



STUDY OF *CLPG* GENE EXPRESSION LEVELS IN LOCAL AWASSI LAMBS IN IRAQ

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Abstract

The present study has been conducted to estimate mRNA expression level of Callipyge (*CLPG*) gene in Local Awassi Lambs in Iraq. It was divided into two groups (Group1: No. 31, age 4 months and live body weight below 30 kg. Group2: No. 24, age 6 months, and live body weight more 30 kg). The results of the study showed the presence of expression of the *CLPG* gene at a rate of 8.7065 in favor of the first group (Group1) versus the second group (Group2) according to the weight group and age. We conclude from the study the presence of expression of the *CLPG* gene according to the weight and age group, and further study may be conducted to ascertain the association of this expression with the growth and carcass meat quality traits.

Keywords: Callipyge gene (*CLPG*), Iraqi Awassi Lambs, qRT-PCR.

Introduction

Modern animal breeding pays a lot of attention to genetics. Molecular science is well developed now, new discoveries are made weekly (Pareek *et al.*, 2011), and there is a huge difference in what scientists knew when they first learned about genetics and what they actually know now. From the times when people started to breed animals, they understood that the traits of offspring are dependent on the traits of the parents (Conner, 2003). By selecting and crossbreeding the best individuals, people for decades produced new and better animal breeds of higher grade, that also lead to the higher interest of heredity phenomenon (Cobb, 2006). Callipyge phenotype in sheep is also known as 'beautiful buttocks'. This phenotype develops only in paternal heterozygous animals (*CLPG*) mutant allele inherited from father and wild type allele inherited from mother. The maternal heterozygous and homozygous genotype pattern does not express the callipyge phenotype in animals. This type of non-Mendelian inheritance pattern is known as 'Polar Over dominance' (Cockett *et al.* 1996 and Vuocolo *et al.* 2007). The best documented mutation for muscle development in sheep is callipyge (*CLPG*), which causes a postnatal muscle hypertrophy that is restricted at the pelvic limbs and loin with little or no effect on anterior skeletal muscles (Cockett *et al.* 1996). The callipyge gene was mapped on ovine chromosome number 18 in the telomeric region within a cluster of imprinted genes (Tellam *et al.* 2012). The callipyge phenotype is inherited in a non-Mendelian mode referred to as Polar Overdominance where only heterozygous individuals inheriting the callipyge allele from the paternal side (paternal heterozygote-NCpat) exhibit the phenotype (Cockett *et al.*, 1996). The causative mutation has been defined as a single-nucleotide polymorphism (SNP) on the distal end of ovine chromosome 18 (Cockett *et al.*, 1994; Freking *et al.*, 1998; Shay *et al.*, 2001). The SNP lies in an intergenic region of the chromosome between the genes *Dlk1* and *Gtl2* (Charlier *et al.*, 2001). In gene expression studies, messenger ribonucleic acid (mRNA) molecules are examined and compared, for example, between different individuals and animal breeds. The development of molecular technology makes it possible to study gene expression, where the sequence of DNA nucleotides converts into RNA and finally protein. The gene expression levels and

differences between breeds can be studied using RNA sequencing technology and bioinformatics analysis. New technology and researches in genetics can be very useful and bring many advantages to agriculture, for example, scientists can genetically compare animals with different economically important characteristics through the sequencing of different points of scientific interest and can distinguish important genes such as high fertility genes or genes that allow reproducing in any season of the year (Dunn and Ryan, 2015). Real time polymerase chain reaction or quantitative PCR (qPCR) can be used for similar analysis. QPCR is used to create additional copies of fragments of DNA and to quantifying the number of copies and quantifying mRNA and miRNA levels in cells and tissues (Novikov *et al.*, 2012).

