

Prediction equations to linear regression analysis of carcass traits on CAPN4 gene expression in Iraqi Local Awassi

Dr. Adil Hussein Radhi Al-Murshidy

Researcher, Department of Quality Control, Ministry of Trade, Iraq.

E-mail: adil.alradhi67@gmail.com

Abstract:

The present study has been conducted to estimate mRNA expression level of CAPN4 gene, and measuring regression coefficient and straight line equation for the expression relationship of the CAPN4 gene with live weight at slaughter, hot carcass weight, and dressing percentage for Iraqi Local Awassi lambs. The results of the study showed the presence of expression of the CAPN4 gene according to the above. We conclude from the study the presence of expression of the CAPN4 gene according to the Dressing percentage, and it can be adopted in developing genetic improvement strategies for sheep to increase the economic return from breeding projects, and that applying the study to a larger sample would give more accurate results to implement the genetic improvement strategy of the native Awassi sheep in Iraq.

Key words: CAPN4 gene, Iraqi Awassi Lambs, qRT- PCR, Regression

Introduction:

Sheep husbandry in Iraq has been a historically important component of rural development and still fulfills a sustainable role in the livelihood of farmers. The country has a tradition of the consumption of sheep products, especially lamb and mutton (Khalaf, et al. 2010 and Al-Khazrji, et al., 2014). Modern animal breeding pays a lot of attention to genetics. Molecular science is well developed now, new discoveries are made (Pareek et al., 2011). The CAPN4 gene encodes the protein of 263–269 amino acids, with the share of more than 90% amino acids identity in humans, mice, pigs, and cattle (Juszczuk-Kubiak et al., 2010). Ovine Calpain small subunit1 gene (CAPNS1), also known as CAPN4, is located on chromosome 14 and contains 12 exons (Chung and Davis, 2014). However there is little known about this gene in sheep .

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).

The regression model is a statistical procedure that allows a researcher to estimate the linear, or straight line, relationship that relates two or more variables. This linear relationship summarizes the amount of change in one variable that is associated with change in another variable or variables. The model can also be tested for statistical significance, to test whether the observed linear relationship could have emerged by chance or not, and expressed by a straight line equation based on a determining factor (R^2). Moreover, these concepts are important when developing improvement plans, especially indirect selection in the different herds of farm animals (Gingrich, 2004).

This study aims to measure Regression coefficient and straight line equation for the expression relationship of the CAPN4 gene with live weight at slaughter, hot carcass weight, and dressing percentage for Iraqi Local Awassi lambs.

Materials and methods:

Animals

Collection of blood sample and RNA isolation. A study was designed to quantitate the mRNA expression levels of CAPN4 gene in Iraqi Awassi lamb. About 3 ml of blood samples were collected randomly from 55 samples comprising Lamb from Iraqi Awassi lamb by jugular vein puncture using disposable needle.

Animals slaughter

Fifty-five Local Awassi lambs were slaughtered randomly selected of the massacre.

Weight of the Animal before slaughter

Record the weight of the animal before slaughtering using a 150 kg ground scale.

Weight of the Animals after slaughter

After completing the slaughtering process, record the weight of the carcass after half an hour has passed since the slaughtering using a 50 kg disc scale, and this was considered the hot weight of the carcass.

The dressing percentage

The dressing percentage was estimated in relation to the Live animal weight body and was calculated by the following formula:

Dressing percentage = hot carcass weight / Live animal weight * 100.

Molecular analysis

The 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 CAPN4 were used in present study.

Gene	Primer sequence (5'→3')	Product size	Reference
CAPN4	F AACATTCTCAACAAAGTGGTG	192	Shahroudi et al. (2006) and Dehnavi et al (2012) .
	R ACATCCATTACAGCCACCAT		
GAPDH	F TGACCTATGGCAACCGATACAA	167	
	R CCGCAAAAGACATCCAGGAT		

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 ddH₂O 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 at the absorbance in Nanodrop spectrophotometer at 260 nm and 280 nm. 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.

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

100 : 10	37	Annealing
00 : 60	60	Elongation
00 : 05	95	Denaturation

qRT-PCR based SYBER Green I Dye Detection

qRT-PCR 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 qRT- PCR PreMix reagent kit is designed for PCR amplification of cDNA for target gene (CAPN4) by using it's 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 Syber 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, qRT- PCR master mixes were prepared for gDNA standard curve, target genes, and GAPDH gene as following (tables 1 and 2).

