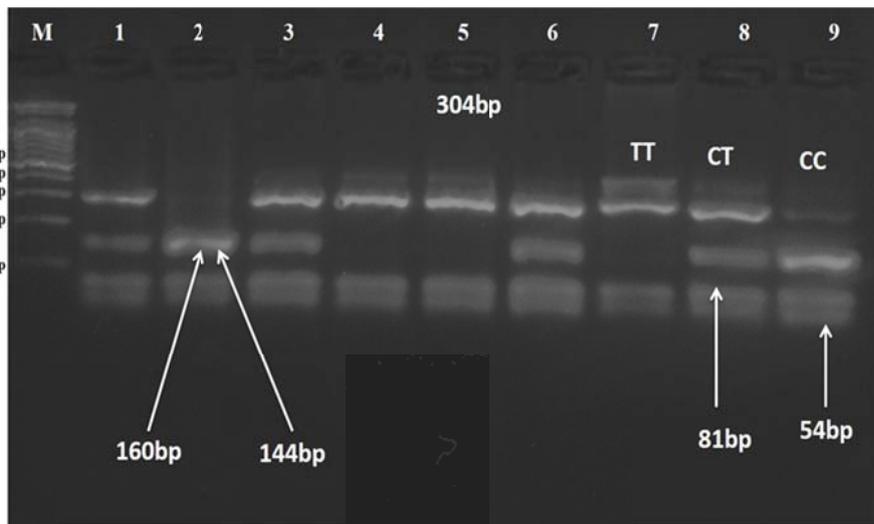


Figure (2): Restriction analysis of avian Prolactin gene 439 bp PCR products digested with *AluI* by 3% agarose gel electrophoresis. Genotype CC (lane 9): Restriction fragments of 160, 144, 81 and 54 bp, genotype CT (lane 8): Restriction fragments of 304,160,144, 81 and 54 bp, and genotype TT (lane 7): Restriction fragments of 304, 81 and 54bp

It was found that the SNP at (C-2402T) in the 5'-flanking region of the length of the study area has been effect on egg production. From the results of gel-electrophoresis there were three genotypes (CC, CT and TT), the

number of bands for each genotype was as follows:-

Genotype CC (homozygous) wild four bands (160,144, 81 and 54 bp)

Genotype CT (heterozygous) five bands (304,160,144, 81 and 54 bp)

Genotype TT (homozygous) mutant three bands (304, 81 and 54 bp)

This is consistent with Cui *et al.* (4) in his research on the polymorphism of chicken prolactin gene since it identified three genotypes as a result of the SNP at (C-2402T) and that are related to the egg production and growth traits in laying hens, as well as the results agreed with several reserches (16,15,17) ,as the size of the piece was 439 bp and determined three genotype (CC,CT and TT) .

Distribution of Prolactin gene polymorphisms

Table 3 showed that the distribution of PRL gene polymorphisms in Brown strain has indicated that CT genotype was higher (56.60 %) than that of genotype TT and CC which were 37.74 and 5.66 % respectively, while in White strain the results indicate a high significant ($p<0.01$) difference of genotype CC (97.75%) compared with the genotypes CT and TT which were 1.12 % in both CT and TT genotypes. A chi-square test was performed to verify if genotype frequencies agreed with Hardy-Weinberg equilibrium expectation. Table 3 revealed that there were highly significant ($p<0.01$) differences between the different genotypes in genotype CT compared with other genotypes TT and CC, this difference may be due to the differences in strain and environmental effect on genotype, as well as the studied sample size. These results were consistent with the findings obtained by Rashidi *et al* (15),who pointed out that the genotype

CT was 0.84 is superiority of other genotypes TT and CC which were 0.06 and 0.10 respectively in indigenous chickens of mazandaran province and agreed with Li *et al* (18), who indicated that there was a highly significant differences of hybrid genotype CT was 0.48 compared to other genotypes CC and TT which were 0.05 and 0.46 respectively in Qingyuan partridge chicken (QY), while the SNP at G8113C with genotype TT was higher 0.47 compared with genotype CC and GC) which were 0.05 and 0.46 respectively in the same strain. In White strain, the genotype CC was highly significant ($p<0.01$) difference compared with the other genotypes TT and CT. These results disagree with Alipanah *et al* (16) who indicated that distribution percentage of genotypes TT was 0.53 compared with other genotypes CC and CT which were 0.20 and 0.27 respectively, when his studies at native chicken (Zabol region) through the prolactin gene relationship in broodiness, but the results disagreed with (2) who confirmed that the genotype TT was higher (71%) compared with CT and CC which were 27 and 2% respectively in Okranian line chicken. Results agreed with Sarvestani *et al* (17) who obtained that the genotype CT in Brown strain was 0.44, while in the other genotypes CC and TT were 0.43 and 0.11 respectively, in the native chickens. Also agreed with Abdi *etal* (19) who found the results of genotype CC was 56% compared with CT and TT which were 44% and 6% respectively in West Azerbaijan native chickens.

Table (3): Distribution percentage of polymorphism (C-2402T) of avian PRL gene in Hy-Line Brown and White Leghorn layer strains.