DNA is the basis of all living organisms: it contains genetic information and instructions for protein synthesis and regulations (Travers and Muskhelishvili, 2015). Instructions for the synthesis of a particular protein are encoded in the DNA fragment, which is called a gene (Gerstein *et al.*, 2007). The specific location of the gene on the chromosome is called the locus. Suitable loci in paired chromosomes may contain the same or slightly different DNA segments, which are called alleles (Simm, 1998). The central dogma of molecular biology states that genetic information from DNA converts to RNA and then to protein (Francis, 1970; Gerstein *et al.*, 2007). Protein is synthesized from DNA in two ways: transcription and translation. Transcription is the process by which RNA polymerase copies the sequence of nucleotides from gene to mRNA (messenger RNA) using DNA as a template, whereas translation is the process by which mRNA is converted into a protein and takes place in the ribosomes (Eugene, 2015). The frequency of functional proteins in the cell production is regulated at different stages of gene expression, mainly at the transcription level. This study aims to quantitate the mRNA expression levels of *CLPG* gene in Local Awassi lambs in Iraq by qRT-PCR.

Materials and Methods

Animals

Collection of blood sample and RNA isolation. A study was designed to identify the genetic polymorphism of callipyge gene in Iraqi awassi Lamb. About 3 ml of blood samples were collected randomly from 55 sample comprising

Lamb from Iraqi awassi Lamb by jugular vein puncture using disposable needle, it was divided into two groups (Group1: No. 31, age 4 months and live body weight below 30 kg. Group2: No. 24, age 6 months, and live body weight more 30 kg).

Molecular analysis

I used a real-time measurement number for a polymerase chain reaction for quantitative reverse transcription Equipped from Bioneer a company, South Korea using the following prefixes for *GAPDH* and *CLPG*.

Gene	Primer sequence (5'→3')		Product size	Reference
<i>CLPG</i>	F	CAGATGCTGTGGACGTGGGGAATC	242	Freking <i>et al.</i> (2002) and Murphy <i>et al.</i> (2006)
	R	GCAAGGGTCTGTTTGGTCCTAA		
<i>GAPDH</i>	F	ACCACAGTCCATGCCATCAC	452	
	R	TCCACCACCCTGTTGCTGTA		

RNA isolation

RNA was isolated from blood according to the protocol described by the kit (Geneaid Biotech - Taiwan).

Assessing RNA yield and quality

There are three quality controls were performed on isolated RNA. The first one is to determine the quantity of RNA (ng/μL) that has been isolated using Nanodrop UV/VIS spectrophotometer (OPTIZEN POP. MECASYS Korea), the second is the purity of RNA by reading the absorbance in spectrophotometer at 260 and 280 nm in the same Nanodrop machine, and the third is the integrity of the RNA by prepared gel electrophoresis, as follows: After opening up the Nanodrop software, the appropriate application was used (Nucleic acid, RNA). A dry Chem-wipe was taken and cleaned the measurement pedestals several times. Then, 1μl of dd H₂O was carefully withdrawn onto the surface of the lower measurement pedestal. The sampling arm was lowered and clicked OK to initialize the Nanodrop, then cleaning off the pedestals and 1μl of the appropriate blanking solution was added as black solution which is same elution buffer of RNA samples. After that, the pedestals were cleaned and 1μl of RNA sample was carefully withdrawn for measurement.

The purity of RNA also determined by reading the absorbance in Nanodrop spectrophotometer at 260 nm and 280 nm, so the RNA has its absorption maximum at 260 nm and the ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, or other contaminants that absorb strongly at or near 280 nm. After that, the integrity of the RNA was determined by prepared gel electrophoresis as following: 1% agarose gel was prepared in 0.5X TBE buffer and heated by using (hot magnetic stirrer) for 2 minutes until disappear all crystals in agarose solution. After cooling, (3 μL) of Ethidium bromide was added to the solution, then the gel was poured in the tray and left until solidifying. Then, it was transferred into electrophoresis machine which contains the same 0.5X TBE buffer. The RNA samples were prepared by mixing 5μl of RNA sample with 1μl of loading dye. Then, all amount was transferred into agarose gel wells, then running the electrophoresis power at 100 Volt for 1 hours, then the RNA bands were seen by U.V light.