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

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

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

Volume	qPCR PreMix
10μL	cDNA template
1μL	Target gene –F Primers
1μL	Target gene –R
8μL	DEPC water
20μL	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 (Table 3). After reaction is completed, data analysis was performed.

Table 3: Set device qRT-PCR for CAPN4 gene.

Cycle	Time	Temp.	Step
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1 10min ° 95C Pre-Denaturation

40 15sec ° 95C Denaturation

30sec

° 60C

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 (CAPN4). 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 (2012) to estimate of linear regression (prediction equations) between difference variables and gene expression in this study.

Results and Discussion:

Quantitative Reverse Transcriptase Real- Time PCR

Data analysis of SYBR®green based reverse transcriptase real-time PCR for CAPN4 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 CAPN4 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 1).

Figure 1: Curves for GAPDH and CAPN4 Genes in Iraqi Local Awasi Lambs.

Weight Regression when slaughtered to the expression of a CAPN4 gene .

It is clear that there is a negative and highly significant regression ($P \leq 0.01$) of live body weight at slaughter on the amount of the expression of CAPN4 gene, and its coefficient was - 0.289 kg, meaning that the live weight when slaughtered for local Awassi lambs decreased by - 0.289 kg when the expression for this gene increased by one unit, and the coefficient of determination for this relationship is 0.02, meaning that the expression of this gene explains 2 % of the live weight when slaughtered for Lambs (Table 4).

Table 4: Regression coefficient and straight line equation for the expression relationship of the CAPN4 gene with live weight at slaughter for Iraqi Local Awassi lambs.

Coefficient of determination (R ²)	Level of significance	Straight line equation	The traits Which on them Regression traits
0.05	*	$Y^{\wedge} = 43.74 - 0.506 X$	Target Ct live weight at slaughter)kg(

0.02	NS	$Y^{\wedge}= 31.82 -0.059X$	House Ct
0.02	**	$Y6^{\wedge}= 31.44 -0.289X$	$\Delta CT= Tct-Hct$
0.02	NS	$Y^{\wedge}= 30.25 +0.724X$	Fold=2 [^] - ΔCT
0.02	NS	$Y^{\wedge}= 30.25 +0.724X$	Ratio= 2 CT (reference) – CT (target)
0.08	NS	$Y^{\wedge}= 32.16 +1.63X1 -1.69X2 +0.138X3$	Target Ct, House Ct , Fold= 2 [^] - ΔCT
Not significant :, NS** (P≤0.01) , * (P≤0.05)			

From table (5) it is clear that there is the regression nonsignificant for hot carcass weight, as its coefficient reached - 0.156, meaning that the weight of the hot carcass of the local Awassi Lambs decreased by - 0.156 kg when the expression of this gene is increased by one unit, whereas, the determination coefficient for this relationship was 0.02, meaning that the expression of this gene explains 2 % of the weight of the hot carcass of the local Awassi lambs.

Table 5: Regression coefficient and straight line equation for the expression relationship of the CAPN4 gene with hot carcass weight for Iraqi Local Awassi lambs.

equation	The traits	Which on them	Regression traits	Coefficient of determination (R2)	Level of significance	Straight line
0.06	*	$Y^{\wedge}= 23.62 -0.274 X$	Target Ct	Hot carcass weight		
		kg(				
0.01	NS	$Y^{\wedge}= 17.19 -0.032X$	House Ct			
0.02	NS	$Y6^{\wedge}= 16.97 -0.156X$	$\Delta CT= Tct-Hct$			
0.02	NS	$Y^{\wedge}= 16.34 +0.391X$	Fold=2 [^] - ΔCT			
0.02	NS	$Y^{\wedge}= 16.34 +0.391X$	Ratio= 2 CT (reference) – CT (target)			
0.61	NS	$Y^{\wedge}= 23.67 -0.302X1 +0.026X2 +0.363X3$	Target Ct, House Ct , Fold= 2 [^] - ΔCT			
Not significant : NS , * (P≤0.05)						

The results of the current study also recorded the presence of a positive and significant regression (P≤0.05) for the The dressing percentage over the amount of the gene expression, and its coefficient reached 0.0028, that is dressing percentage increased by 0.0028 when increasing the expression for this gene one unit, and the coefficient of determination for this relationship is 0.05, meaning that the expression

of this gene explains 5 % of the dressing percentage when slaughtered for Lambs (Table 6).