Genotype	Brown strain	White strain
	No. (%)	No. (%)
CC	3 (5.66 %)	87 (97.75%)
CT	30 (56.60 %)	1 (1.12 %)
TT	20 (37.74%)	1 (1.12 %)
Total	53 (100%)	89 (100%)
Chi-square value (χ^2)	11.092 **	14.163 **

** (P<0.01)

Table (4): Allele frequency of avian PRL gene (C-2402T) in Hy-Line Brown and White Leghorn layer strains.

Genotype	Brown strain	White strain
C	0.34	0.98
T	0.66	0.02

Allele frequency of PRL gene

Results shown in Table 4 revealed that the allele frequency of C allele was 34% lower than T allele 66% in Brown strain, while in White strain allele frequency of C allele was higher 98% compared with T allele 2%. The results obtained by Cui *et al* (4) who found that frequency of C allele was 1% and higher than T allele in White leghorn and disagreed with the results for White breed for same researchers when his studied for Yangshan breed noted that the frequency of C allele was 0.05 compared with T allele which was 0.95 but this results consistent with results in Red breed, as well as the results were comparable with the same researcher at the White Rock 30 breed was a frequency of the T allele has reached 0.65 and C allele was 0.35 .

The results of present study were consistent with previous studies (16, 2) that the frequency of T allele was higher than C allele in Brown strain by using the same restriction enzyme and primers for prolactin gene. The results agreed

with pohnke *etal* (20) in his study on the allelic frequency of allele T was higher in the local Chinese chicken and said the reason that there is interaction between the locations of SNPs and T allele leading to enhance the production of the hormone prolactin, and thus reduce egg production. In White strain the results were consistent with some reports (2, 17, 19) as they had noticed that the allelic frequency of C allele was higher than T allele in different breeds (Ukranian line A , West-Azerbaijan native chicken and Fars native chicken).

Egg weight (EW)

The results of Table 5 showed that EW vary according to genotype, as the results indicate that there are significant effect (P<0.05) between genotypes CC, CT and TT on EW at different ages. The genotype CC were 71.27 ± 4.94 gm, while the lowest estimate was for the genotype CT and TT were reached $66.06 \pm 0.81, 64.70 \pm 1.27$ gm respectively at 34 weeks of ages in Brown strain ,while the other ages were not

significant. Whereas, in White strain the genotype CT was 58.13 ± 0.00 gm achieved greater EW than the other genotypes at 30 weeks of age, as well as, the differences were not significant for the others. This may be due to positive correlation between BW and EW, as well as, the EW trait was affected by a number of genes on chromosome Z. The presence of the negative relationship between egg production and the EW led to differences in the effect of genotypes of PRL gene on egg EW. These results agreed with previous studies (2, 19) on the Ukrainian and West Azarbaijan chicken, as they had noticed that the genotype CC has been higher than CT and TT for EW.

Results also agreed with Bhattacharya and Chatterjee (21) who found that the genotype AA was higher than the genotypes AB and BB that may be due to the genetic variation resulting from

the effect of more than one pair of genes on EW in the naked neck chicken.

The results coincided with Fathi and Zarringhobaie (22) who found that the mutation in 24-bp indel that DD genotype was higher estimate of genotypes II and ID in turkey. The results were not consistent with Li *et al* (18) who noticed that the genotype BB was superiority than others using *DraI* restriction enzyme in the intron 1 in ducks. Lotfi *et al* (23) did not found any significant correlation of genotypes with egg weight. Also it is not consistent with Li *et al* (18) who noticed that SNPs at (T8052C and G8113C) were not significantly associated with EW in two breeds of Chinese chicken. The reason may be due to differences in mutations impact on this trait which genes are responsible for the variation in traits, so the genes affect the traits and SNPs of the PRL gene have effects on chicken egg production (13).

Table (5): Effect of avian PRL gene polymorphism (C-2402T) on egg weight trait (EW) in Hy-Line Brown and White Leghorn layer strains (Means \pm SE)

Age (weeks)	Egg weight (gm)					
	Brown strain			White strain		
	CC	CT	TT	CC	CT	TT
30	66.03 \pm 6.72 a	61.81 \pm 1.69 a	60.37 \pm 2.41 a	54.16 \pm 0.84 b	58.13 \pm 0.00 a	50.33 \pm 0.00a
32	63.32 \pm 3.29 a	61.28 \pm 1.16 a	60.79 \pm 1.33 a	53.51 \pm 2.37a	53.71 \pm 0.00a	51.42 \pm 0.00a
34	71.27 \pm 4.94 a	66.06 \pm 0.81ab	64.70 \pm 1.27 b	59.68 \pm 0.78 a	59.45 \pm 0.00 a	58.25 \pm 0.00a
38	70.29 \pm 1.71 a	68.44 \pm 0.85 a	69.65 \pm 1.09 a	61.84 \pm 0.72 a	63.80 \pm 0.00 a	61.09 \pm 0.00 a
40	61.72 \pm 0.00 a	68.12 \pm 0.95 a	66.90 \pm 1.82 a	59.72 \pm 1.31 a	60.35 \pm 0.00 a	66.89 \pm 0.00 a

Means with the same superscripts of each breed within each row are significantly ($p < 0.05$) different.

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