DNase inactivation (DNase I) Treatment

The extracted total RNA samples were treated with DNase I enzyme to remove the trace amounts of genomic

DNA from the eluted total RNA by using samples (DNase I enzyme) and done according to method described by Genaid – Taiwan company.

cDNA synthesis

DNase-I treatment total RNA samples were used in cDNA synthesis step using AccuPowerR RocketScript RT PreMix kit that provided from Bioneer company, Korea and done according to company instructions as follows: All DNase-I treatment total RNA samples were adjusted at the same concentrations that measured by nanodrop by DEPC water. RNA was converted to cDNA by preparation of reverse transcription PreMix reaction. 20 μL of RT PreMix was added into AccuPower RocketScript RT PreMix tubes that contain lyophilized reverse transcription enzyme. The lyophilized pellet were dissolved completely by vortex and briefly spinning down. The RNA was converted into cDNA under the following thermo cycler conditions. Finally, the samples were stored at -20 C° until performed qRT-PCR.

Cycles	Time	Temp. °C	Steps
1	10 : 00	37	Pre-Denaturation
	60 : 00	60	Denaturation
	05 : 00	95	Annealing

qRT-PCR based SYBER Green I Dye Detection

qRTPCR was performed using AccuPower® Greenstar™ qPCR PreMix reagent kit (Bioneer, Korea) and Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer, Korea). According to a method described by Cheon *et al.* (1999). The Syber Green based qRTPCR PreMix reagent kit is designed for PCR amplification of cDNA for target gene (*CLPG*) by using its primers and (*GapdH*) Housekeeping gene, as well as for quantification of PCR amplification copy numbers comparatively to copy numbers of Genomic DNA qRT-PCR standard curve. The SYBR Green dye that was used in this kit is DNA binding dye which reacted with new copies of amplification specific segment in target and housekeeping gene, then the fluorescent signals were recorded in Real Time PCR thermocycler.

Experimental design of qRT- PCR

For quantification of gene expression in treatment and control samples at duplicate, internal control gene as housekeeping gene (*GAPDH*) was used to normalization of gene expression levels, therefore, qRTPCR master mixes were prepared for gDNA standard curve, target genes, and *GAPDH* housekeeping gene as following tables (1 and 2).

Table 1: qRT-PCR Master Mix for target genes.

Volume	qPCR PreMix	
10 µL	cDNA template	
1 µL	Target gene –F	Primers
1 µL	Target gene –R	
8 µL	DEPC water	
20 µL	Total	

Table 2 : qRT-PCR Master Mix for GAPDH housekeeping gene.

Volume	qPCR PreMix	
10 µL	cDNA template	
1 µL	Target gene –F	Primers
1 µL	Target gene –R	
8 µL	DEPC water	
20 µL	Total	

After that, qPCR PreMix were added into *AccuPower* GreenStar qPCR PreMix tube. Then, real-time PCR tubes sealed by the optical adhesive film and mixed by vigorous vortexing for resuspension of PreMix pellet. The tubes centrifuge at 3,000 rpm, for 2 min, then Exicycler™ 96 Real-Time Quantitative Thermal Block instrument was started and loaded the following program according to the kit instruction. After reaction is completed, data analysis was performed.

Cycle	Time	Temp.	Step
1	10 min	95 °C	Pre-Denaturation
40	15 sec	95 °C	Denaturation
	30 sec	60 °C	Annealing/ Extension
			Detection(Scan)
1	-	-	Melting

Data analysis of qRT-PCR

The housekeeping gene (*Gapdh*) was represented as a normalize gene that can be used for calculation of the relative gene expression or fold change in target gene (*CLPG*). Therefore, the quantities (Ct) of target gene were normalized with quantities (Ct) of housekeeping gene (*Gapdh*) by the relative quantification gene expression levels (fold change) using Livak method (Livak and Schmittgen, 2001). The relative quantification method, quantities obtained from q RT-PCR experiment must be normalized in such a way that the data become biologically meaningful. In this method, one of the experimental samples is the calibrator such as (Control samples) each of the normalized target values (CT values) is divided by the calibrator normalized target value to generate the relative expression levels. After that, the Δ CT Method with a Reference Gene was used as following equations:

$$\Delta CT (\text{calibrator}) = CT (\text{target, calibrator}) - CT (\text{ref, calibrator})$$

$$\text{Ratio (target / reference)} = 2^{CT (\text{reference}) - CT (\text{target})}$$