Table 6: Regression coefficient and straight line equation for the expression relationship of the CAPN4 gene with dressing percentage for Iraqi Local Awassi lambs.

equation	Coefficient of determination (R2)	The traits	Which on them	Regression traits	Level of significance	Straight line
0.08	*	$Y^{\wedge} = 54.16 + 0.0018 X$	Target Ct	Dressing percentage		
(%)						
0.05	*	$Y^{\wedge} = 54.02 - 0.00117X$	House Ct			
0.05	*	$Y^{\wedge} = 54.34 + 0.0028X$	$\Delta CT = Tct - Hct$			
0.06	*	$Y^{\wedge} = 54.00 - 0.0048X$	Fold = $2^{\wedge} - \Delta CT$			
0.06	*	$Y^{\wedge} = 54.00 - 0.0048X$	Ratio = 2 CT (reference) – CT (target)			
0.61	*	$Y^{\wedge} = 54.07 + 0.0077X_1 - 0.0072X_2 + 0.135X_3$	Target Ct,			
			House Ct , Fold = $2^{\wedge} - \Delta CT$			
			) *P≤0.05(			

We conclude from the study the presence of expression of the CAPN4 gene according to the above results, and further study may be conducted to ascertain the association of this expression with the growth and carcass meat quality traits.

References :

Al-Khazrji, W. J., Al-Samari, W. A. and Ajil, A. M. (2014). The effect of some genetic and non-genetic factors on fertility and growth traits in Awassi sheep. The Iraqi J. Agric. Sci. 45: 205–211.

Cheon, M., Park, D., Kim, K., Park, S. and Ryu, K. (1999). “Homologous Upregulation of GnRH Receptor mRNA by Continuous Endocrine, 11(1): 49-55 .

Chung, H.Y. and Davis, M., (2014). Effects of genetic variants and mapping assignments of the ovine calpain regulatory subunit gene on chromosome 14. Genes Genom. 36, 465–473 .

Dehnavi, E., Azari, M. A., Hasani, S., Nassiry, M. R., Mohajer, M., Khan Ahmadi A.R., Shahmohamadi L and Yousefi S. (2012). Association between yearling weight and calpastatin and calpain loci polymorphism in Iranian Zel sheep. Iranian Journal of Applied Animal Science 2(2): 131-135.

Dunn, C., and Ryan J. (2015). The evolution of animal genomes. Current Opinion in Genetics & Development, 35, pp: 25–32.

Gingrich, P. (2004). "Regression." Introductory Statistics for the Social Sciences 834–84.

Juszczuk-Kubiak, E., Flisikowski, K. and Wicińska, K. (2010). A new SNP in the 30UTR.Region of the bovine calpain small subunit (CAPNS1) gene. Mol. Biol. Rep. 37, 473–476.

Khalaf, A. I., Said, S. I. and Edriss, S. M. (2010). Role of some genetic and environmental factors in growth traits of Turkish Awassi, local and crossbred lambs. The Iraqi J. Agric. Sci., 41: 12-22.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(- $\Delta\Delta CT$) method. Methods 25(4): 402-8.

Novikov, V., Lebedev, U., Saharnov, A., Utkin, V., Balmusova, A., Alyasova, V .

.(2012) Polymerase chain reaction for detection and quantitative comparison of

FAS mRNA. Vestnik Nizhegorodskogo Universiteta imeni Lobachevskogo 2 , pp: 211-215.

Pareek, C. S., Smoczynski, R. and Andrzej, T. (2011). Sequencing technologies and genome sequencing. Journal of Applied Genetics, 52(4), pp: 413–435.

SAS. (2012). Statistical Analysis System, User's Guide. Statistical. Version 9.1th ed. SAS. Inst. Inc. Cary. N.C. USA.

Shahrudi, F. E., Nassiry, M. R., Valizadh, R., Moussavi, A. H., Tahmoorespour, M. and Ghiasi, H. (2006). Genetic polymorphism at MTNR1A, CAST and CAPN loci in Iranian Karakul sheep. Iranian Journal of Biotechnology, 4(2): 117–122.