First, normalize the CT of the reference (ref) gene to that of the target gene, for calibrator sample. Second,

normalize the CT of the reference (ref) gene to that of the target gene, for the test sample:

$$\Delta CT (\text{Test}) = CT (\text{target, test}) - CT (\text{ref, test})$$

$$\Delta \Delta CT = \Delta CT (\text{test}) - \Delta CT (\text{calibrator})$$

$$\text{Fold change} = 2^{-\Delta \Delta CT}$$

$$\text{Ratio (reference/target)} = 2^{CT (\text{reference}) - CT (\text{target})}$$

So, the relative expression was divided by the expression value of a chosen calibrator for each expression ratio of test sample.

Statistical analysis

Results were expressed as All statistical analysis were carried out using SAS (SAS Institute, Inc., USA).

Results and Discussion

Figure (1) shows the result of RNA extraction process through the electrophoresis product on the Agarose gel.

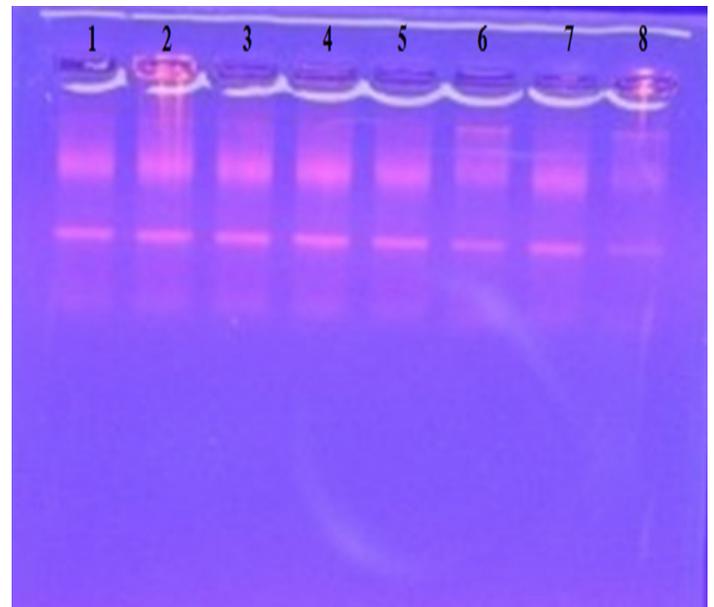


Fig. 1 : Electrophoresis relay product for RNA. Agarose gel concentration: 1%, dye used: ethidium bromide, electric current: 70 volt, numbers 1-8 refer to RNA samples of the Local Awassi Lambs in Iraq.

Quantitative Reverse Transcriptase Real- Time PCR

Data analysis of SYBR® green based reverse transcriptase real-time PCR for *CLPG* gene expression levels were presented as a relative quantification normalized by housekeeping gene expression (*GAPDH*). The data of threshold cycle numbers (Ct) were calculated from amplification plot of real-time PCR detection system, during exponential phase of fluorescent signals of SYBR® green primer of *CLPG* gene that react with complementary DNA (cDNA) of Lambs mRNA, where, the amount of PCR product (DNA copy numbers) in master mix reaction is approximately doubles and more in each PCR cycle. First prepared series dilution of blood cDNA was used with the primer of gene to draw the amplification plot of gene, and then from this plot, threshold cycle was used to calculate a linear regression based on the data points, and inferring the efficiency of primer from the slope (figures 2).

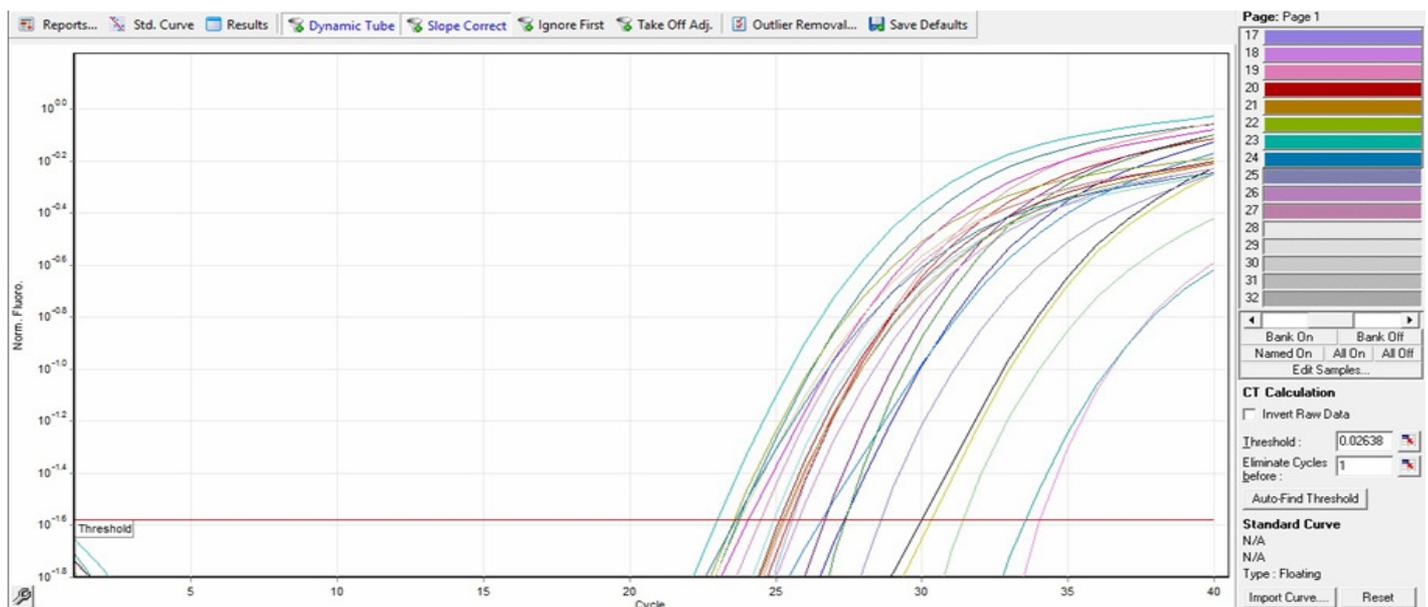


Fig. 2 : Curves for *GAPDH* and *CLPG* Genes in Local Awassi Lambs in Iraq.

The results of the study showed the presence of expression of the *CLPG* gene at a rate of 8.7065 times in favor of the first group (Group1) versus the second group (Group2) according to the weight group and age. Table (3), and these results were consistent with what was stated by White *et al.* (2008), as it indicated the presence of a

differentiated genetic expression without being affected by the presence of a *CLPG* mutation in sheep muscles. In the same vein, Murphy *et al.* (2006) explained that the *CLPG* gene cannot be the cause of phenotype alone, because it is expressed in the muscles in which the *CLPG* occurs.

Table 3 : $\Delta\Delta$ CT and Fold change values for a *CLPG* gene by weight group and age in Iraq.

Fold change	$\Delta \Delta$ CT	Δ CT (Group2)	Δ CT (Group1)	Weights / kg		
				Group2	Group1	
8.7065	-3.1221	1.2725	-1.8496	33.9771	27.7629	Mean
1.76	1.02	0.93	2.66	0.31	0.26	SE
-	-	24	31	24	31	No.

We conclude from the study the presence of expression of the *CLPG* gene according to the weight and age group, and further study may be conducted to ascertain the association of this expression with the growth and carcass meat quality traits.

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